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CAMBIA

CENTER FOR THE APPLICATION OF MOLECULAR BIOLOGY TO INTERNATIONAL AGRICULTURE

14 FEB 1994

20635

REF: Contract No: 93/200/IR

23 September 1993

Mr M Kohonen,.
Chief Contracts Section
General Services Division
Department of Administration
UNIDO
PO Box 300
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Austria

BY FAX: +43 1 230 8272

Dear Mr Kohonen

Subject: Project GE/GLO/90/004 - Five Year Work Programme of ICGEB
Practical Course on "Tissue Culture and Beyond"
9 - 21 October, 1993, Cairo Egypt

Please find enclosed Final Report and invoice for the above project. Should you have any queries please do not hesitate to contact Dr Richard Jefferson or myself.

Yours sincerely

Narelle Dryden
Narelle Dryden
Administrative Assistant

PLEASE NOTE CHANGE OF ADDRESS: CAMBIA, GPO BOX 3200, CANBERRA ACT 2601, AUSTRALIA

0923KOHO.DOC

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Practical Course:

"Plant Biotechnology: Tissue Culture and Beyond"

UNIDO Project Number GE/GLO/90/004

Final Report

December 29, 1993

UNIDO/ICGEB Contract number 93/200/IR

Contractor:

CAMBIA
GPO Box 3200
Canberra ACT 2601,
Australia

Course Director:

Dr Richard A. Jefferson,
Director
CAMBIA

Course Venue:

National Research Centre, Cairo, Egypt

Course Duration:

October 9-21, 1993

Table of Contents

Introduction and Background

CAMBIA was approached in February of 1993 by Mr George Tzotzos, ICGEB, Vienna regarding conducting a course on behalf of ICGEB to be held from October 9 - 21, 1993 in Cairo, Egypt, with the title: "Plant Biotechnology - Tissue Culture and Beyond". Upon verbal agreement, and following substantial preliminary discussion with ICGEB Vienna, Dr Richard Jefferson, Director of CAMBIA, attended a meeting in August, 1993 at the National Academy of Scientific Research and Technology, in Cairo, Egypt, to coordinate the course with the local organizer, Professor Hamdy Abdel-Aziz Moursy, and to work together with the local organizers, and the representative of ESCWA, Mr Hassan Charif, to select students and inspect the venue for the course, and arrange for local involvement in course planning and execution.

At this time, it was determined that the course had been advertised by UNIDO/ICGEB under the agreed title "Plant Biotechnology: Tissue Culture and Beyond", but had been subsequently advertised as "Tissue Culture Applications in Industry and Agriculture" and variants on that name by the local organizers, and the applications to prospective students had been sent with that title, and a syllabus established that had not been harmonized with UNIDO/ICGEB, ESCWA or CAMBIA. Thus all the candidates who applied, and from whom the participants were selected, were informed of one course, whereas the financial supporters and the implementing agency for the course had planned another.

On inspection of the proposed laboratory, it was suggested that it would not be capable of supporting an intensive tissue culture component, as there were only two poorly-functioning hoods - neither with sterile capacity - and little physical space for students, and only one quality dissecting microscope. Assurances were made that the tissue culture facility would be upgraded for the course in time. The syllabus planned, agreed upon and referred to in the Terms of Reference (Annex 3), was modified as well as possible to encompass the interests of the accepted participants, including a more substantial component of tissue culture than had been agreed to previously. Specialized faculty were therefore recruited by CAMBIA to achieve the modified aims of the course. Assurances were also made to the CAMBIA and ESCWA representatives that the National Research Centre would be the venue of the lectures, with good projection facilities, and immediate proximity to the laboratory.

After communications during the period leading up to the course it was decided that due to the modest infrastructure (see Annex ....) it would be necessary for CAMBIA to provide the majority of the equipment, disposables, consumables and reagents for the course. Upon arrival for the setup prior to the course, the lecture venue had been changed without prior consultation to the top floor of the Raja Hotel, at which the students were staying. This proved inadequate, with poor projection facilities, inadequate ventilation, poor acoustics (road noise was substantial), and a generally unprofessional ambience. No reasons were offered why the venue had shifted from the NRC, which would have been immediately adjacent to the laboratory. Nonetheless, the course was conducted under these conditions, following as closely as possible the agreed format. The course was, from the perspectives of the students, a success (see enclosed evaluations).

Proposed Lecture Topics

Professor Wayne Parrott, Department of Agronomy, University of Georgia

I. Overview of plant tissue culture
- A. History
- B. Definition of terms
- C. Uses
  - 1. Proposed uses
    - a. Selection at the cellular level
    - b. Production of "instant inbreds"
    - c. Recovery of hybrids not otherwise possible
    - d . Mass propagation
    - e. Production of novel traits
    - f. Germplasm storage
  - 2. Realized uses
    - a. Mass propagation
    - b. "Clean" germplasm
    - c. Callus assays

II. Micropropagation
- A. Clonal multiplication
- B. Virus elimination
- C. Germplasm storage/transfer

III. Organogenesis
- A. Induction
- B. Uses

IV. Somatic embryogenesis
- A. Induction
  - 1. Biology
- B. Uses
  - 1. Mass progagation
    - a. Bioreactors
    - b. Synthetic seed
  - 2. Gene transfer

V. Anther culture/haploid production
- A. Phylogenetic limitations
- B. Somaclonal limitations
- C. Use in breeding programs

VI. Protoplast techniques
- A. Somatic hybrids
  - 1. Phylogenetic considerations .
    - a. Evaluation of published results
  - 2. Alternatives
- B. Cybridization
  - 1. Resistance of chloroplast genome to recombination
- C. Cytoplasm exchange
- D. Assymetric fusion
- E. Use in gene transfer
  - 1. Somaclonal limitations
  - 2. Alternatives

VII. Somaclonal variation
- A. Causes
- B. Types
- C. Approaches
  - 1. Random occurrence

2. *In vitro* selection

D. Evaluation of published results
   1. In ornamental industry
      a. Economic losses
   2. In breeding programs
      a. Source of novel variation?
      b. Bottleneck: screening or variation?
   3. Lindsey Withers' stages
      a. Viewed as a problem
      b. Viewed as an opportunity
      c. Disappointment
      d. Balance

VIII. Techniques

Professor Ry Meeks-Wagner, Institute of Molecular Biology, University of Oregon, Eugene

"DEVELOPMENTAL GENETICS" Much of what is expected from new genetic engineering technologies being applied to plant improvement is based on existing concepts on the genetic control of development. Many of these concepts have been directly assumed from the genetic analysis of animal development. However, there is now good evidence from the analysis of plant and animal development that some of these concepts need to be modified, or updated, to reflect the integration of physiology and development. These "modified" concepts have enormous implications for the use of genetic engineering as a tool to alter crop plant growth and development.

"*ARABIDOPSIS THALIANA*: MORE THAN A MODEL SYSTEM?" *Arabidopsis thaliana* has emerged as a powerful plant species for the genetic analysis of many processes. The international effort to coordinate these genetic analyses with the physical mapping of the Arabidopsis chromosomes makes possible the opportunity to understand, in great detail, the genetic and molecular basis of Arabidopsis growth and development. The information derived from this work should benefit researchers working with a variety of crop plants.

"cDNA LIBRARY CONSTRUCTION: CLONING GENES BASED ON TRANSCRIPTIONAL EXPRESSION PATTERNS" In many situations it is desirable to isolated genes that are transcriptionally expressed in particular cell types or at particular times during plant development. For example, it may be of interest to isolate the transcriptional regulatory regions of a gene that is highly transcribed specific tissue in order to "drive" the expression of a foreign gene in that tissue. The isolation of genes for such a purpose can be accomplished by the construction and screening of cDNA libraries.

"POLYMERASE CHAIN REACTION (PCR): ANALYSIS AND CLONING OF TRANSCRIPTS FROM LIMITED AMOUNTS OF TISSUE" Often it is difficult to obtain sufficient tissue for traditional methods of transcript analysis and cDNA cloning. In such situations it is possible to use PCR-based techniques to provide either qualitative or quantitative information related to the transcriptional expression of particular genes, and to generate adequate amounts of cDNA for the construction and screening of cDNA libraries. Specific examples of these methods will be discussed to illustrate the potential, and the pitfalls, of PCR-based transcript analysis and cDNA cloning.

Dr. Richard A. Jefferson, Director, CAMBIA, Australia

I. Gene Fusions and Transgenesis
   A. GUS system
   B. Promoter and Cellular and Developmental Analysis

II. Gene Transfer technology: Methodology. Theory. Application and Practice

A. Strategies for variety-independent gene transfer into dicotyledonous species *Agrobacterium tumefaciens*, Particle Bombardment

B. Strategies for variety-independent gene transfer into monootyledonous species

III. Assessment of current transformation methodologies: Reality vs Fiction

Professor Steven G. Hughes, Director of Biotechnology, Unileve: Plant Breeding International and Unilever Plantations

I. Integration of Biotechnology into Commercial Plant Improvement
II. The Oil Palm Experience; a cautionary tale in plant biotechnology

Roundtable Discussions and lectures

Convenor: Dr Sujata Lakhani, ICGEB, New Delhi
1. 'Science on a Shoestring'
dealing specifically with the difficult issues of running productive scientific programs in less developed countries,
2. 'Women in Biotechnology' -
addressing gender issues involved in the successful integration of women into both research and production.
3. 'Farmers First: Priority Setting in Plant Biotechnology.

Additionally there will be lectures on basic aspects of molecular genetics and biotechnology given informally to ensure all students are at the appropriate level for the course material.

Laboratory Topics:

Part I - Tissue Culture
see lecture topics, above.

Part II - Molecular Biology
Preparation of Plasmid DNA
Bacterial Transformation
Preparation of Plant DNA
Restriction Endonuclease Digestion
Agarose Gel Electrophoresis
Southern Transfer
Hybridization Probing of RFLPs using chemiluminescence detection
Analysis and Interpretation of RFLPs

Polymerase Chain Reaction (PCR)
Amplification of single copy plant genes
Random Amplification of Polymorphic DNAs (RAPD)

Part III Transgenesis
Agrobacterium infection of potato/tobacco
Particle Bombardment (Helium GUN)
Promoter Analysis of GUS fusions
Cellular analysis of transformation

Actual Course Programme:

Plant Biotechnology: Tissue Culture and Beyond

SATURDAY, OCTOBER 9, 1993

Registration (Raja Hotel)		
Welcoming Address		Professor Hamdy Abdel-Aziz Moursy Vice President Academy of Scientific Research & Technology Cairo, Egypt
Introductory Remarks		Dr. Richard Jefferson Director, CAMBIA Canberra, Australia
Lecture:	History of Tissue Culture	Professor Hamdy Abdel-Aziz Moursy
Lecture:	Overview of Tissue Culture	Dr. Wayne Parrott Dept. of Crop and Soil Sciences University of Georgia
Laboratory and discussion session		Molecular biology review

SUNDAY, OCTOBER 10, 1993

9:00 - 10:00	Developmental Genetics	Dr. Ry Meeks-Wagner Institute of Molecular Biology University of Oregon
Lecture:	Polymerase Chain Reaction (PCR) for cloning genes	Dr. Ry Meeks-Wagner
Lab discussion session and lab experiments		(RMW)

MONDAY, OCTOBER 11, 1993

Lecture:	Running a Tissue Culture Lab	Dr. Wayne Parrott
11:00 - 18:00	Lab discussion session and lab experiments (Lunch during lab time)	
19:00	Round table discussion	Dr. Sujata Lakhani ICGEB New Delhi, India

Tuesday October 12

Lecture:	Somatic Embryogenesis	(Wayne Parrott)
	Secondary Products	(Ragai Ibrahim]
Lab:	Bacterial Transformation	[Ry Meeks Wagner]
	Embryo Lab I	[Mohammed Aly / Wayne Parrott]

Wednesday October 13

Lecture:	Plant Transformation	[Wayne Parrott]
	Vectors and Gene Fusions	[Richard Jefferson]
Lab:	Blue/white Plates	[Ry Meeks Wagner]
	Agrobacterium cocultivation	[Wayne Parrott]
	Picking Colonies for mini-preps overnights	[Ry Meeks Wagner]
	Embryo Lab II	[Mohammed Aly / Wayne Parrott

Evening: Sound and Light Show at the Pyramids

Thursday, October 14

Lecture:	None	
Lab:	Minipreps of cloning	[RMW]
	Run Gel	[RMW]

Afternoon Free

Friday, October 15

Free

Saturday, October 16

Lecture:	RFLP/RAPDs	[RMW & Salma Talhouk]
Lab:	RAPDs	[RMW & ST]

Sunday, October 17

Lecture:	GUS & Transgenics	[RAJ]
Lab:	Transfer *Agrobacterium*	[WAP]
	Gene Gun	[RAJ & WAP]

Monday, October 18

Lecture:	Anther Culture	[MA]
	Embryo Rescue	[MA & WAP]
Lab:	Anther Culture	[MA & WAP]
	Section t-genic tobacco	[RAJ]

Tuesday October 19

Lecture:	Ideas into reality: The difficulties of translating biotechnology ideas into practical applications.	[Dr. Steven Hughes]
Lab:	Analysis of GUS+ tobacco	[RAJ]
	GUS assay Agro + shots	

Wednesday, October 20

Lecture:	Oil palm case studies	[SGH]
Lab:	Analysis of GUS+ transgenics	[RAJ]
Round Table:	Genetic basis of instability	[WAP, RAJ, SGH]

Thursday, October 21

Discussion:	Technology choice
	Misc. Problems with TC and Biotech.

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Course Evaluations:

The following form was printed and distributed to all official course participants. Virtually all were returned and are reprinted anonymously - at the request of many of the students. The transcriptions are within the report, photocopies of the originals, with the names masked, are appended (Annex 1).

Much of the content of the Contractor's Evaluation is mirrored in the comments made by the course participants. The transcription made only minor grammatical and spelling ammendments.

Plant Biotechnology: Tissue Culture and Beyond

National Research Centre
Cairo, Egypt
October 9 - 21, 1993

CAMBIA Course Evaluation

This evaluation is a confidential document that will help CAMBIA in planning and executing future courses. Please provide as much detail and as honest an assessment as you can. We are particularly interested in areas where improvements need to be made. The topics listed are only suggestions - all areas of the course need to be critically assessed. Feel free to use space on the back of the form, or additional sheets of paper. If you wish, your evaluation can be anonymous - but please included your name on the form so that we can ensure only participants provide the information. Thank you for your assistance. Please return this form directly to the Course Director for CAMBIA or one of the International Staff.

Name

Course Content (Syllabus)

Lectures

Practicals

Instructors

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization

Laboratory Infrastructure

Accomodations

Financial Arrangements

General Comments and Suggestions

1.

Course Content (Syllabus)

Lectures -

The level and quality of lectures were exceptionally good. There was a genuine effort to convey information about the latest techniques and discuss their drawbacks and advantages, with respect to their potential applications, especially as potential to expand the use of tissue culture. Handouts from lectures were constantly lacking due to the fact that the non-registered NRC staff were always taking copies for themselves before all registered students did.

Practicals

Laboratory sessions were severely handicapped by the lack of infrastructure at NRC as well as the absence of coordination in the local organizing committee. In addition, the lab sessions were constantly disrupted by NRC staff insisting on taking part of the practicals. The lack of organization from the host country was such that despite daily reminders that only registered students can participate in practicals, this issue was a daily problem.

Course Organization

The organization of the course had to be constantly changed due to the lack of facilities and supplies.

Laboratory infrastructure

NONE PRESENT. As for the "tissue culture facility" it was in the form of a very small room with contaminated hoods (X2). This set up was by no means fit for a course. I personally feel that the choice of the location was a fraud especially when I know that there are much better tissue culture facilities in Egypt.

Accommodations

The hotel was dirty, bathroom tub was plugged, the door knob was not functional. The atmosphere made me feel insecure such that I felt the need, and I did, put a table before my door at nights. In addition I felt my privacy was invaded as I noticed that food and water that I purchased and stored in my room was being partly consumed by the staff in my absence. The absence of a lounge area prohibited any group interaction in informal settings which in my opinion are very important. As a result, I personally felt deprived from the opportunity to set up possible collaborative research projects with fellow Arabs.

Financial Arrangements

When comparing the Raja rates with much better hotels, here again I could only personally conclude that it was highly overrated.

General Comments and Suggestions

If it wasn't for the compassion and the dedication of the International Instructors, I would have left 2 days after my arrival.

2.

Course Content (Syllabus)

Mostly dealt with molecular biology although the title was "Plant Tissue Culture Techniques". But after all, the topics were chosen very carefully to give us the hints of all major techniques of molecular biology that we did not know before.

Lectures

Very effective; especially considering the poor facilities you had. You would have asked for more contributions from the audience to make the lectures more dialogues (To prevent from being passive listeners)

Practicals

Very good but too many to digest (understand) in a short time. It was easy to mix them up. Not enough numbers of experts to help and show everybody sufficiently.

Instructors

Brilliant! Very patient! Spoken clearly but many people had some difficulty in understanding Wagner's speech.

Course Organization

BAD! Especially local part of it. Two unrelated titles for the same course; Course Venue and Practical Venue were simply intolerable.

Laboratory Infrastructure

Insufficient at all! Small, dirty, unorganized, unhygenic, etc.

Accommodations

Not too bad, but very expensive; having the same breakfast every single morning was a nightmare.

Financial Arrangements

UNJUSTIFIABLE VARIATIONS among participants. Everybody had the same replies (letters) from the ESCWA on behalf of the sponsoring agencies, but some had to pay participation fee while some did not. I personally lost $200 out of my pocket.

General Comments and Suggestions

A lighter schedule over a longer period could have been more effective and fruitful. Also excursions, field trips, etc. might have been useful. Most of the participants" language abilities were not enough to follow the course efficiently!

3.

Course Content (Syllabus)

The contents of the course were good. The makers of the syllabus have done their best to include all the modern techniques which are now being used in all labs around the world. The syllabus contained to topics on tissue culture, plant breeding, genetic engineering, molecular biology, etc. I think it was a good idea not to stick just on one technique but give an idea about all modern techniques. In my opinion that was an excellent syllabus for a two weeks course.

Lectures

The preparation of lectures was also excellent. The lectures were prepared ina a way to give the whole idea about problems, benefits and also limitations one can face during the handling of techniques.

Practicals

The practicals were designed in such a way to provide an opportunity to each participant to learn most modern techniques during two weeks course. I think after doing practicals for two weeks everyone now have some idea about each techniques. Even I have in one week.

Instructors

All the instructors have done an excellent job during the whole period of the course. Everyone has tried his best to transfer the knowledge as much as he can. They were friendly and helped each of us during practicals, lectures, and in all other problem. These types of instructors are very good for students in such a type of course. My best wishes to all of them.

Course Organization

Course organization was very poor as host institute is concerned but the invited speakers have done wonderful job to make these two weeks very happy, knowledgeable for everyone. In my opinion the host institute have organized this course just to generate money for their institute otherwise they have no experience how to organize a course, because no one was in touch with participants to solve their problems.

Laboratory Infrastructure

I am very sorry to say that the institute has no laboratory infrastructure to organize such type of a course. The labs were most of the time remain very dirty. The most of the instruments were brought by the invited speakers with them. I can't understand where all the money gone which was supposed to be given to those institute for organization of this course. How can an institute organize such a course which even has no auditorium for lectures??

Accommodations:

That was burning issue among the participants during whole period of the course. The accommodations were arranged very expensively. I think 45$US for one day for such a dirty hotel was too much. Hotel have no air-conditioned auditorium so why selected that hotel? During the stay in hotel, they provide same type of breakfast. Everyone of us was given a written statement that hotel will provide transportation from and daily to the institute but when they were asked everyday about transportation they simply refuse. Even our instructors were living in a four star hotel and they were simply paying 42$US per day. Why did we pay 45$?

3....cont.

Financial Arrangements

As financial arrangements are concerned, I am very grateful to sponsoring agencies to give me the opportunity to participate in this course. All the financial support seems to be given to us was taken away by host institute. Why they arrange such an expensive accommodation (US$45/day) in such a dirty hotel for us. During corresponding host organization has not told to any of us about terms and conditions. When we arrived in Cairo instead of getting US$900 we got 200 - 240 dollars and they cut 45$ from our daily accommodation charges. WHY?

General Comments and Suggestions:

I would like to suggest the sponsoring agencies PLEASE before giving money to host institutes it should be confirmed whether they have facilities to host the course. These facilities at least includes, laboratory infrastructure, transportation and a good auditorium for lectures. Its my request to sponsoring agencies to ask from host institute about money which they gave them for us as DSA and if possible return the money to each participant because it has created a lot of frustration among participants. Thanks.

4.

Course Content (Syllabus)

As far as I know, the name of this course was "Plant Tissue Culture and its Application to Agriculture and Industry" But I have learned here, that the name is "Plant Biotechnology: Tissue Culture and Beyond" . So, this course include mostly from molecular biology. But after all, I have seen the major techniques of molecular biology.

Lectures:

The lectures were very effective, they included the latest (new) developments and all details. Although my first foreign language is German, and I can't understand English very well, the instructors held the lectures very clearly and I have learned more things than I expected.

Practicals

They were the best under this limited conditions. But it was easy to mix them up. At the same time we made three treatments sometimes.

Instructors

They are very brilliant. A good team, they made personally efforts to run a successful course.

Course Organization

The building and this institute has no facilities to do a course like this. Course program was very intensive. We didn't see the other institutes and universities. We have not any idea about the science of Egypt. Are all the laboratories and institutes so bad? No excursion no field trips.

Laboratory Infrastructure

Insufficient at all!! Dirty and unorganized.

Accommodations

Not too bad. But very expensive. During two weeks we had same breakfast and same lunches.

Financial Arrangements

I have no problem regarding finance. (Thanks to IDB!)

General Comments and Suggestions

A better place could be chosen for the practicals.

5.

Course Content (Syllabus)

The lectures generally covered molecular biology very well, but I've come to Egypt to learn more about tissue culture techniques, the two subject are important for me, but tissue culture is more relevant.

Lectures

The lectures were well done, and there was a big effort to make the lectures up to that high scientific level.

Practicals

I've learned many new techniques, and few techniques also I was expecting to see more and learn more.

Instructors

All the instructors are good and qualified and specially patient and I want to "soulever le chapeau" for Sujata because she always was able to manage and keep smiling all the time.

Course Organization

The organization was not satisfying; We were not treated right. I've received such a program before I came and I was very excited to come, but when I came it was totally different.

Laboratory Infrastructure

This is the catastrophic situation. We were waiting all the time. We wait to get tools and supplies from other candidates. I'm interested in seeing all of the equipment of tissue culture laboratory because I want to equip my one lab, but I have not seen many!

Accommodations

I was not satisfied in this hotel, and I think that we paid a lot of money for nothing. Dust everywhere, the food not good, the air conditioners don't work, I can't sleep from heat.

Financial Arrangements

The rate for the hotel is high.

6.

Course Content (Syllabus)

The lectures covered many points in molecular genetics and some in tissue culture, so did the practicals. I thought that the course will be mainly in tissue culture with some knowledge in genetics. I think for a person who wants to gain knowledge in molecular genetics the syllabus could satisfy him.

Lectures

The lectures is designed for a person who had pre-information in tissue culture and not for that who want to know the basics in tissue culture. For me, I have no problem with genetics, but my knowledge in tissue culture is not enough to proceed all things. Lecturers speak slow enough so we can follow and they are very patient

Practicals

I think the time for practicals is not enough to begin some experiments from the proper point. We did not prepare a media, and I think we should do so, to gain experience if we hadn't and to improve our skills if we had.

Instructors

I think they are existent enough to find whenever we need, and they are helpful and patient.

Course Organization

Official participants come all on time, but the day is very filled so sometimes we are very tired and cannot proceed efficiently. The time for entering the class is not fixed so some participants who came late cause interruption.

Laboratory Infrastructure

Lab infrastructure is not suitable ror such work. The area is very small compared to the number of participants. We are working in one small unit of tissue culture so a lot of time was lost in waiting to begin the work. Also pipettes were not enough. The lecture room also is small so we cannot sit comfortably.

Accommodations

the hotel is relatively comfortable and near the NRC, but there is no need for such and expensive hotel. The same food every day and I think the quality is not coinciding with the money paid. There is no regular transportation to and from the hotel and the NRC.

Financial Arrangements

This is the most bad thing during the course. We are forced to stay in Raja Hotel and pay 45$ per day, and this is very costly for us. We receive 60$ per day from the sponsor and we are not coming in a tour trip to be resident in such hotel. Also we pay for dinner out of this fee. so it is very expensive hotel for us, and the organizing committee did not allow us to arrange our settlement by ourselves, and I think we can do that.

General Comments and Suggestions:

To receive the syllabus of the course before coming. The length of the course is very short compared to the syllabus. The organizing committee should be away from our financial support and every one must be free in his residence whenever he attend the course.

7.

Course Content (Syllabus)

The title of the course is Tissue Culture Applications in Industry and Agriculture, but most of the syllabus was molecular biology and I need more information more lectures about micropropagation.

Lectures

Lectures were very useful, but I didn't understand too much about molecular biology because I don't have good background about it, and also the lecture room was not very helpful.

Practicals

Anther culture, somatic embryogenesis, Bacterial transformation, Agrobacterium transformation, GUS and transgenics and gene gun were very useful for us and we were pleased to know about PCR RAPDs ..but in fact we didn't use them in our lab in Jordan, somebody else may do it.

Instructors

Instructors are very helpful, and we can go to them and ask them for anything any time.

Course Organization

The course was not well organized, the lecture room was very bad even in the hotel or in the NRC, also the course didn't include any tour program, so we had to go by ourselves and everyone wants a lot of money when he knows that we aren't Egyptian.

Laboratory Infrastructure

The tissue culture lab was very small, and also molecular biology was very small for about 25 participants, and I want to tell you that they painted the walls when they knew that we are coming, so the labs are not equipped very well.

Accommodations

The hotel was not good. The air conditioner in the room is not good and sometimes I found people in my room watching TV while they clean the room and also every day for 14 days we had the same breakfast.

Financial Arrangements

Bad financial arrangements. I faced a problem with registration fee, but it was solved, and also the hotel is not good enough, they took also for our transportation and w didn't go with them more than 3 - 5 times.

General Comments and Suggestions

I hope if we can make a true tissue culture course in any country rather than Egypt!

8.

Course Content (Syllabus)

The course content was very educative and met the needs of all tissue culturists who at present handle tissue culture in the 'artistic' manner. The molecular concept met the modern scenario and high tech concept.

Lectures

Excellent. Presentation excellent. But how I wish Richard, Wayne and Ry could have divided us into three groups and the facilitators or mentors for 2/3 nights tuition in the first week to update several genetic concepts. Then we have a bench mark examination to level the group off. Then we go all out for the RAPDs, RFLP's PCR and GUS. Then I am sure everyone will go back and open up Molecular Laboratories in all the countries. You know, when I go back I will ask my Director to send me to a short course at the local university to explore the presence of all the enzymes and probes locally. During the lectures there are so many people coming in and out and it is very disturbing. Another complaint is the keeping of time is very poor. We should be more strict so that any latecomers should stay out.

Practicals.

As for practicals - we are so many of us. There should be demonstrators for several groups. Say Group I and Group II will always be under Richard, g. 3-4 under Ry, G5,6 under Wayne. Arabs who do not understand English will be under Dr Mohammed Ali with Arabic translations. Sujata will help in personal demonstrations. Then fro another practical we change instructors.

Instructors

Instructors Ry, Richard, Wayne, Sujata and also Steven Hughes were excellent.

Course Organization

The course organization is very sloppy because of the distances between the laboratories and the lecture hall. We are practically running about the place. The course do not consider prayer time at all which upset me very much. Sometimes when I go down very late for prayer the mosque downstairs was closed. I know it is up to us to leave anytime but it hurts when something going on is out of my scope.

Laboratory Infrastructure

Is not very spacious to cater for the many students. The molecular laboratory has good facilities like pipettes, etc., but I believe these are from CAMBIA.

Accommodations

Raja Hotel is rather a distance to the laboratory. This course should be carried out in an area with accommodation nearby. This running and moving about caused a lot of time to be wasted. Finally at the end of the course we have little time together with the international experts. The food is the same for many days. Breakfast is boring.

Financial Arrangements

Excellent. The sponsor paid a substantial amount of money which is very much better than other courses that I have gone to.

8....cont.

General Comments and Suggestions

This course should be carried out again for other students but let it be better organized. As a post-mortem I hope the Course lecturers will visit us at our research stations maybe in a year's time so that if we are not applying what we are learning now, then your presence will help solve some problems like a misunderstanding Director or dictatorial director who finds it hard to understand molecular biology. The presence of any of the International speakers would make our Dictators see things more clearly.

9.

Course Content (Syllabus)

The program was fine and continuous linkage was existing between all parts. However, the only comment I DO have is related to a lack of concordance between the two circulars (which indicated and promised a great part of tissue culture techniques) while in reality it was more molecular biology (which is fine for me). Maybe for other trainees it was confusing.

Lectures

Excellent lectures were given by the *international* faculty. However the lecture room - either in Raja or in NRC was not adequate and poorly equipped for such meetings.

Practicals

Again, nothing to reproach the international faculty, they did their best with the existing equipment. Without their willingness and hard work, the techniques could never be taught to students.

Instructors

I take this opportunity to congratulate all international instructors. They were terrific, taught us with an efficient and clear way. They were always available for questions and round-table discussions.

Course Organization

The local organization was lacking a lot of coordination. All trainees were everyday surprised with a matter (in general it was always a *financial* aspect) which disturbs the climate of such a course. Hope this won't happen again!

Laboratory Infrastructure

I think it is too late to discuss such matters, because the CAMBIA company should have made a decision prior to the course (not to hold the course due to insufficient infrastructure). Anyway don't make the same mistake twice!

Accommodations

Here again, we suffered (financially) from the arrangements made prior to our arrival. A US$45 daily rent for a hotel that doesn't deserve US$10 is a joke. Rooms ere dirty, meals not reasonable etc..

Financial Arrangements

CAMBIA should get in contact with the sponsors in order to make for the damage made to trainees. Some received their perdiem for 17 days, others, for 12 days. Furthermore, the registration free had disturbed most of us, regardless of the way trainees were treated (as *kids*!) I think that's enough.

General Comments and Suggestions

Besides all the above mentioned problems, and discomfort caused by some organizing people, I think the course was successful. Thanks of course to the International faculty!

10.

Course Content (Syllabus)

Lectures were more about and very high rate molecular biology. Although to learn for me lastly news, techniques and applications from specialists is very useful as is a beginning for future.

Lectures

Good

Practicals

Good

Instructors

A good team with a perfect leader (specialists, friendly .....)

Course Organization

Laboratories and facilities were not sufficient

Laboratory Infrastructure

Accommodations

Good

Financial Arrangements

Good

General Comments and Suggestions

The course could have been held at a university. The social activities could have been done.

11.

Course Content (Syllabus)

The course content was concentrated on the few topics related with Agrobacterium, gene transformation and genetic manipulation and lack of tissue culture tech which all the participants were expected to be. the course was condensed and to many information provided within the short time.

Lectures

The lectures were very good and interesting. The lecturers were able to send the message clearly and smoothly and the materials itself were useful to all the participants whom they work with tissue culture type of work.

Practicals

Too many participants was one of the limitations of practicals part of this course which didn't allow to do so much work besides the LAB was not able to fit with the all participant.

Instructors

Good is not enough word to describe the instructors whom they were friends before every thing. They wanted to give as much knowledge as they could.

Course Organization

(1) Scientific part was well organized in terms of lectures and instructors.

(2) the other part of course dealing with finishing touches and preparations to make the course run smoothly was not in the same rank as the first part which make participants feel uncomfortable.

Laboratory Infrastructure

Poor

Accommodations

Not good. they didn't give us many choices in choosing whatever we like such as the way we want to live, the kind of food we like most, the suitable hotel we would like and so on.

Financial Arrangements

Financial arrangements makes me feel bad towards the course because I came without any money because I was expected to be paid upon arrival, so I end up with more than 4 days without any money and after I send fax to the sponsor personally they gave me payment for just 12 days while I stayed for 15 days and the most important part was Nobody Care!

General Comments and Suggestions

Generally, whenever we try to organize such a training course we should have one leader to make a decision to direct the course and should have everything prepared before the starting day and should have expert people help in making the course well organized. At least they should have experience in dealing with people.

12.

Course Content (Syllabus)

The course content is complete and can help to choice way of research in tissue culture according to the problems that each country is now facing.

Lectures

A background in basic genetics is necessary to take advantage of the lectures in molecular biology. Reviews are briefly done but it will be better if we were more informed on the content of the course before coming and prepare by our own reviews.

Practicals

Practicals would be more beneficial to participants if topics were separated (not two or three topics and lectures in the same session). Participants tend to mix sequences of processing.

Instructors

No special comment about Instructors

Course Organization

Quite good but the lab is not very convenient for lectures. The classroom was always crowded. Important documents miss for participants whereas observers have those documents.

Laboratory Infrastructure

The laboratory is spacious and we have discovered new machines like the PCR one and others, but we would like to process more and understand really how they work. We would like also to have more information on those machines (technical specifications, costs, etc.)

Accommodations

Quite good, but the food would be more diversified. We've always eaten the same meal at lunch.

Financial Arrangements

$60 per day/per diem is probably good according to the living cost in Egypt but everyone knows that the $15 left to participants are not enough to finish the day - the best dinner costs nearly 27 pounds at the hotel.

General Comments and Suggestions

I think that being a first course, it is a successful one, in spite of some little imperfect points.

13. (Observer)

Course Content (Syllabus)

Is beneficial for plant research and not dealing with Microbiology except those in relation to plants.

Lectures

Are very clear and simplified, regarding to the different levels of participants.

Practicals

Sometimes, we did not see the result of experiment, i.e. Southern Blot analysis and electrophoretic bands of RFLP test.

Instructors

Very kind people. they can simplify any unclear knowledge. A!so, they answer any questions till complete understanding. but sometimes they were wearing shorts and this is refused in Egyptian society.

Course Organization

Very well

Laboratory Infrastructure

We needed to know more applications on these instrument we had trained on (PCR and electrophoresis), beyond that application on the field of plant tissue culture. Are these instruments used for Microbiology or animal tissues??

Accommodations

N/A

Financial Arrangements

N/A

General Comments and Suggestions

Repeated each 3 years to make third world in relation to modern techniques.

14.

Course Content (Syllabus)

Comprehensive for both biotechnology aspect in wide case and narrow case according to tissue culture.

Lectures

It was very rich and important and advanced given in excellent mood.

Practicals

All the effort used for succeeding the course regardless the was very much crowded.

Instructors

Without doubt all the instructors. They are genteel and help every one without hesitation and without late.

Course Organization

I think the organizers forgets the freedom of the trainees and they not left any options for us.

Laboratory Infrastructure

Accommodations

Not too much good, not too much bad, very weak service, and bad treatment.

Financial Arrangements

Some organizers put obstacles in the way of some trainees.

General Comments and Suggestions

15.

Course Content (Syllabus)
Excellent

Lectures
It's more interesting but one lecture in "Secondary Metabolite Production in Cell Culture" It is not enough.

Practicals
It's very good and I learnt advanced techniques in Genetic engineering and biotechnology, without CAMBIA I don't believe learnt it.

Instructors
Excellent

Course Organization
It's very good, but 15 days it is not more enough for this condensed program.

Laboratory Infrastructure
Very good

Accommodations

Financial Arrangements

General Comments and Suggestions
The course "Plant Biotechnology: Tissue Culture and Beyond". It's more interesting for me and I learnt many advanced techniques. But the hours for practical part is not more enough.
The suggestions:

(1) Another course in this field

(2) More and more contact in future between CAMBIA and my lab, Plant Cell and Tissue Culture, National Research Centre

Finally: All thanks to CAMBIA

16.

Course Content (Syllabus)
Very good: but you can increase the time of practical in the syllabus to make an extensive background for the participants about the techniques which used.

Lectures
The time of lecture is good to discussed and suggested any problem which we have.

Practicals
Very very good, but the time not enough. I suggested that the practical time must be a large time in the course time.

Instructors
Excellent. they help the participants for understanding the goal of these course by the discussion.

Course Organization
I thank them for this course, we need more and more training course to get a good experience and to exchange information and maintain continuing dialogue on the developments in the field for mutual benefit and use.

Laboratory Infrastructure
The laboratory was poor to help in that course. The laboratory must be improved and must have a modern machines which made the worked of research is easier.

Accommodations

Financial Arrangements

General Comments and Suggestions
(1) The time of practicals must be increasing

(2) The laboratory which used must be exchanged and improved to help in the work

(3) we need more of these courses to improve our carrier.

17.

Course Content (Syllabus)
Concentrated on Genetics - a few on Tissue Culture

Lectures
Good

Practicals
Good

Instructors
Good

Course Organization
Good

Laboratory Infrastructure

Accommodations

Financial Arrangements
Not good arrangement

General Comments and Suggestions
I suggest the future courses should include more about tissue culture.

18.

Course Content (Syllabus)
It is very interesting and I learnt many advanced new techniques.

Lectures
It is very good but I wish to be more in tissue culture.

Practicals
It is good but it should be more in tissue culture.

Instructors
Very good especially Dr Richard and Dr Mohammed

Course Organization
It is very good

Laboratory Infrastructure
It is very good but laboratory is more narrow.

Accommodations
It is very good

Financial Arrangements
It should be arranged better than this.

General Comments and Suggestions
I wish to be more lecture more in plant tissue culture. it is very good time to know many of different persons from different countries in the same field.

19. (Observer)

Course Content (Syllabus)
Sufficient

Lectures
Well balanced and correlated

Practicals
Didn't strictly followed the prescribed course.

Instructors
Competent, language of some was not clear

Course Organization
Above average

Laboratory Infrastructure
Not really outstanding

Accommodations
Not applied (observer)

Financial Arrangements
Not applied (observer)

General Comments and Suggestions
Generally it was interesting specially assays related bother plant and animal. Would have preferred a programme devoted to animal biotechnology.

20.

Course Content (Syllabus)
Contents are good, but I feel that is should be conclude:
1. Concerns about the dangers of unrestricted gene cloning
2. More applications of PCR
3. Roll of pulsed-field gel electrophoresis in Molecular biology

Lectures

Practicals

Instructors

Course Organization

Laboratory Infrastructure

Accommodations

Financial Arrangements

General Comments and Suggestions
If the topics and program arrived to us shice month or two weeks at least, we will useful from this course very much.

21. (Observer)

Course Content (Syllabus)
Good

Lectures
Good

Practicals
Very good

Instructors
Very good, very helpful and efficient

Course Organization
Well organized, but still it could be better.

Laboratory Infrastructure
Good

Accommodations
I had no accommodations

Financial Arrangements
I had no financial arrangements. But still as an official observer from NRC, I believe we had more rights and we should have been treated better by the local organizer.

General Comments and Suggestions
Generally the course was very beneficial from the practical side. But I wanted to hear more about how to solve a problem, i.e. to raise a problem and through solving it we go through practical side of the course. Still I did benefit a lot from the course, although it was not exactly my field of Human Genetics.

Something else I would like to say, I was very upset by the troubles of some of the international participants or staff have met. I believe much of these things could be avoided by more arrangements and communication between international and local staff members. I wanted each of them to go back home with the best impression of Egypt, which is a real fact. We have a beautiful country, so some arrangements should have been done for them from the touristic point of view. So when we arrange such a course the scientific arrangement should go hand in hand with the accommodation and touristic arrangements.

In a future course I would like to hear and learn more about molecular biology and have more practical and applications in this field.

22. (Observer)

Course Content (Syllabus)
The main subject of the course is Biotechnology which includes up-to-date methodology.

Lectures
The lectures started at 9.00am and ended at 1.00pm. daily. The lectures lasted two weeks. They treated in detail the theoretical and practical background of Biotechnology. the lectures were fruitful up-to-date and to the point.

Practicals

Instructors
Dr Professor Richard Jefferson was more than excellent and practically. He did his best to know the problem we face and to give us valuable suggestions and solutions without imposing himself on our minds. Moreover, he enjoys modesty and decency. He was generous enough to invite us to see the light and sound program at his own expense.

Dr Professor Wayne Parrott was full of vitality and activity. He had his constructive suggestions during the course. He has his own role in lab apparatuses and the discussions of the round table.

Dr Professor Sujata Lakhani was dynamic and she was the part and parcel of the lab apparatuses.

Course Organization
The course was disciplined and well organised. It was based on sound and firm ground. It covered all the points concerend.

Laboratory Infrastructure
the apparatuses were adequate and up-to-date.

Accommodations
The accommodation was expensive and beyond my financial ability.

Financial Arrangements
I wasn't supported financially by any body. The whole financial arrangement was at my own expense.

General Comments and Suggestions
I hope to attend any coming training course held by CAMBIA, otherwise I am right when I ask to restore money spent on that course.

Contractor's Evaluation of Course Activities

More than 20 students from 13 countries participated in the course **Plant Biotechnology: Tissue Culture and Beyond**. held from October 9 - 21 in Cairo on the campus of the National Research Centre, with lectures held at the Raja Hotel. The course covered many aspects of modern biotechnology related to plants including genetic mapping, tissue culture, gene characterization, genetic transformation and priority setting.

Given certain infrastructural limitations, and some miscommunications and misunderstanding pertaining to the organization and content, the course was a great success. The students, by and large, found the course useful, innovative, stimulating and challenging (see Evaluations).

ICGEB Vienna can be proud of having initiated and funded and thus made possible a professional level international course on such a timely topic. ESCWA and the IDB can be proud of their sponsorship of the participants to take part in this course. The students - on the whole - worked extremely hard, and proved themselves to be able learners and participants in all aspects of the course, from practicals, to lectures and discussions, to informal round-tables. The course typically started early and ended late in the day - sometimes going well into the night, and yet the students were invariably cheerful and full of energy.

Points which should be kept in mind to improve such courses in the future:

- **There should only be one principal organizer, responsible for decision-making**. In this case, there were several organizations involved in various aspects, and thus the accountability, and distribution of responsibilities was sometimes obscure. With at least three funding agencies (UNIDO/ICGEB, IDB, ESCWA), one executing agency (CAMBIA), and more than one host organization (the National Research Centre and the National Academy of Scientific Research and Technology having changed management), the lines of communication were severely stretched

- **The course advertised internationally should be the course offered and implemented**. In this case, the local organizer advertised a course with a different name and content from that published by ICGEB and contracted to CAMBIA.

- **Courses should only be held in circumstances where sufficient local infrastructure can be assured from the outset, and the degree of commitment of resources by the local organizers can be spelled out clearly.** In this case, the laboratory and lecture infrastructure was absolutely insufficient for even a course of a quarter of the size or sophistication, and constant negotiations were undertaken during the course to obtain suitable facilities, thus undermining the continuity and function.

Annex 1

Final Budget

"Plant Biotechnology: Tissue Culture and Beyond"

The budget for this course is broken up into four components, which are to be paid separately as indicated in the Fax from Dr George Tzotsos to Dr Richard Jefferson dated 4 October 1993 (copy attached).

1) Preparatory trip August 1993 by RAJ

Sub-total (1) - Total expenditure:	**US $ 1,863.00**

2) Main course budget: expenses incurred by CAMBIA:

Statement of Expenditure:	**Total US $**
INCOME CATEGORIES	
UNIDO: 1st installment	19,200.00
EXPENSE CATEGORIES	
Expendables	3,114.27
Management Costs	632.62
Non-expendable accessories	3,326.73
Secretarial assistance	2,000.00
Subsistence costs	6,923.08
Travel	8,255.50
Expense - Unassigned	500.00
Total expenditure:	US $ 24,752
Total income received from UNIDO:	US $ 19,200
Balance outstanding:	US $ 5,552

Sub-total (2)	**US $ 4,800**
[Balance agreed to by UNIDO]	

3) Money paid on behalf of Dr Sujata Lakhani

As indicated in the Fax dated 4 October, all of Dr Lakhani's costs are to be met by UNIDO / UNDP. However, due to constraints of timing, CAMBIA had to advance money to cover costs incurred by Dr Lakhani to enable her to travel and participate in the course. This money should therefore now be reimbursed directly to CAMBIA.

Airfare plus terminal charges and visa	US $ 1111
DSA: 18 days at US $106/day	1908
Sub total (3) -Total expenditure:	**US $ 3019**

4) Expenses incurred by Richard Jefferson during the course (October 1993)

As stated in the Fax of October 4, an additional US $1,000 plus full DSA were to be paid for Richard Jefferson for running the course in October.

Sub-total (4) -Total expenditure:	**US $ 2,908**

Thus the outstanding balance owed to CAMBIA is:

Sub total (1) - RAJ August trip	US $ 1,863
Sub-total (2) - Main Course Budget	US $ 4,800
Sub-total (3) - Dr Lakhani's expenses	US $ 3,019
Sub-total (4) - RAJ October trip	US $ 2,908
Total payable by UNIDO	**US $ 12,590**

Annex 2:

Original Student's Course Evaluation Sheets

Prefab - Cambria

United Nations Industrial Development Organization
International Centre for Genetic Engineering and Biotechnology

Vienna Office, V.I.C. P.O Box 300, A-1400 Vienna, Austria
Tel: (+43 1) 2113I ext 4336 Fax: (+43 1) 230 7355

FAX MESSAGE

TO:

Dr. Richard A. Jefferson
Attn: Ms. N. Dryden
CAMBIA
CSIRO Division of Plant Industry
Fax: (0061-6) 246 5303 or 246 5000

DATE: October 4, 1993

FROM:

Dr. George Tzotzos
ICGEB Vienna Office
UNIDO, Vienna/Austria
Fax: (43-1) 230 7355
Tel: (43-1) 21131 ext 4336

Dear Richard,

In the response to Narelle's queries regarding the ICGEB grant to support the Cairo course, please note the following:

1 - Right from the beginning, I had informed you and ESCWA that our support exceeds US$ 25,000. In fact, generally we do not provide more than US$ 20,000.

2 - The funds are meant to cover the travel and subsistence costs of the lecturers as well as expendables.

3 - In view of the difficulties that you have encountered, we have already issued a contract for US$ 24,000.

4 - In addition we are issuing a contract for you to cover the costs of your preparatory trip (August 1993), the US$ 1,000 that are needed for your October trip as well as your DSA for both periods of stay.

5 - Furthermore, we have supported, in full, the costs for Dr. Lakhani (US$ 2,534).

6 - Our total contribution for the course exceeds well over US$ 30,000 which is unprecedented for ICGEB.

I hope this explanation clarifies the issue.

Best regards,

George Tzotzos (Ph.D.)
ICGEB Science Coordinator

Plant Biotechnology: Tissue Culture and Beyond

National Research Centre
Cairo, Egypt
October 9 - 21, 1993

CAMBIA Course Evaluation

This evaluation is a confidential document that will help CAMBIA in planning and executing future courses. Please provide as much detail and as honest an assessment as you can. We are particularly interested in areas where improvements need to be made. The topics listed are only suggestions - all areas of the course need to be critically assessed. Feel free to use space on the back of the form, or additional sheets of paper. If you wish, your evaluation can be anonymous - but please included your name on the form so that we can ensure only participants provide the information. Thank you for your assistance. Please return this form directly to the Course Director for CAMBIA or one of the International Staff.

Name

Course Content (Syllabus)

Lectures -

Practicals

Instructors

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II

Cairo - October 1993

(name).....

Course Organization The organization of the course had to be constantly changed ~~[illegible]~~ due to the lack of facilities and supplies.

Laboratory Infrastructure [Very poor] ~~[illegible]~~ ~~[illegible]~~

As to the "tissue culture facility" it was in the form of a very small room with a laminar flow hood (?) [illegible]. This set up was by no means fit for a course. I personally feel that the choice of the location was ill planned especially when I know that there are much better [illegible] facilities in Egypt.

Accomodations

The hotel was dirty, bathroom [illegible] was clogged, the door knob was not functional. The atmosphere made me feel insecure such that I felt the need, and I did put a table before my door at nights. In addition I feel very unhappy ~~[illegible]~~ that I purchased noticed that food and water [illegible] partially consumed by the staff and stored in my room ~~[illegible]~~ in my absence. etc →

Financial Arrangements

When comparing the [illegible] with much better hotels [illegible] ~~[illegible]~~ here again I came to an unpleasant conclusion that it was highly overrated.

General Comments and Suggestions

If it wasn't for the compassion and the dedication of the international instructors I would have left ~~[illegible]~~ 2 days after my arrival.

absence of a [illegible] prohibited any significant interaction in informal settings which in my opinion are very important. As a result [illegible] I personally felt deprived from the opportunity to set up possible collaborative research projects with fellow Arabs.

Plant Biotechnology: Tissue Culture and Beyond

National Research Centre
Cairo, Egypt
October 9 - 21, 1993

CAMBIA Course Evaluation

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Name

Course Content (Syllabus) Mostly dealt with molecular biology although the title was "Plant Tissue Culture Techniques". But after all, the topics were chosen very carefully to give us the hints of all magor techniques of molecular biology, that we did not know before.

Lectures Very effective; especially considering the poor facilities you had. You would have asked for more contribution from the audience to make the lectures more dialogous. (To prevent from being passive listener?).

Practicals Very good but too many to digest (understand) in a short time. It was easy to mix them up. Not enough numbers of experts to help and show everybody sufficiently.

Instructors Brilliant! Very patient! Spoken clearly but many people had some difficulty in understanding Wagih's speach.

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CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization BAD! Especially local part of it. Two unrelated titles for the same course; course venue, and practicals venue were simply intolerable.

Laboratory Infrastructure INSUFFICIENT at all! Small, dirty, unorganised, ~~as~~ unhygienic etc.

Accomodations Not too bad, but very expensive; having the same breakfast every single morning was a nightmare.

Financial Arrangements UNJUSTIFIABLE VARIATIONS among participants. Everybody had the same replies (letters) from the ESCWA on behalf of the sponsoring agencies, but some had to pay participation fee while some did not. I personally lost $200.— out of my pocket!

General Comments and Suggestions

~~[illegible]~~ A ~~shorter~~ lighter schedule over a longer period could have been more effective and fruitful. Also excursions, field trips etc. ~~[illegible]~~ might have been ~~included~~ useful.

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Most of the participants' language ability [illegible] to follow the course efficiently/

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Name.

Course Content (Syllabus) The content of the course was good. The number of the lectures was enough to have a good idea about all the modern technologies which are now being used in all labs around the world. The lecturers understand the topics in basic science, Plant breeding, Genetic Engineering, Molecular biology etc. I think it was a good idea not to stick just on the lectures we had gave us ideas about all modern technologies. In my opinion that was an excellent syllabus for a two weeks course.

Lectures The preparation of lectures was an excellent. The lectures were prepared in way to give the whole idea about problems, benefits, and the limitations we can face during the handling of a technology.

Practicals The practicals were arranged in such a way to provide an opportunity to each participant to learn most modern technologies during two weeks course. I think after doing practicals for two weeks everyone now have some idea about each technology even I have in my work.

Instructors.. All the instructors have done an excellent job during the whole period of the course. Every one has tried to improve the knowledge of everyone as far as they were friendly and helped each others during practicals, lectures, and in all other problems. Every type of instructors are very thank for himself, in such type of course. My best wishes to all of them.

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(name)......

Course Organization :- Course organization was very poor as that institute [illegible] concerned. But the invited speakers have done wonderful job to bring these two weeks very happy, [illegible] in every [illegible]. In my opinion the institute [illegible] organized this course just to generate money for their institute rather than they have no experience how to organize a course, because no one was in touch with participants to solve their problems.

Laboratory Infrastructure :- I am very sorry to say that the institute has no laboratory infrastructure to organize such type of a course. The lab [illegible] very dirty. The most of the instruments were brought by the invited speakers with them. I could understand [illegible] organization of this course. However the institute was supposed to [illegible] organize [illegible] course [illegible]. No audio-visual aids for lectures.

Accomodations :- That was burning issue among the participants [illegible]

Financial Arrangements

General Comments and Suggestions

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Name ________

Course Content (Syllabus)

As far as I know, the name of this course was "Plant tissue culture and Its Application to Agriculture and Industry" but I have found here, that the name is "Plant Biotechnology: Tissue culture & Beyond", this course included mostly from Molecular Biology. But after all, I have seen the major techniques of molecular biology.

Lectures

The lectures were very effective, they included the last (new) developments and all details. Although my first foreign language is German, and I can't understand English very well; the instructors held the lectures very clearly and I have learned more things than I expected.

Practicals

They were the best, under the limited conditions. But it was easy to mix them up. At the same time we mixed three treatments sometimes.

Instructors

They are very brilliant. A[illegible] them; they made personally efforts to run a successful course.

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CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization

* The building and the material has no facilities to do a course like this
* Course program was very [illegible]
* We didn't see the [illegible] and their [illegible]. We have no any idea about the [illegible] of Egypt for the [illegible] laboratories and institutes [illegible]. No excursion, no field [illegible]

Laboratory Infrastructure

Insufficient [illegible] and equipment

Accomodations Not the best

But very expensive. During the weeks we had some breakfast and some [illegible].

Financial Arrangements

[illegible]) I have no problem regarding finance
([illegible] [illegible] [illegible])

General Comments and Suggestions

A better place should be chosen for the [illegible]

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Name

Course Content (Syllabus)

The lectures generally covered molecular biology very well, but I've came to Egypt to learn more about tissues cultures techniques, the two subject are important for me but tissues cultures is more relevant.

Lectures The Lectures was well done, and there was a big effort to make the lectures up to that high ~~to~~ scientific level.

Practicals I've learned many ~~the~~ new techniques, and few techniques also I was expecting to se more and learn more

Instructors All the instructors are good and qualified and specially patient and I want to "Soulever le chapeau" for Sujata because ~~et~~ she allways was able to manage and keep smilling all the time.

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization

the organization was not satisfying ; we was not the treated right. I've received such a program before I came and I was very exited to come, but when I came it was totaly different.

Laboratory Infrastructure This is the catastrophic situation, we was waiting all the time, we wait to get tools and supplies from other candidats. I'm interested in seeing all of the equipment of tissuss cultuns liaberatory because I want to equip my one lab, but I've haive not see many.

Accomodations I was not satisfied im this hotel and I Think that we ~~paied~~ payed a lot of money for nothink. Dust every where, the food not good, the air conditioner dont work, I ~~can~~ can't sleep from heat,

Financial Arrangements

(The rate for the hotel is high)

General Comments and Suggestions

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Name

Course Content (Syllabus)

The lectures covered many points in molecular genetics and some in tissue culture, so did the practicals. I thought that the course will be mainly in tissue culture with some knowledge in genetics. I think for a person who want to gain knowledge in molecular genetics the syllabus could satisfy him.

Lectures

The lectures is designed for a person who had a pre-information in tissue culture and not for that ~~or~~ who want to know the basics in tissue culture - for me I have no problem with genetics, but my knowledge in tissue culture is not enough to proceed all things.
Lectureres speak slow enough so we can follow and they are very patient.

Practicals

I think the time for practical's is not enough to begin some experiment from the proper point. We did not prepare a medium and I think we should do, to gain experience if we had'nt and to improve our skills if we had.

Instructors

I think they are existent enough to find whenever we need - And they are helpfull and patient.

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(name)...

Course Organization

efficient participant come all in time, but the day is very filled so some times we are very tired and cannot proceed efficiently. The time for entering the class is not fixed so some participant who came late cause interuption.

Laboratory Infrastructure

Lab infrastructure is not suitable for such work, the area is very small compared to the number of participant. We are working in one small unit of tissue culture so alot of time was lost in staying to begin the work. also pipettes is not enough. The lecture room also is small so we cannot sit comfortably.

Accomodations

The hotel is relatively comfortable and near the NRC but there is no need for such expensive hotel. the same food every days and I think the quality is not coincided with money paied. There is no regular transportation to and from the hotel and the NRC.

Financial Arrangements

This is the most bad thing during the course we are forced to stay in Raga hotel and pay 45 $ per day and this is very costly for us. we recieve 60 $ per day from the sponsour and we are not coming in a tour trip to be resident in such hotel. also we pay for dinner out of this fees. so it is very expensive hotel for us. and the organising commitee did not allow us to arrange our settlement by our self, and think we can do that.

General Comments and Suggestions

1. To recieve the syllabus of the course before coming.
2. The length of the course is very short compared to the syllabus.
3. The organising committee should be away from our financial support and every one must be free to in his residence wherever he attend the course.

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Name

Course Content (Syllabus)

The title of the course is tissue culture application in industry and Agriculture, but most of the syllabus was molecular biology, and I need more informations, more lectures about micropropagation

Lectures

Lectures ~~[illegible]~~ were very useful, but I didn't understand too much about molecular biology because I didn't have ~~[illegible]~~ good back ground about it, and also the lecture room [illegible] [illegible].

Practicals

[illegible] culture, somatic embryogenesis, Bacterial transformation [illegible] agrobacterium, Gus & Transgenics and gene gun were very useful for us, and we ~~[illegible]~~ were pleased to know [illegible] PCR, RAPD [illegible] [illegible] but in fact we didn't [illegible] [illegible] [illegible] [illegible] [illegible] else very [illegible].

Instructors

Instructors are very helpful, and we [illegible] [illegible] them and ask them for [illegible] [illegible] [illegible].

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(name).

Course Organization

The course wasn't well organized, the lecture room was original room in the hotel or in NRC, also the course didn't include any tour programme, so we had to go by our selves and every one was a lot of money when he knows that we are Egyptian.

Laboratory Infrastructure

The tissue culture lab was very small, and also Molecular biology was very small for about 25 participants, and I want to ~~make~~ tell you that they painted the walls when they knew that we are coming, so the labs are ~~are~~ not equipped very well.

Accomodations

The Hotel was not good. The air conditioner in the room is not good and sometimes I found people in my room working to work they clean the room and also every day for 10 days we had the same breakfast

Financial Arrangements

Bad financial Arrangments, I faced problem with Registration fee but it was solved, and also the Hotel is not good enough, they took also for our transportation and we should go to them more than 3 times

General Comments and Suggestions

I hope if we can make tissue culture course in any country rather than Egypt.

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Name

Course Content (Syllabus)

The course content was very educative and met the needs of all in the tissue culturist who at present handle tissue culture project tobe artistic manner.
The Molecular concept met the modern scenario and high tech concept.

Lectures

Excellent. Presentation excellent. But ~~I~~ how I wish Roberts, Wayne and Ry could have divide us into 3 groups and be the facilitators or mentors for 2/3 nights tuition in the 1st week to update several genetic concepts. Then we have a bench mark examination to level the group off. Then we go all out for the RAPDS, RFLP's, PCR and GUS. Then I am sure everyone will go back and open up Molecular Laboratories in all the countries.

Practicals

You know when I go back I will ask my Director to send me to a short course at the local university to explore the presence of all the enzymes and probes locally → [look at the back page.]

As for Practicals - we are so many of us. There should be demonstrators for several groups. Say group I and group II will always be under Roberts, 9.3 - 4, under Rye, 95 and 6-6 under Wayne. Arabs who do not understand English will be under DR Mohammed Ali & Arabic translation SUJATA will help in personal demonstration. Then for another practical we change instructor.

Instructors

Instructors Ry, Roberts, Wayne Sujata excellent and also Steve Hughes

BO

CAMBIA

lectures. During the lectures there are so many people coming in and out and it is very disturbing. Another complain is the keeping of time is very poor. We should be more strict so that any late comers should stay out.

CAMBIA COURSE EVALUATION FORM Part II
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(name)

Course Organization The course organisation is very sloppy because of the distances between the laboratories and the lecture hall. We are practically running about the place. The course do not consider prayer time at all which upset me very much. Sometime when I go down very late for prayer the mosque downstairs was closed. I know it is up to us to leave anytime but it hurts when something going on is out of my scope.

Laboratory Infrastructure is not very spacious to cater for the many students. The molecular laboratory does good facilities like pippette, centrifuge but I believe these are from CAMBIA.

Accomodations Raya Hotel is rather a distance to the laboratory. This course ~~did~~ be should be carried out in the an area with accomodation nearby. Thus running and moving about cause a lot of time wasted. Finally at the end of the course we have little time together with the international experts. The food is the same for many days. Breakfast is boring.

Financial Arrangements Excellent.
The Sponsor paid a substantial amount of money which is very ~~eg~~ much better than other courses that I have gone to.

General Comments and Suggestions This course should be carried out again for other students but let it be better organised. Like as a post mortem I hope the Course Lecturers will visit us at our research stations maybe in a year's time so that if we are not applying what we are learning now, then your presence will help solve some problems like a misunderstanding Director or dictatorial director who find it hard to understand molecular Biology. The presence of any of the International Speakers would make our Dictators see things more clear.

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Name

Course Content (Syllabus)

The program was fine and continuous linkage was existing between all parts. However, the only comment I do have is related to a lack of concordance between the two curricula (which included on a promised a great part of tissue culture techniques) while in reality it was more molecular biology (which is fine for me) May be for other trainees it was confusing)

Lectures

Excellent lectures were given by the international faculty. However the lecture room (either in [illegible] or in NRC) was not adequate & poorly equipped for such meetings.

Practicals

Again, [illegible] have to refer to international Faculty: they did their best with the existing equipment; [illegible] their willingness & hard work [illegible] were [illegible] to student.

Instructors

I take this opportunity to congratulate all [illegible] They were terrific, [illegible] They were always [illegible] efficient [illegible] available for questions & [illegible] discussions

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(name)..

Course Organization

The [illegible] organization was lacking a lot of coordination. All trainers were everyday surprised with a matter [illegible] which disturb the [illegible] of the course. Hope this [illegible] again!

Laboratory Infrastructure

I think it is too late to discuss such matter because the CAMBIA company should had made a decision prior to the course [illegible] the available [illegible].

Accomodations

Here again, we suffered (financially) from the [illegible] arrangement made prior to our arrival. A 45 US$ [illegible] daily [illegible] for a hotel that [illegible] Clean [illegible]

Financial Arrangements

CAMBIA should get into contact with the [illegible] in order to make for the [illegible] trainees [illegible] 19 days [illegible] for 12 [illegible] furthermore [illegible] trainees were [illegible]. I think that is enough.

General Comments and Suggestions

Beside all the above mentioned problems [illegible] Thanks of course to the [illegible] the course was [illegible]. Thanks a million.

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Name

Course Content (Syllabus)

Lectures were more about and very high rate molecular-biology. Although to learn for me lastly news, techniques and applications from specialists is very useful and is a beginning for future.

Lectures

Good

Practicals

Good

Instructors

A good theam with a perfect leader (Specialists, friendly ...)

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II
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(name).....

Course Organization

Laboratories and facilities were not sufficient

Laboratory Infrastructure

Accomodations

Good

Financial Arrangements

Good.

General Comments and Suggestions

The course could have been held at a university. The social activities could have been done.

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Name

Course Content (Syllabus) comprehensive for both bio technology aspect in wide case and narrow case acording to tissue culture.

Lectures it was very rich and important and advanced given in excellent mood

Practicals all the effort used for succeeding the course regardless the was very much crowded.

Instructors whithout doubt all the instructors are talented and help every one without hesitation and without Late.

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(name).....

Course Organization

I think the organizers forgets the freedom of the Trainees and they left any option for us

Laboratory Infrastructure

Accomodations

not too much good, not too much bad, very week servise, and bad treatment

Financial Arrangements

Some of organizers put obstacles in the way of Som Trainees

General Comments and Suggestions

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Name

Course Content (Syllabus)

Lectures

Practicals

Instructors

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(name).....

Course Organization

Laboratory Infrastructure

Accomodations

Financial Arrangements

General Comments and Suggestions

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Name

Course Content (Syllabus)

Lectures

Practicals

Instructors

CAMBIA

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(name).....

Course Organization

Laboratory Infrastructure

Accomodations

Financial Arrangements

General Comments and Suggestions

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Name

Course Content (Syllabus)

The course content was concentrated on the few topics related with Agrobacterium, gene transformation and genetic manipulation and Lack of tissue culture Tech. Which all the participant were expected to be. The course was condence and ~~too~~ to many information provided within short time.

Lectures

The Lectures were very good - interesting the lecturers were able to send the message clearly and smothly. and the materials itself were very usefule to all the participant whom they work in tissue culture type of work.

Practicals

to many participant was ~~the~~ one of the limitation of practicals part of this course which did not allow to do so much work. besides the LAB was not enable to fit with the all participant

Instructors

good is not enough word to describe the instructors whom they were friendly before every thing. they want to give us much knowledge as they can.

CAMBIA

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(name).....

Course Organization

1) Scientific part was well organized in term of ~~lect~~ lectures, and instruct

2) The other part of course dealing with Finishing touches and preparatio
to make the course run smoothly was not in the same
rank as the first part which make participant fell uncomfortable

Laboratory Infrastructure poor ~~good~~

Accomodations not good - They didn't give us many choices
in choosing what ever we like such as the way we want to live
the kind of food we like meet the suitable hotel we would
like and soon

Financial Arrangements

Financial arrangements makes me fell bad towards the
course because I came without any money because I was expected
to be paid ~~as soon~~ as per arrival, so I end up with more than
11 days without any money and after I ~~sent~~ Fax to the sponser person
they gave me payment for just 10 days while I stayed for
(15) days - and the most important part was No body
care care!

General Comments and Suggestions

Generally when ever we ~~are~~ plan to organiz such ~~a~~ training course
we should have one leader to make decision ~~to~~ direct the course
and should have every thing prepared before the starting day
and should have an expert people helping ~~in~~ making the course
well organized at least they should have experience dealing with the people.

CAMBIA

Plant Biotechnology: Tissue Culture and Beyond

National Research Centre
Cairo, Egypt
October 9 - 21, 1993

CAMBIA Course Evaluation

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Name

Course Content (Syllabus) Excellent

Lectures It's more interesting, but one lecture in "Secondary Metabolite Production in Cell Culture" it's not more enough.

Practicals It's very good, and I learnt advanced techniques in Genetic engineering and biotechnology, what CAMBIA don't believe I learnt it.

Instructors Excellent

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).

Course Organization It's very good, but 15 days's it's not more enough for this combined program.

Laboratory Infrastructure very good

Accomodations

Financial Arrangements

General Comments and Suggestions The course "Plant Biotechnology: Tissue Culture and Beyond. It's more interesting for me and I learnt many advanced techniques. but the hours for practical part it's not more enough.
The suggestions: 1. Another courses in this field.
2. More and more contact and future between CAMBIA and my labs Plant Cell & Tissue culture. National Research Centre

finally: Alot of thanks to

CAMBIA

Plant Biotechnology: Tissue Culture and Beyond

National Research Centre
Cairo, Egypt
October 9 - 21, 1993

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Name

Course Content (Syllabus)

Concentrated on Genetic
aspects in Tissue culture

Lectures

Good

Practicals

Good

Instructors

Good

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization

Good

Laboratory Infrastructure

Accomodations

Financial Arrangements

Not good arrangement

General Comments and Suggestions

I Suggest the future courses ~~the~~ should include more about tissue culture

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Plant Biotechnology: Tissue Culture and Beyond

National Research Centre
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Name

Course Content (Syllabus)

very good, but you can increase the time of practical in the syllabus to make an extensive background for the participants about the techniques which used.

Lectures

The time of lectures is good to discuss and suggested any problem which we have.

Practicals

Very very good, but the time not enough. I suggested that the practical time must be change. Time in the course time.

Instructors

excellent - they help the participants for understanding all used of these in course.

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).

Course Organization 9 thanks -Him for this course, we need more and more training course to get a good experience and to exchange information and maintain continuing dialogue on the development in the field for mutual benefit and use.

Laboratory Infrastructure

The laboratory was poor to help in that course. the laboratory must be improved and must have a modern machines which made the work of research is easier.

Accomodations

Financial Arrangements

General Comments and Suggestions

(1) the time of practicales must be increas
(2). the laboratory which used must be exchange and improved to help in the work
(3). we need more of these courses to improve CAMBIA and carrier.

Plant Biotechnology: Tissue Culture and Beyond

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Name

Course Content (Syllabus)

It is interesting and I learnt
Many advanced. new teqtch

Lectures

It is very good
but I wish to be more
in tissue culture

Practicals

It is good but it
should be more in tissue
culture

Instructors

very good espizally Dr
Richard and Dr Mohamned

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization

It is very good

Laboratory Infrastructure

It is very good
but Laboratory it is more
narrow

Accomodations

It is very good

Financial Arrangements

It should be arranged
better than this

General Comments and Suggestions

I wish to be more
lecture more in plant
tissue culture
It is very good time to know
many of diffent Persons, same field
from diffent countrys in the

Plant Biotechnology: Tissue Culture and Beyond

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Name

Course Content (Syllabus)

Sufficient.

Lectures

Well balanced and correlated.

Practicals

Didn't strictly followed the prescribed course.

Instructors

Competent, language of some was not clear.

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CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name)....

Course Organization

Above average

Laboratory Infrastructure

Not really outstanding.

Accomodations

Not applied [observer].

Financial Arrangements

Not applied [Observer].

General Comments and Suggestions

Generally it was intresting specially assays related to both plant and animal.
Would have prefered a programme devoted to animal biotechnology.

Plant Biotechnology: Tissue Culture and Beyond

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Cairo, Egypt
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Name _____

Course Content (Syllabus)

Contents are good, but I feel that it should be included
1. Lectures about the dangers of unrestricted gene cloning.
2. More applications of PCR
3. Use of pulsed field gel electrophoresis in molecular biol.

Lectures

Practicals

Instructors

CAMBIA

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name)

Course Organization

Laboratory Infrastructure

Accomodations

Financial Arrangements

General Comments and Suggestions

- If the [illegible] and program arranged for us [illegible] months or [illegible] weeks at least, we will [illegible] from this course very much.

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Name

Course Content (Syllabus)

good

Lectures

good

Practicals

very good

Instructors

very good, very helpful and efficient

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CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization

well organized, but still it could be better.

Laboratory Infrastructure

good

Accomodations

I had no accomodations

Financial Arrangements

I had no financial arrangements
But still as an official observer from NRC, I believe, we had more rights and we should have treated better by the local organizer.

General Comments and Suggestions

Generally the course was very beneficial from the practical side. But I wanted to hear more about how to solve problems e.g. to raise a problem and thoroughly solving it through the practical side of the course, not [illegible] although

something else I would like to say. Some
very upsetting trouble some of the international
participants or staff have met. I believe much
of these things could be avoided by more communication
& communication between the international &
local staff members. I was sad each of them
to go back home with this impression of
Egypt, which is a real pity. But unfortunately
we have a beautiful country & some of things
should have done better from the tourists
point of view. So when arranging such a
course the scientific arrangements should go hand
in hand with the accommodations & touristic
arrangements.

For a future course I would like more to
learn & learn more about molecular
Biology and have more practical applications
in this field.

Plant Biotechnology: Tissue Culture and Beyond

National Research Centre
Cairo, Egypt
October 9 - 21, 1993

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Name

Course Content (Syllabus) The main subject of the course is Biotechnology which includes up to date methodology.

Lectures The lectures started at 9 A.M. and ended at 1 P.M. daily. The lectures lasted two hours. They treated in detail the theoretical and practical background of Biotechnology. The lectures were fruitful up to date and to the point.

Practicals

Instructors Dr. Professor Richard Jefferson was more than excellent theoretically and practically. He did his best to know the problems we face and to give us valuable

CAMBIA

suggestions and solutions without imposing himself on our minds. Moreover, he enjoys modesty and decency. He was generous enough to invite us to see the light and sound program at his own expense.

2. Dr. Professor Engle was full of vitality and activity. He took his constructive suggestions during the course. He has his own role in fair-appraisal and the discussions at the round table.

3. Dr. Professor Seigle was dynamic and she was the part and parcel of the fair-appraisal.

CAMBIA COURSE EVALUATION FORM Part II
Cairo - October 1993

(name).....

Course Organization

The course was disciplined and well-organized. It was based on sound and firm ground. It covered all the points concerned.

Laboratory Infrastructure

The apparatus were adequate and up-to-date.

Accomodations

The accomodation was expensive and beyond my financial ability.

Financial Arrangements

I wasn't supported financially by any body. The whole financial arrangement was at my own expense.

General Comments and Suggestions

I hope to attend any coming training course held by Cambia, otherwise I would like to ask to reduce my spent on that course

CAMBIA

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Contracts Section
P.O. Box 300, A-1400 VIENNA, AUSTRIA
Telex: 135612 Telegraphic: UNIDO VIENNA
Facsimile: (1) 2308272

FACSIMILE TRANSMISSION

PLEASE DELIVER IMMEDIATELY TO ADDRESSEE

IN CASE OF INCOMPLETE TRANSMISSION, PLEASE CALL THE CONTACT PERSON BELOW BY PHONE OR BY FAX CARON/GSI

Drafted by/Contact person: [illegible]	Authorized by: Mr.M.Kohonen, Chief
Our reference: GE/GLO/90/004	Telephone/Extension: 4836 21131/
Your reference:	Date: 20 September 1993
This page No. 1 of 4 pages	TRANSMISSION No. 7938

To:
CAMBIA
G.P.O.Box 3200
Corner of Clunies Ross St. and
Barry Drive, Black Mountain
Canberra ACT 2601
AUSTRALIA
att.: Dr.Richard Jefferson
Dr. Ry Meeks Wagner

Facsimile No.: 00616 246 5303

Subject: Project GE/GLO/90/004 - Five-Year Work Programme of ICGEB
Practical Course on "Tissue Culture and Beyond"
9 - 21 October 1993, Cairo, EGYPT

Dear Sirs,

We are pleased to advise that Cambia, Australia, has been selected by UNIDO for execution of services for the above-mentioned project at a total all-inclusive cost of United States Dollars twenty-four thousand (US$ 24,000) payable in this currency. The present award is based on the Terms and Conditions of the Substantive Terms of Reference dated 17 September 1993 (copy attached for easy reference).

Payment under this Contract will be made according to the following payment schedule and subject to receipt of your invoices on the letter-head of your institute according to the attached sample invoice:

		US$
aaa)	Upon acceptance of present award, the sum of	19,200
bbb)	Upon UNIDO's receipt and acceptance of the Contractor's Final Report to be submitted no later than 30 November 1993, the sum of	4,800
	TOTAL US$	24,000

Subject to our prompt receipt of your telefax confirmation of acceptance of the present award and pending finalization of the formal Contract the present communication constitutes full authority for Cambia, Australia to carry out work and incur costs up to the above total amount of United States Dollars twenty-four thousand (US$ 24,000).

In all future correspondence relating to this project please quote UNIDO Contract No. 93/200/IR.

Kindly let us have your confirmation together with your first invoice at your earliest convenience.

Yours sincerely,

M. Kohonen
Chief
Contracts Section
General Services Division
Department of Administration

cc: Mr. Tzotzos

C.93/200
Annex A
17 September 1993

Terms of Reference

INTERNATIONAL CENTRE FOR GENETIC ENGINEERING AND BIOTECHNOLOGY

Practical Course "Tissue Culture and Beyond"

9-21 October 1993, Cairo, Egypt

AIM OF THE PRACTICAL COURSE

The aims of this practical course, which is organized as part of ICGEB's programme of activities for 1993, are given on pages 3-5 of the Terms of Reference.

BUDGET AND SCOPE OF THE CONTRACTING SERVICES

In accordance with the submitted proposal for the above course, which has been approved by the Director of ICGEB, the Contractor should provide the following services:

		US$
1.	Ad-hoc Travel and DSA of international participants	24,000.
	TOTAL	24,000

GENERAL TIME SCHEDULE

- Upon conclusion of the course, the Contractor should submit a report, including:

  a - the course programme and abstracts;
  b - evaluation of the course activities;
  c - financial statement.

- The report along with the contractor's invoice shall be submitted to the Contracts Section, UNIDO, Vienna International Centre, P.O. Box 300, A-1400 Vienna/Austria (Tel.: 211 31 Ext.: 4838, Fax: 230 82 72)

- Upon receipt by UNIDO, the report will be evaluated by the substantive UNIDO/ICGEB office(s).

LANGUAGE REQUIREMENTS

Lecturers should be proficient in English, which will be the working language used for the project. The report will be submitted in English.

PAYMENT TERMS

Eighty percent (80%) of the project funds will be transferred to the Contractor upon signature of the contract, the remainder upon submission of the requisite report, including a financial statement, countersigned by the chairman and the secretaries of the organizing committee. In both cases, the contractor shall submitt to the Contracts Section, UNIDO an invoice for payment.

EXPERTS/CONSULTANTS

No individual experts are to be recruited by UNIDO. The course organizer and lecturers are expected to have proven research experience in the scientific area(s) of the course.

COURSE ORGANIZERS

Dr. Richard Jefferson
Dr. Ry Meeks Wagner

CONTRACTOR

CAMBIA
GPO Box 3200
Canberra ACT 2601
Australia

Telephone: (0061-6) 246 5303
Facsimile: (0061-6) 246 5303

Letterhead of your Institution

INVOICE

Chief
Contracts Section
General Services Division
Department of Administration
UNIDO
P. O. Box 300
A-1400 Vienna
Austria

Date: .....

UNIDO/ICGEB Project: GE/GLO/90/004

UNIDO Contract No.: .....

Purpose of Payment: * as appropriate

US$ .....

(Upon acceptance of Award)
~~(Upon signature of Contract:)~~
* ~~(Upon acceptance of Annual Progress Report:)~~
~~(Upon acceptance of Terminal Evaluation Report:)~~
(Upon acceptance of Final Report)

Kindly make payment to: (indicate your Institution's bank account number or any other method of payment such as check payable to your Institution).

(Bank)

(Address)

Account No.:

Owner of the bank account is:..Institute .....

Please indicate authorized person(s) to administer funds under the above account:

.....

(signature(s) of responsible official(s)

Manual prepared for the CAMBIA
UNIDO/ICGEB/Course

Plant Biotechnology:
Tissue Culture And Beyond

Cairo, Egypt October 9 - 21, 1993

Part I

Hosted by the

Academy of Scientific Research and Technology
Arab Republic of Egypt

CAMBIA

CENTER FOR THE APPLICATION OF MOLECULAR BIOLOGY TO
INTERNATIONAL AGRICULTURE

GPO Box 3200
CANBERRA ACT 2601
AUSTRALIA

TEL: + 61 6 246 5302
FAX: +61 6 246 5303
Internet Email: cambia@cambia.org.au

REFERENCES FOR TISSUE CULTURE

Parrot, W. A. Class notes. Advanced plant genetics. Gene transfer in plants.

Parrot, W. A., S. A. Merkle, and E. G. Williams. 1991. Somatic embryogenesis: Potential for use in propagation and gene transfer systems, pp. 158-200. *In* D. R. Murray (ed.), Advanced methods in plant breeding and biotechnology, CAB International.

Merkle, S. A., W. A. Parrot, and E. G. Williams. 1990. Applications of somatic embryogenesis and embryo cloning, pp. 67-101. *In* S. S. Bhojwani (ed.), Development in crop science 19, Plant tissue culture: Applications and limitations, Elsevier, Amsterdam.

Parrot, W. A., R. E. Durham, and M. A. Bailey. Somatic embryogenesis in legumes.

Advanced Plant Genetics
AGY/BOT 890
Section III
Gene Transfer in Plants

I. Barriers to interspecific gene transfer
  A. Pre-fertilization barrriers
    1. Physical
    2. Unilateral
    3. Genetic
    4. Gametocidal chromosomes
  B. Post-fertilization barriers
    1. Pre germination barriers
      a. Affecting the embryo
        (1) Hybrid inviability
        (2) Lethal genes
        (3) Differential zygote lethality
        (4) Chromosome elimination
          (a) Effect of nuclear architecture
      b. Affecting the endosperm: Endosperm Balance Number
        (1) Triploid block
        (2) Imprinting
        (3) Haploid production
    2. Post germination barriers
      a. Hybrid inviability or weakness
        (1) Genome incompatibility
        (2) Plastid/genome incompatibility
      b. Failure of Flowering
      c. Hybrid sterility
        (1) Chromosomal
        (2) Genic
      d. Inviability and weakness of the F_2
      e. Lack of gene expression
      f. Genes that prevent homoeologous pairing
II. Overcoming barriers to gene transfer
  A. *In planta* pollination
  B. *In vitro* hybridization techniques
    1. *In vitro* pollination
    2. Ovule/ovary culture
    3. Embryo rescue
    4. Regeneration
    5. IAA treatments
    6. Protoplast fusion
      a. Phylogenetic considerations
      b. Uses
        (1) Recovery of hybrids

(a) Complementation
(b) Resistance
(c) Metabolic inhibitors
(d) Mechanical
(2) Cybridization
(3) Cytoplasm exchange
(4) Assymetric fusion
(5) Electroporation
(6) By-passing seedling lethality
c. Limitations

C. Single-gene transfer
1. Methods
a. *Agrobacterium*-mediated
(1) Ti-plasmid
(a) Components
(b) Transfer into plants
(2) Vector construction
(a) Components
(3) Genes amenable to transfer
b.Direct DNA uptake
c. Electroporation
d. Microinjection
e. DNA injection into reproductive organs
f. Pollen-mediated
g. Silicon carbide fibers
h. Microprojectile bombardment
2. Antisense RNA
3. Problems with expression of transgenes
a. Cosuppression
4. Somaclonal variation
a. Types
b. Causes
c. Uses

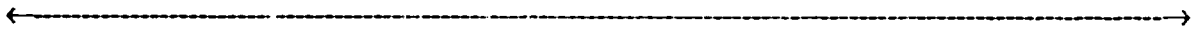
D. Lawyers, guns, and money.
1. Effects of intellectual property rights
a. Impact on germplasm exchange
b. Technology access contracts
2. Effects of regulations
a. Government agencies
b. Public perception
(1) Special interest groups
(2) Lack of knowledge
(3) The role of the press

A. Barriers to interspecific gene transfer

Concept of a biological species, formulated in 1940 by Ernst Mayr:

> *"Groups of actually or potentially interbreeding populations that are reproductively isolated from other such groups."*

- Inherent in the definition of species is fact that barriers to gene transfer exist between species.
- Nevertheless, breeders often desire traits that do not exist in the species being bred, hence the desire to cross different species together.
- The less related two species are, the tougher they are to cross:



Compatibility — Incompatibility — Incongruity

1) Prefertilization barriers

a) Physical

- time of flowering
- floral
  - style length
  - style osmotic pressure
- ecological
- reproductive modes
  - cleistogamy
  - apomixis

b) Unilateral incompatibility

- Found in crosses between self compatible and self incompatible species.

♀	×	♂	
SI		SC	→ fails
SC		SI	→ successful

- result of gametophytic SI alleles
- nevertheless, failure is on stigma, not in style
- unilateral effect implicates the S allele

c) Single genes

♀	× ♂	
popcorn	dent or flint	→ fails
dent or flint	popcorn	→ successful
dent × popcorn	dent	→ successful

- due to the action of a single gene on chromosome 4:

Popcorn Ga_1Ga_1	×	Dent ga_1ga_1	→ Cross fails
(Dent × popcorn) Ga_1ga_1	×	Dent ga_1ga_1	→ Crosss OK

- However, there is preferential transmission of the chromosome carrying Ga_1 over ga_1:

(Dent × Popcorn) × (Dent × Popcorn)
Ga_1ga_1 Ga_1ga_1

↓

1 Ga_1Ga_1 : 1 Ga_1ga_1

- get a 1:1 ratio instead of the expected 1:2:1 ratio.

Rashid & Peterson, 1992: Unilateral cross incompatibility in maize:

cif cif × *cim1 cim1 cim2 cim2* = incompatible

Kein, 1943; Lange & Riley, 1973: Crossing of wheat and rye is prevented by the action of Kr_1 and Kr_2:
- homoeologous loci on chromosomes 5A and 5B
- most wheat is *Kr1 Kr1*
  - 'Chinese Spring' is *kr1 kr1 kr2 kr2*
- slow down and prevent the pollen tube from reaching the wheat ovule

d) Gametocidal chromosomes (Endo, 1990) **& segregation distorters** (Marois, 1992)

- Also called pollen killer, gametocidal gene, gamete eliminator, and gamete aborter.
- Found in progeny derived from interspecific crosses between wheat and *Aegilops* species in the sections Polyeides and Sitopsis
  - E.g., in *Thinopyrum distichum* and *T. ponticum*, the *Sd_1* and *Sd_1d* alleles linked to leaf rust resistance
- During breeding, alien chromosomes get eliminated, except for one of them.

- Gametes with this extra chromosome survive
- Gametes without this chromosome abort
    - If the gametocidal gene goes to the micropylar megaspore, the chalazal megaspre cannot function
    - Some breakage is evident at the first post meiotic mitosis
    - Have also been called "cuckoo" chromosomes, so named after a bird that lays its eggs in the nests of other birds and abandons them, leaving the foster parents to raise them
- Postulate the existence of a dominant gene(s) on the chromosome that cause abortion of gametes lacking the alien chromosome:

Sporophyte	Gametophyte	Gamete
21 II (W) + 1 I (A)	21 I (W) + 1 I (A)	Functional
	21 I (W)	Abortive

- Result is partial sterility combined with preferential trasmission of alien chromosome
- Effect can be modified by several wheat genes, so magnitude of effect depends on wheat background
- Differs from zygotic lethals in that the homozygote is viable
- Whatever factor is responsible can get translocated onto wheat chromosomes, thus giving this behavior to a wheat chromosome.
- Three types of gametocidals identified: homoeologous groups 2, 3, or 4.
- The longissima 1 and sharonensis 2 (group 4) cause chromosomal deletions in the zygote, but only when transmitted through the pollen
- Similar effects described in wheat, maize, tobacco, tomato, lima beans, and rice.
- Evolutionary significance: isolating mechanism
    - ⇒ those that cause chromosomal deletions in the embryo and endosperm can lead to death
    - ⇒ any hybrids formed would suffer from 50% sterility
    - ⇒ provoke genomic rearrangements in hybrids, leading to rapid speciation

2. Post fertiliztion barriers (Post Zygotic Barriers)

a. Pregermination barriers

(1) Barriers that affect the embryo

(a) Hybrid inviability (Stebbins, 1958)

- Embryo dies due to incompatibility between the genes and chromosomes of one species with those of the other.

(b) Lethal genes (Gerstel, 1958; Halloran, 1981)
- Cause the death of developing embryos

E.g.,
Triticum durum × *Aegilops umbellulata*

L^e = lethality
L^l = lethality
l = inviability

In cowpea:

Portuguese white	L_1	Lethal combination
Light red	L_3	
Light red	L_3	Lethal combination
N.I. 31	L_4	

Crepis tectorum × *C. capillaris*:
- *ll* combination kills the embryo at the cotyledon stage.

Cotton - "red lethal"
- RL_a from *G. arboreum* and RL_b from *G. hirsutum* are lethal together.

Triticale (**Ren and Lelley, 1989**):
- B genome of wheat has Ne_1 and Ne_2
- Rye genome has Ner_1 and Ner_2
- Any two of these will cause necrosis in triticale
- Expression of necrosis genes can be suppressed by adding another genome (A,B D, or R) to the triticale

(c) Differential zygote lethality (Rick, 1963)
- also called post syngamic elimination
- preferential recovery of parental genotypes
  - "more hybrid" genotypes are less viable than "less hybrid" genotypes
- found in tomato, corn, cotton, sweet clover, and snapdragon
- RFLP data now finding this is not an unusual event

e.g., *Lycopersicon esculentum* × *L. chilense*
↓
L. esculentum × F_1

Gene	+	mutant	χ^2 vs. 1:1
Anthocyanin deficiency	242	285	3.51
Potato leaf	159	190	2.75
Dwarf	193	280	16.00
Lutescent	174	104	22.84

(d) Chromosome elimination (Subrahmayan and Kasha, 1973)

- Originally found in crosses of *Hordeum vulgare* × *H. bulbosum* (2n = 2× = 14):

Age (days)	Cells with chromosome # of:								Ave. # of cells/ embryo
	7	8	9	10	11	12	13	14	
3	3		1		2		1		37
4		3		2	2	1	2	1	75
5	10	6	4	4	1	1	1	1	199
6	26	14	5	3			1	1	370
7	68	16	10	3	1				772
8	160	11	2	2		1			1178
9	177	41	11						2306
10	218	13	7	2	1				4710
11	431	22	7						7430

- Above is result of crosses between diploids
- Results differ in interploidy crosses: (data from **Kasha, 1974**)

♀	♂	F_1
VV	BB	V
BB	VV	V
VV	BBBB	VBB
BBBB	VV	VBB
VVVV	BB	VV
VVVV	BBBB	VV
BBBB	VVVV	VV

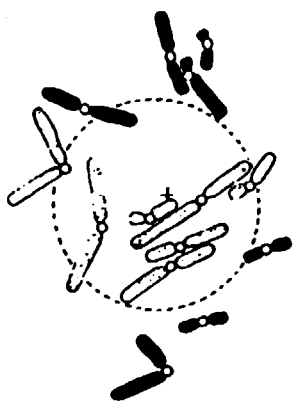
- Only 1V:2B ratios are stable.
- Studies with trisomics: (Ho and Kasha, 1988):

VV + chromosome 1 × BBBB	→	stable
VV + chromosome 2 × BBBB	→	elimination
VV + chromosome 3 × BBBB	→	elimination
VV + chromosome 4 × BBBB	→	stable
VV + chromosome 5 × BBBB	→	stable
VV + chromosome 6 × BBBB	→	stable
VV + chromosome 7 × BBBB	→	stable

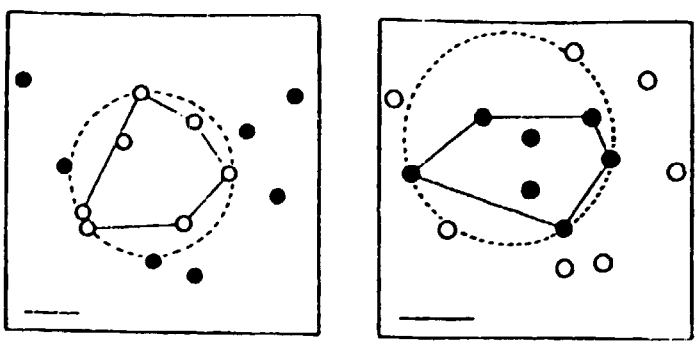
-Factors on both arms of chromosome 2 and short arm of chromosome 3 control chromosome elimination.

-Hypothetical mechanisms of chromosome elimination:

1) Kasha, 1974: Asynchrony of mitotic cycles
2) Bennett, 1984; 1987: Nuclear domains-- Chromosomes of different species occupy concentric spheres within the nucleous. Those on the outer sphere get lost:



from Bennet, 1984



Hordeum (○) & *Secale* (●) centromeres R: *H. vulgare* x *S. africanum* L: *H. chilense* x *S. africanum*

-Ciba-Geigy, which started a breeding program from scratch, used this method used to develop commercial barley varieties for Canada, including "Mingus", which was the highest yielding variety of its time.

(2) Barriers affecting the endosperm: Endosperm Balance Number

(a) Triploid block -Death of triploid embryos following 2×-4× crosses
-Results from breakdown of endosperm

-Was puzzling, as nothing is intrinsically wrong with 3× embryos. In fact, can do embryo rescue and recover vigorous plants.

History:
-Müntzing, 1930: -2:3:2 embryo:endosperm:maternal genome ratio was necessary
-Watkins, 1932: -3:2 endosperm:embryo genome ratio was necessary
-Valentine, 1954: -2:3 maternal:endosperm genome ratio was required

All these hypotheses were plagued by exeptions.

-Nishiyama and Inomata, 1965: -2♀:1♂ ratio is necessary in the endosperm. Everything else is irrelevant. Worked with *Brassica*.

Lin, (1975) 1984: Provided evidence while working with maize:

- used *ig* to get central nuclei ranging from 2× to 8×

-(The *indeterminate gametophyte* mutation is marked by loss of control of the 3 mitotic divisions that form the megagametophyte, permitting a greater number of mutations to occur.)

- crossed with 2× and 4× males

Genomic constitution and development of maize endosperm at maturity:

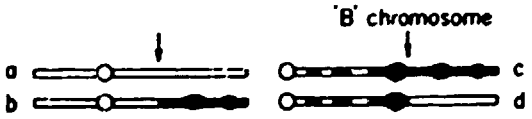
Genomic constitution of endosperm (♀:♂)	Endosperm development			Source
	Normal	Suboptimal	Abortive	
1×:1×			+	*ig/ig* × *Ig/Ig*
2×:1×	+			
3×:1×		+		
4×:1×			+	
5×:1×			+	
6×:1×			+	
7×:1×			+	
2×:2×			+	*ig/ig* × *Ig/Ig/Ig/Ig*
3×:2×			+	
4×:2×	+			
5×:2×			+	
6×:2×			+	
2×:1×	+			*Ig/Ig* × *Ig/Ig*
2×:2×			+	*Ig/Ig* × *Ig/Ig/Ig/Ig*

Notes:
1) Only 3× or multiples of 3× endosperm developed normally, and then only if 2♀:1♀
2) Now need to show that passage through ♀ or ♂ gamete alters gene expression

Background Information:

-Used translocations (breaking 10L at various points) with B chromosome
- 38 such 10^{tt} and B^{10} translocations are known
- Derived from X-ray induced mutations

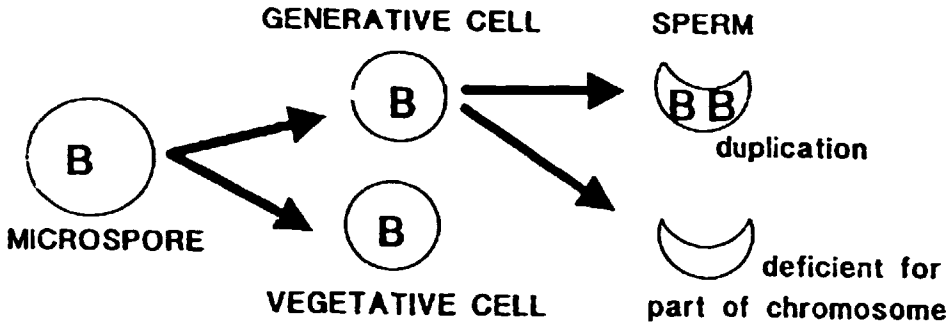
a = chromosome 10 of maize



b = a 10^{B} translocation

d = a B^{10} translocation

- Sometimes, nondisjunction of B chromosomes occurs during microgametogenesis:



-Depending on which sperm fertilizes what, will get an embryo or an endosperm either lacking part of a chromosomal arm, or having a duplication of that arm.
- Preferential recovery of B chromosomes also occurs on female side.

-Kermicle, 1975: Concept of "imprinting" in plants.

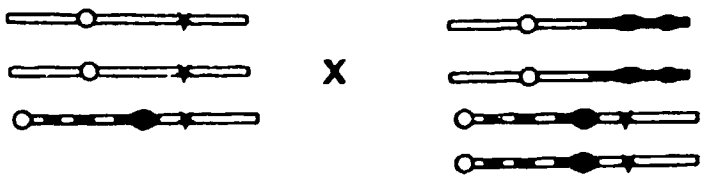
-Used *R* locus in maize:

♀	♂		
RR	*rr*	→	Solid red color
rr	*RR*	→	Weak, mottled red color

-Used to think was due to dosage effect, with *RR/r* > *rr/R*
-Using 10^{B} translocations, got double dose of *R* through the male gamete
-Found that ♀ or ♂ origin of allele determines its function
-Called "epiallele"

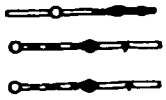
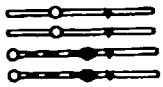
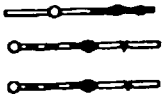
-10^{B} translocations used to show that imprinting affects normal endosperm development:

Cross: 10 10 B^{10} x 10^{B} 10^{B} B^{10} B^{10}



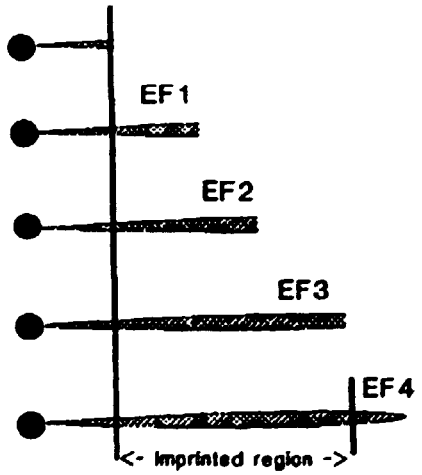
where the star represents the imprinted region.

Number of imprinted regions that come through the:

♀	♂		♀	♂
2	2	normal		
4	0	subnormal		
2	0	subnormal		
4	2	normal		

NOTE: The shaded types have the same number of imprinted regions, yet the phenotype differs.

-Found 6 10^{B} translocations that affected endosperm development, all near the centromere:



-Because these had a cumulative effect, postulate that 3 factors were involved:

- As greater amounts of 10L are broken off, there is a greater effect on endosperm development.

- Conclusion is that the indicated region is the imprinted region

- As all breaks had even a minor effect, postulate a factor, EF_4, on the end of 10L.

- At this point, the hypothesis explains the triploid block:

1) Endosperm development depends on ratio of 2♀:1♂ genomes

2) ♀ or ♂ identity of genome is determined by imprinting of genes (endosperm factors)

In summary, looking at the genomes in the endosperm:

Cross	♀	♂
2×-2×	2	1
2×-4×	2	2
4×-2×	4	1

-Shaded combinations all abort.

-This also works in some interspecific crosses, e.g.:

2× *Trifolium pratense* × 2× *T. diffusum* → 2× hybrids

but it does not work in others, e.g.:

2× *T. pratense* × 2× *T. pallidum* → cross fails due to endosperm breakdown, leading to death of the embryo. This is reminiscent of the triploid block.

- The breakdown of endosperm in interspecific crosses was called Somatoplastic sterility, by **Cooper and Brink, 1940**

- However, other interspecific combinations do not appear to follow the 2♀:1♂ genome rule:

e.g., 2× *T. pratense* × 4× *T. pallidum* → 3× hybrids

- in this case, the genomic constitution of the endosperm is 2♀:2♂.

Johnston et al., 1980

- Working with interspecific crosses of potato

- Explained apparent discrepancy by explaining that it is not the genomes that have to be in a 2♀:1♂ ratio, but the Endosperm Balance Number (EBN)

- Each species is assigned an EBN based on its crossing behaviour to a standard species, e.g.:

Take *T. pratense* as a standard:

- It is 2×, so arbitrarily assign it an EBN of 2 (= 1 EBN per haploid genome)

T. diffusum: crosses readily with it, so it too must be 2×, 2EBN.

T. pallidum: the 2× form does not cross to 2× *T. pratense*, but the 4× form does. Therefore, 4× *T. pallidum* must be 2EBN

- Successful crosses are those that match EBN values, e.g.:

2x, 2EBN *T. pratense* × 2x, 2EBN *T. diffusum*
2x, 2EBN *T. pratense* × 4x, 2EBN *T. pallidum*

1) Notice that EBN is a property of a chromosome set (x):

- If 2×, 2EBN ⇒ 1×, 1EBN
- 2×, 1EBN ⇒ 1×, ½EBN

Looking at EBN ratios:

Cross	Genetic ratio	EBN	♀ : ♂ EBN Ratio
2x *pra* × 2x *diff*	2♀ : 1♂	2♀ : 1♂	2 : 1
2x *pra* × 2x *pall*	2♀ : 1♂	2♀ : ½♂	4 : 1
2x *pra* × 4x *pall*	2♀ : 2♂	2♀ : 1♂	2 : 1

- Thus, it is the EBN, not the genomes, that must be in a 2♀:1♂ ratio.

2) Also note that EBN is additive:

Trifolium pallidum: 2x, 1EBN --> Colchicine doubling --> 4x, 2EBN

Solanum tuberosum: 4x, 4EBN --> Anther culture --> 2x, 2EBN

3) EBN has predictive value:

Given that 2x *pra* = 2EBN
2x *diff* = 2EBN
2x *pall* = 1EBN

And you want to get a hybrid between *T. pallidum* and *T. diffusum*, then

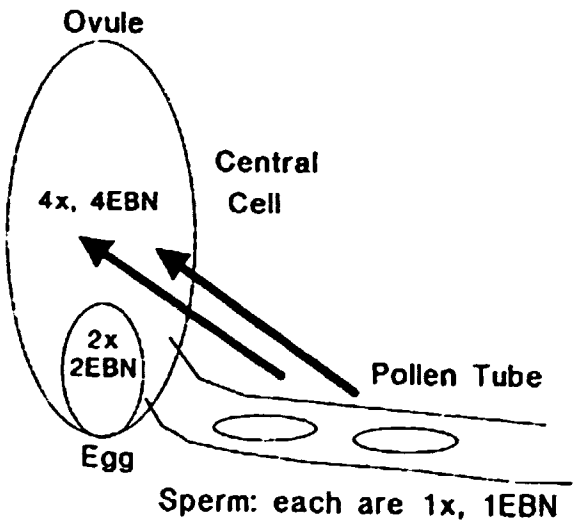
- You would immediately know that the cross between the two diploids will not work
- But the cross between 2x *T. diffusum* and 4x *T. pallidum* will.

- The world potato germplasm collection has now been assigned EBN's to all the species in its catalogue. Within the genus *Solanum*, find the following EBN's:

2x, 1EBN
2x, 2EBN
4x, 2EBN
4x, 4EBN
6x, 4EBN

- So far, the EBN concept has been used to explain both the triploid block and interspecific crossing behaviour. It also explains the origin of haploids following 4x-2x pollinations, a process called haploid extraction:

e.g., *Solanum tuberosum* ssp. *tuberosum* (4x, 4EBN) × *S. tuberosum* ssp. *phureja* (2x, 2EBN)



-The sperm fuse together prior to fertilization, and then fertilize the central cell. This produces an endosperm that is 4♀:2♂ genomic ratio and 2♀:1♂ in EBN. Thus the endosperm develops normally, and the egg develops along with it.

-The ability of the sperm to restitute is under genetic control. In potato, types have been selected that do so at a high rate, thus giving large numbers of haploids.

-This goes to show that what really matters is whether or not the endosperm gets fertilized and is able to develop. It really does not matter if the embryo is fertilized or not.

- **Nishiyama and Yabuno, 1979:** Independently described the equivalent phenomenon in oats and related species:

  - Reaction of ♂ = Activating value (AV)
  - Reaction of ♀ = Response value (RV)

  For an interspecific cross to be successful, then :

$$\frac{AV}{2RV} = 0.5$$

  - Numerically, this is identical to the 2♀:1♂ ratio of EBN.
  - Each species has its own AV assigned to it based on crosses to a standard species.
  - In this case, 2x *Avena strigosa* was arbitrarily assigned AV = 1. Other AV values were assigned relative to this.

- The EBN has also been reported in alfalfa, clover, and impatiens.

- Endosperm barriers are overcome by:

  1) matching EBN values --- may result in odd ploidies
  2) embryo rescue

- Genetic control of EBN (**Ehlenfelt & Hanneman, 1988**)

  - Suggest 3 genes are involved, based on an exceptional inter-EBN hybrid between *Solanum commersonii* (1EBN) and *S. chacoense* (2EBN)
  - Call the genes *E, B*, and *N*

POST GERMINATION BARRIERS (Stebbins, 1958)

(1) Hybrid inviability or weakness resulting from:

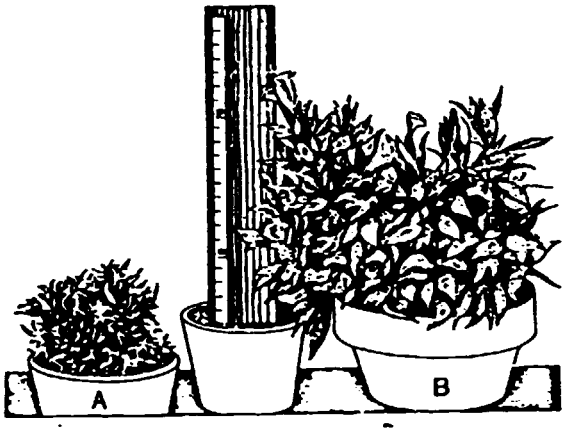
(a) Genome incompatibility = incompatibility between parental genomes. There is no way to overcome this. Originally tried protoplast fusion, but the rule of thumb is that if sexual crosses won't work, neither will protoplast fusion [see review by **Negrutiu, 1989**]. ∴ Resort to assymetric fusion or single-gene transfer.

(b) Plastid/genome incompatibilities: e.g.,

Trifolium pratense × *T. diffusum*	→ normal
reciprocal	→ albino

B) *Epilobium luteum* × *E. hirsutum* → normal
A) reciprocal → sterile

- *Epilobium* = fireweed
-Note: Cytoplasmic male sterility is normally a manifestation of this phenomenon.



(c) Gene interactions (**Shii et al., 1980**)

- Cross Andean × Mesoamerican beans (*P. vulgaris*) → weak F_1

- Conditioned by two genes, *Dl1* and *Dl2*

-Stands for *Dosage-dependent lethal*
- *Dl1* is found in Mesoamerican beans & affects root development
- *Dl2* is found in Andean beans and affects shoot development
  - homozygotes at both loci = lethal
  - heterozygotes at both loci = lethal at high temperature
  - homozygous dominant at 1 locus, heterozygous at other = sublethal
  - homozygous recessive at either locus = OK

(2) Failure of flowering
(3) Hybrid sterility

i) <u>chromosomal or haplontic:</u> - Inability of chromosomes to pair.
- Overcome by doubling of chromosome number:
- e.g., 2x *T. pratense* × 2x *T. diffusum* → sterile 2x F_1 → colchicine or NO_2 → fertile 4x
- Some pairing mutations may be confused for chromosomal sterility.

ii) <u>genic or diplontic:</u> - Chromosome doubling does not restore fertility
- Probably due to a milder form of genomic incompatibility.

(4) Inviability and weakness of the F_2
(5) Lack of gene expression (Heslop-Harrison, 1990)

- E.g., stem rust resistance from AABB not expressed in AABBDD
- Due to nuclear architechture and/or suppressor genes (in this case, on 7D)

(6) Genes that prevent homeologous chromosome pairing eg, *Ph* in wheat

OVERCOMING BARRIERS TO GENE TRANSFER

*** Always try simpler approaches first! ***

1. In planta pollination: - Use to overcome prefertilization barriers. e.g., inability of pollen tube to reach egg; premature flower abscision.

- Mentor pollen - best of poplars (Michurin, 1948)
- Use of growth regulators to prevent flower abscision
- Apply substances to the stigma to increase germination of pollen on stigma e.g., H_3BO_3, organic solvents, extracts from compatible pollen.
    - e.g., ethylacetate, n-hexane, nonpolar solvents (Willing & Prior, 1976: TAG 47:141-151)
- Mixture of irradiated and non-irradiated pollen (Shintaku et al., 1988: TAG 76:293-298)
- Growth regulators to enhance pollen tube growth
- Shorten the style (use gelatin block as artificial stigma)
- Stylar graft with a compatible style
- Inject pollen directly into the ovary

2. In vitro hybridization techniques

a. In vitro pollination

Techniques:

- Explant the ovules: whole flowers <----------> isolated ovules

    - in general, the more tissue present, the better the chances are for success.

- Dust pollen on ovules
    - remove part of ovary wall to expose ovules, or
    - isolate ovules on placenta

- timing, maturity, and plant genotype are all important.

b. Ovule culture - similar to in vitro pollination, but wait for fertilization to occur in situ before culturing the ovules. (review by Collins, Taylor, and DeVerna, 1984)

- Use when embryos can be rescued without isolating them
- Aka "ovary culture", "in ovulo embryo culture", and "fertilized ovule culture"
- Old technique, going back to 1942.
- Used to bypass endosperm-related post fertilization barriers.
    - Permits embryo to obtain nutrients from medium rather than endosperm.
- Range: Intact flowers <----------> isolated ovules
- Need preliminary investigations to determine:
    - Tissue (whole or part of flower)
    - Best time to explant
    - Optimize media: osmoticum, salts, vitamins, etc.

c. Embryo rescue

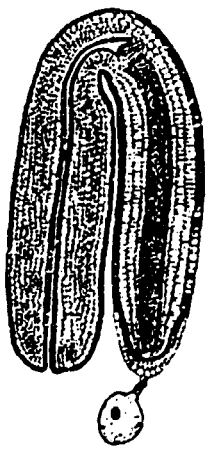
- First attempt reported in 1754
- Overcomes endosperm barriers
- Hybrid embryo must survive long enough to reach a culturable size
- The older and larger an embryo is, the simpler its growth requirements
- Must optimize the medium

-Stages:

1. Globular stage

2. Heart stage

3. Torpedo stage



4. Cotyledon stage

- Monocot stages = globular, scuttelar, and coleoptilar

- Ovule culture vs. embryo rescue

    - Sometimes one technique will succeed where the other fails
    - Endosperm breakdown
        - Can release toxic compounds as it dies
        - Can allow the overgrowth of maternal tissues (i.e., Cooper and Brink's somatoplastic sterility), leading to embryo death

    - In general, ovule culture is easier than embryo rescue, so should try it first.
    - Especially true if embryo abortion occurs at very early age, before it is culturable

d. Regeneration - Can sometimes bypass hybrid inviability, e.g.:

Nicotiana suavelens × *N. tabacum* → seedlings die
N. tabacum × *N. amplexicalis* → seedlings die

- can take seedlings before they die → callus → regenerate plants → grow to maturity

e. IAA - (Zhou et al , 1991; Sharma et al., 1990; Z. Pflanzenzucht. 85:248-253)

- *Nicotiana repanda* × *N. tabacum* seedlings die due to hybrid inviability
- 2 mg/l IAA in growth medium allows recovery of mature plants that set seed.
- reciprocal cross is sterile

- *Solanum melongena* × *S. khasianum*
- IAA needed at an early stage to prevent inviability

- suggests that hybrid inviability is due to altered auxin synthesis in hybrid

f. Protoplast fusion

<u>i) Recovery of hybrids</u>

- The development of tissue culture techniques led to the belief that any two species could be hybridized, e.g.:

    - corn/soybeans
    - tobacco/chicken

- The latter example led to speculations that meat would be produced in large tanks exposed to sunlight. Chloroplasts from the tobacco would provide the photosynthetic energy. Even appeared in *Time* magazine.

- The *National Enquirer* took this one step further- crossing people with trees, producing trees that would harvest their own fruit, then walk south for the winter, and set another crop.

- More likely scenarios featured the creation of "pomatoes", featured in *National Geographic*
  - Hybrids that have been obtained from plants that are normally not compatible. All have been sterile:

    - pomato: was to give tomatoes and potatoes, but gave neither
    - Arabidobrassica (Gleba & Hoffman, 1979: Natur. 66:547)
    - tomato/night shade (Guri et al., 1988: MGG 212:191)

- In retrospect, the view that protoplast fusion would bypass barries was very naive. If genomes are incompatible, they are incompatible no matter how they are brought together. There is a limit to how closely related 2 species must be if they are to be crossable. (Negrutiu, 1989)

- Bates, 1992: "The work of the past two decades suggests this is not a realistic goal."

- If 2 species are too divergent, genetic information of one becomes incompatible with genetic information of the other.

    - E.g., 1 may be trying to flower while the other is trying to go dormant.
    - or, one may produce compounds toxic to the other

- The great majority (2 or 3 exceptions only) of <u>fertile</u> hybrids that have been obtained by protoplast fusion can also be obtaine sexually. The first example was

  Solanum tuberosum × *S. brevidens* (Austin et al., 1988)
  Brassica napus × *Eruca sativa* (Fahleson et al., 1988)

- In this case, embryo rescue was never attempted. That could potentially have been a much simpler way to get the hybrids.

SELECTION OF HETEROKARYONS:

- Get intra- and interspecific protoplast fusion. Must have a way to separate the hybrid protoplasts from the nonhybrid ones.

1) Complementation:
   - eg. tobacco, nitrate reductase: Use 2 lines each with a different mutation
     - grow on medium with nitrate as the only N source.
   - Only the hybrids with restored nitrate reductase activity will survive.

- eg., *Petunia hybrida* (regenerator) susceptible to actinomycin D × *P. parodii* (nonregenerator) resistant to actinomycin D

2) Resistance eg., *Nicotiana sylvestris*

S-2-aminoethyl-cysteine
5-methyl-tryptophan } Fuse lines resistant to 1 amino acid analogue

- Select on medium with both compounds. Only hybrids survive.

3) Metabolic inhibitors E.g., *Solanum nigrum* × *Petunia hybrida*

- Treat one with diethylcarbonate, the other with iodoacetate
- Both are irreversible inhibitors of metabolism, but at different points

4) Mechanical
   a) Identification of heterokaryons
      - use of dyes
      - use of meshophyll cells (green) with suspension cells (white)

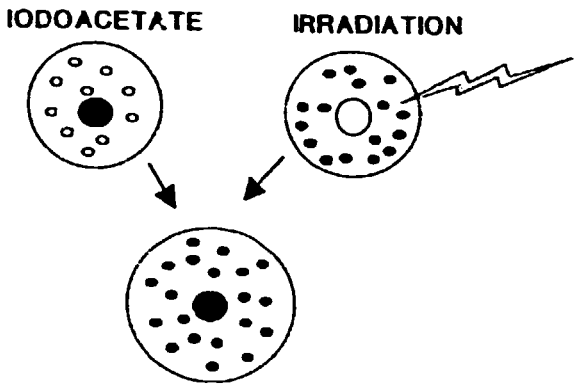
   b) Procedures
      - serial dilutions
      - micropipettes
      - micromanipulators
      - flow cytometry

ii) Cybrids The nucleous of one species with "hybrid cytoplasm"

- Hoped could get hybrid cytoplasms
- Recombinations in chloroplasts has turned out to be extremely rare-- only 1 or 2 reported cases.
  - Chloroplasts sort themselves out, ending up with those of one species or the other
  - Mitochondria do recombine
- May be a source of cytoplasmic male sterility
- Inactivate nucleous of one species with radiation, or count on chromosome elimination to occur after fusion.

iii) Cytoplasm exchange

- The nucleous of one species with the cytoplasm of another:
  - Inactivate organelles of one species with iodoacetate
  - Inactivate nucleous of other species with radiation
    - Only viable combination is through fusion of protoplasts from each species:



Note: Sometimes, will recover the parental type + one trait from the irradiated parent. E.g.:

- Nitrate reductase deficiency corrected after fusion of *Nicotiana* with *Datura* and *Nicotiana* with *Physaris*.
- Nuclear albino restored to green after carrot × parsely fusion

iv) Assymetric fusion

- First suggested by Gleba & Hoffman, 1979
- X-ray or γ-ray protoplasts from one parent only, then do fusion
  - loss ranges from 1-2 chromosomes to almost complete loss of genetic material
- Can get some traits from the irradiated parent into the other parent
  - Transfer in form of chromosomes or chromosomal segments
  - Less transfer of undesireable material
  - The material transferred is random

-E.g., Hinnisdale et al., 1991

- Kanamycin-resistant petunia @ 100 krad + *N. plumbaginifolia*
  - Got 24 kanamycin-resistant plants
    - 6 had petunia chromosome fragments
    - 4 were euploid 2x (as opposed to aneuploids or 4x)
      - kanamycin segregated 3:1
- Availability of a screen helps

-First done by Gupta et al., 1984 (MGG 197:30-35)

- albino *Datura* + X-rayed *Physallis* → green *Datura* (had *Physallis* chromosomes)

vi) transformation via electroporation

- Obsolete technology

vii) Bypassing seedling lethality

Nicotiana nesophila	× N. tabacum →	seedlings die
N. stocktonii	× "	seedlings die
N. repanda	× "	seedlings die

- However, hybrids obtained from protoplast fusion will survive.

LIMITATIONS OF PROTOPLAST TECHNOLOGY

- Protoplasts are extremely unstable, creating several large mutations.
- Complicated technology, requiring tedious procedures, special facilities, etc.
- Few species regenerate easily from protoplasts: alfalfa, tobacco, canola, carrot, rice, fescue, birdsfoot trefoil, tomato, potato, *Prunus*, citrus, lettuce, rudbeckia
- Our major crops are all difficult to work with: corn, wheat, soybean, peas, beans

Thus far, all the techniques discussed aim at obtaining wide hybrids. This brings in a lot of unwanted germplasm along with the desired trait. This also creates a lot of problems with sterility. Consequently, emphasis has shifted towards single-gene transfer

- Wide hybridization still necessary if trait to be transferred has undefined genetics
- Combination of RFLP technology with wide hybridization will reduce problems of linkage drag, i.e., introgressing unwanted traits

SINGLE GENE TRANSFER

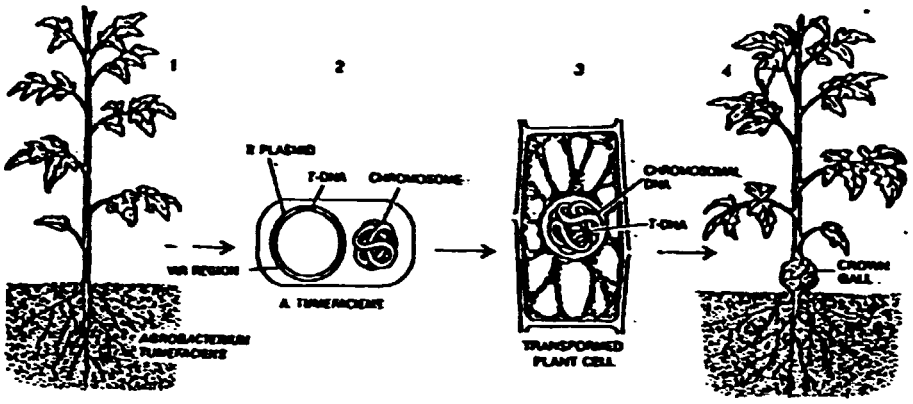
1. *Agrobacterium*-mediated transformation

- Derived from crown gall disease, caused by a bacterium called *Agrobacterium tumefaciens*:

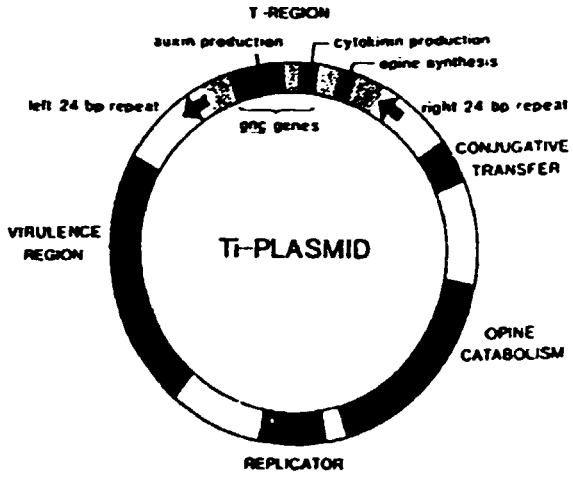
  - Agro → Soil
  - bacterium → Bacterium
  - tume → Tumor
  - faciens → Maker

- In other words, this is a bacterium found in the soil and which can cause tumors.
- Related species is *Agrobacterium rhizogenes* (root creator)
- The Ti plasmid (for tumor inducing) is responsible for tumorigenesis

Hooykaas & Schiperoort, 1992



- Ti plasmids have 3 important regions:



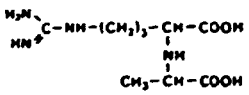
Hooykaas & Shilperoort, 1992

- VIR = virulence region- necessary for gall formation
- VIR region: has 8 operons with 24 genes that code for the trasfer functions. These are called VirA through VirH
- VIR is thought to have evolved from bacterial conjugation process.

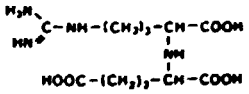
- T-DNA = transfer region- actually transferred and integrated into plant DNA
- Opine catabolism = allows bacterium to metabolize opines as carbon and nitrogen source
- Conjugative transfer activated by opine synthesis

- T-DNA: Both single and split types exist.

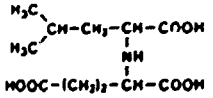
- Single: have one gene for auxin production & 2 genes for cytokinin production -these hormones cause cells to multiply and form a gall
- 1 gene for nopaline or octopine production
- Split types: -T-DNA is split in the middle with DNA that gets spliced out. Has 4 borders + 1 gene for mannopine or agropine



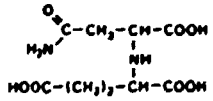
OCTOPINE



NOPALINE

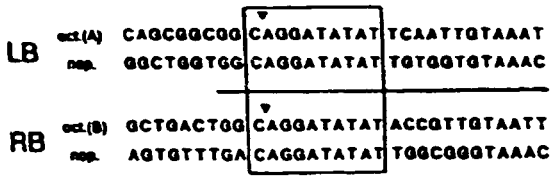


LEUCINOPINE



SUCCINAMOPINE

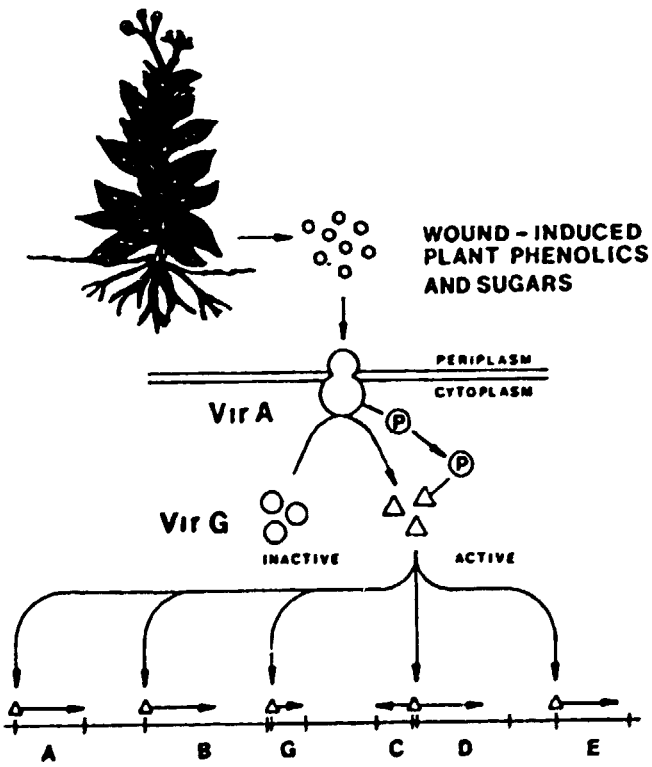
- T-DNA is flanked by imperfect 24 bp repeats:



THE T-DNA TRANSFER PROCESS:

1) Bacteria attach to plant cell walls, using 2 chromosomal virulence genes: - *chvB* = β-1, 2 glucan = rhicadhesin, and *chvA* = export of *chvB* gene product

2) Plant is wounded.
   - Wounded cells begin to repair themselves.
     -Secrete acetosyringone and other phenolic compounds used in cell wall synthesis.
       - Solanaceous plants secrete derivatives of benzoic acid & cinnamic acid
     - Secrete sugars which are either known plant metabolites or components of major cell wall polysaccharides
     - Bacteria respond by invading and colonizing wounded cells



Nestser, 1991

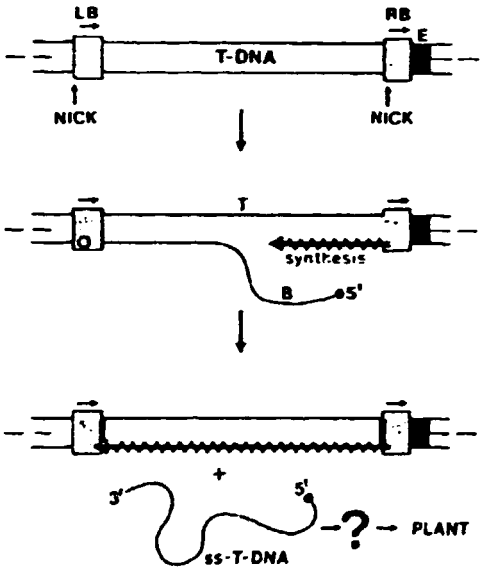
3) The VirA gene product is in the cell wall of the bacterium. It recognizes acetosyringone and becomes autophosphorylated. Then it transfers the phosphate group to VirG, thus activating it.

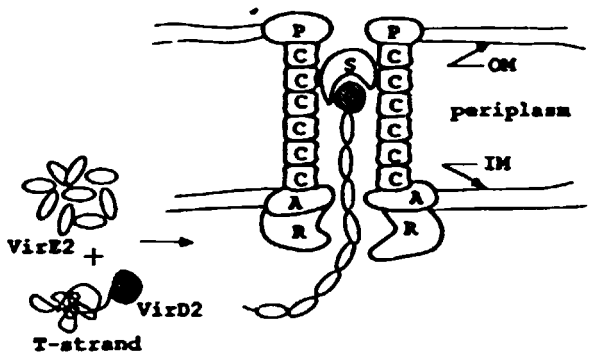
- VirA has 2 promoters. One gets activated by plant phenolics and phosphate starvation, the other by acidic conditions.
- VirA also recognizes sugars. Upon doing so, it activates *Chv*B, a chromosomal gene that codes for a periplasmic glucose-binding protein.

4) VirG protein then activates the rest of the VIR region.

5) VirD codes for an endonuclease that recognizes the 24 bp repeats and nicks them.

-DNA replication occurs, generating the T-strand:





Zambryski, 1992. P = plant-cell binding proteins; S = T-complex shuttle protein; C = channel (VIRB4); A = ATPase (VIRB11); R = T-complex receptor.

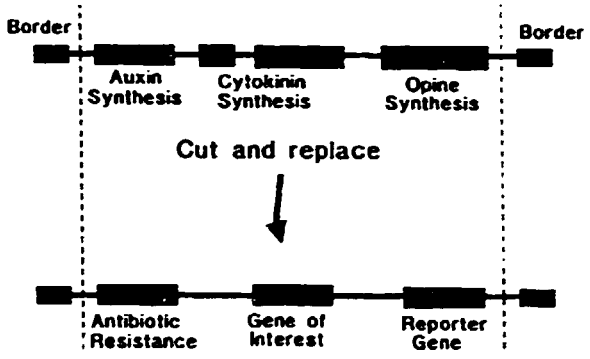
◂ Hooykaas & Schilperoort, 1992

6) T-strand covered by protein from VirE2 locus.
- -VirD2 protein attaches to 5' end.
  - -Help with nuclear uptake
  - -Protect from degradation?
  - -Help with integration?
- Functions of VirC and most of VirB remain unknown.
  - VirB codes for 11 peptides. Most are membrane bound. Two have ATP binding sites. Speculate that help create pores in the membrane for export of T-DNA.
- VirC affects host range.

7) What happens next is still unknown. Three schools of thought exist:

1) Bacteria conjugate with plant cells
2) Bacteria inside wounded cells disintegrate, releasing contents into cytoplasm
   - Speculate that T-DNA passes through plasmodesmata and enters adjoining cells where it becomes integrated into the plant's DNA.
3) Is a viral feature, with the protein covering the T-strand analogous to the protein coat of a virus.

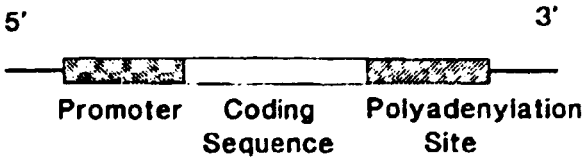
NOTE: Because the genes that control T-DNA are not on the T-DNA itself, it is engineerable. In fact, only the borders are necessary for DNA transfer into plants. Everything in the middle can be replaced:



1) **The antibiotic resistance gene** is known as a selectable marker. Cells that have been engineered can be identified on the basis of their acquired resistance.
e.g., kanamycin, hygromycin, streptamycin, bleomycin, gentamycin, etc.

- As these genes are of bacterial (or fungal) origin, they are not expressed in plants. Consequently, their promoter must be replaced with a plant promoter, and a terminator added (polyadenylation site), creating a chimeric gene:

  - From the Greek *chimera*, a monster consisting of parts of many animals.



- Some available promoters:

Constitutive:	Cauliflower mosaic virus 35S; nopaline synthase
Leaf-specific:	Rubisco
Tapetum-specific:	TA29
Embryo-specific:	ß-phaseolin
Inducible:	Heat shock, alcohol dehydrogenase

2) The reporter gene - it easily tells that the T-DNA is integrated and working properly in a plant

-e.g.'s: GUS (ß-glucuronidase), luciferase, nopaline synthase.

3) The gene of interest:

-First must realize that some traits are not amenable to genetic engineering techniques, eg., yield, drought tolerance, salt tolerance, etc. These are best tackled through interspecific hybridization in conjunction with marker-assisted selection.

-Genes that have thus far been amenable: See handout.

Preparing the gene:

-Right promoter

-Right terminator

-Right coding sequence

- Can have problems with codon bias. The genetic code is universal, but all organisms do not have the necessary tRNAs to transcribe codons. Eg:

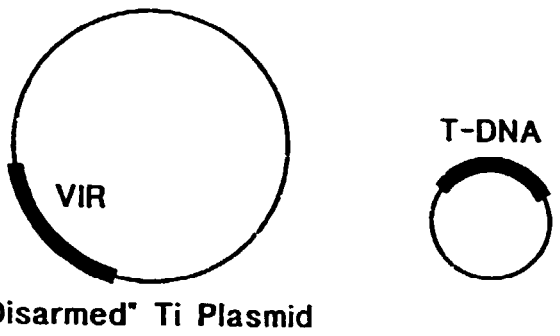
Serine Codon	Frequency of Use	
	E. coli	Human
UCU	2.81	0.45
UCC	2.07	2.09
UCA	0.06	0.26
UCG	0.00	0.68

- In the above example, a human gene with UCG would not be expressed in *E. coli*. One with UCA would be expressed poorly.

-This was the case with the *Bt* genes. Codons not used by plants had to be replaced with synonymous codons more commonly used by plants.

Preparing the vector:

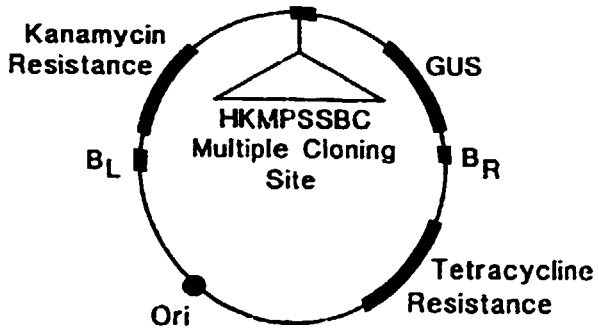
- The Ti plasmid is ~ 250 kb long. Technology can engineer plasmids of only ~ 20 kb. Hence, strategy is to separate the T-DNA from the VIR region, in a system known as <u>binary vectors</u>:



- Works as VIR genes are effective in trans they do not have to be on the same plasmid.

- A Ti plasmid without T-DNA is said to be <u>disarmed</u>.

- The diagram below gives a closer look at a binary vector. Several variations on the theme are possible:

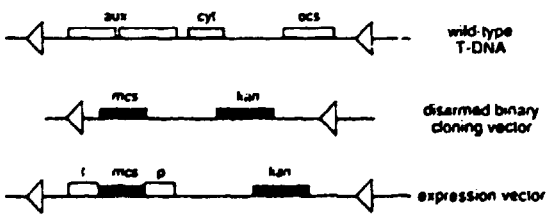


Note the following:

- The tetracycline (or other antibiotic resistance) allows you to separate bacteria containing this plasmid from bacteria that don't have it.

- The origin of replication is necessary to allow replication of the plasmid in the bacteria.

- The multiple cloning site makes it easy to insert the desired gene into the vector.



- While *Agrobacterium*-mediated transformation has been successful for some species, this has not been universal. *Agrobacterium* is a plant pathogen, and for it to work on a given species, the species must be within the host range of *Agrobacterium*. This has excluded most major crops.

-Yes: cotton, walnut, tobacco, tomato, potato, canola, potato, alfalfa
- Difficult: soybean, peanut
- No: corn, wheat, rice

-Notice that this list closely follows the protoplast list!

NOTE: There is a difference between gettin occasional transformed plants after much effort and the ability to obtain transgenic plants at will.

- Several alternatives to *Agrobacterium*-mediated transformation have been attempted.

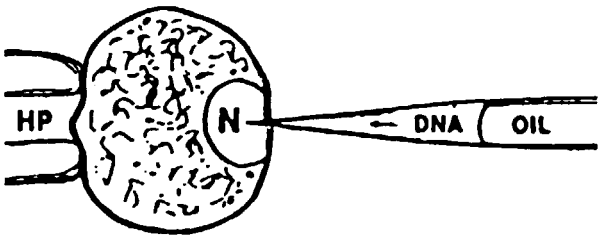
2. Direct DNA intake: Makes use of a protoplast's ability to up take anything (e.g., glass beads, macromolecules, etc.)

- Mix protoplasts with DNA
- Grow protoplasts on selection media
- Very low efficiency
- Can increase efficiency by adding compounds such as PEG (Polyethylene glycol)

3. Electroporation: Use of an electric discharge to create holes in a protoplast's cell membrane, thus increasing the efficiency of transformation.

- There is now evidence that electroporation may be used with intact tissues.

4. Microinjection: Literally inject DNA into single protoplasts.
- Requires a very expensive micromanipulator



All of the protoplast-mediated systems have several limitations:
- Few species are easily regenerated from protoplasts.
- Protoplasts are inherently genetically unstable

5. DNA injection into reproductive organs: DNA is injected into ovules, pollen tubes, etc. Has not been reproducible. False positives have most likely been the result of transformation of bacterial endophytes, rather than the plant itself. Not yet proven to everynone's satisfaction.

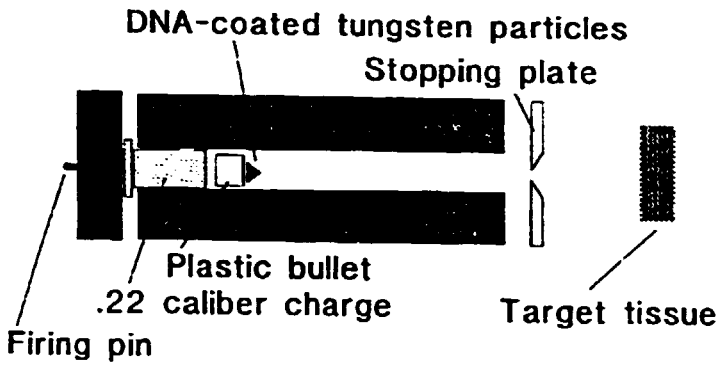
6. Pollen-mediated: Mix pollen with DNA
- Not yet proven to everyone's satisfaction

7. Silicon carbide fibers: Mix cells, silicon carbide fibers, and DNA, then vortex.
- The fibers act as spears, penetrating the cell wall and making an opening for DNA to enter the cell. (Kaeppler et al., 1990)

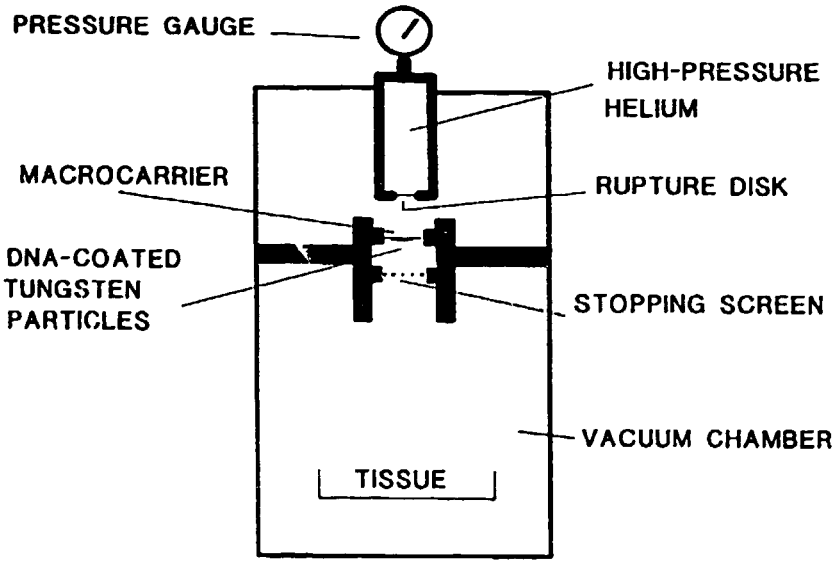
8. Microprojectile bombardment: --also known as the "Gene gun"

- Literally shoots DNA-covered tungsten or gold particles into plant cells.

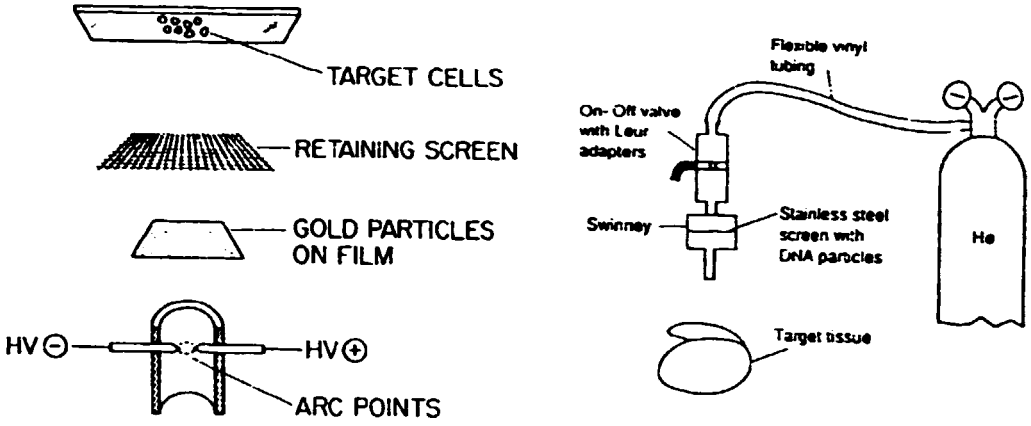
- Extremely simple concept:



A new model simply uses compressed air:



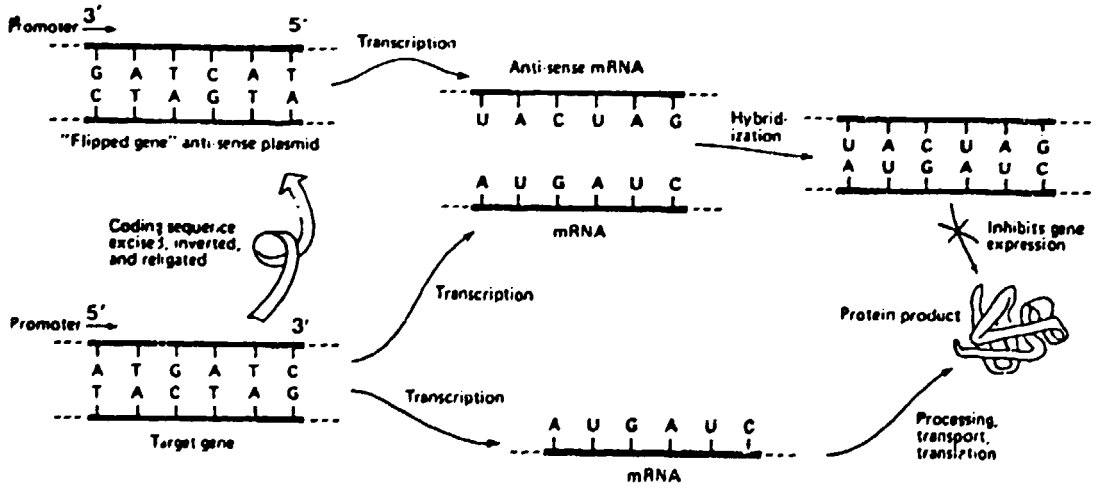
- There is also another model that uses electrical discharges to propel the microprojectiles, and yet another that uses low-pressure helium. In short, there are many ways to accelerate microprojectiles.



Finer et al., 1992

Antisense RNA:

- Adding genes to an organism is a way to confer new traits onto that organism. However, at times the objective is not to express a new trait, but to get rid of expression of an existing trait.
- In lower organisms, site-directed mutagenesis is a way to eliminate gene expression, but this currently does not work well in plants.
- Antisense RNA is an alternative:



Uses of antisense technology:

- Calgene's 'FlavrSavr' tomato. Antisence polyglucorinidase prevents cell wall degradation, so tomato will not soften. Therefore, it can be vine-ripened and still survive shipping.

- Florigene/DNAP's 'Flori-ant' white mum. Took a pink mum (which does not sell well) but had excellent commerial characteristics (quick flowering, long life after cutting), and made it white using antisense chalcone synthase.▸

Problems with transgene expression:

- Gene expression varies with different transformation events. I.e., for the same gene, independent transformation events of the same genotype can vary greatly. Attritubuted to several causes:

  - Position effects: Integration is a random event. Know from classical cytogenetics that genes translocated near heterochromatin decrease their level of expression. There is some evidence against this concept for transgenes.

  - Copy number: As copy number of genes increases, the overall expression decreases. There is experimental evidence for and against this. In the cases where gene expression is decreased, methylation appears to be involved.

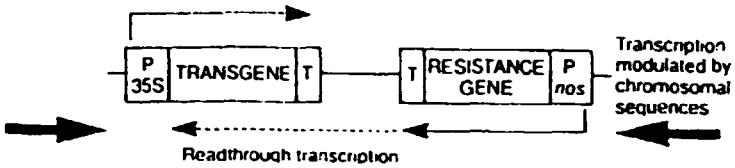
  NOTE: Gun-mediated transformation (and other methods of direct DNA transfer) tends to result in a high copy numbers of the transgene at the site of insertion. In contrast, *Agrobacterium*-mediated transformation usually results in single or few-copy (<10) insertions.

- Co-suppression (Jorgensen, 1990):

Anthocyanin biosynthesis:

Coumaryol-CoA + 3 Malonyl-CoA -CHS→ chalcone →→ dihydroflavonal -DFR→ →→ anthocyanin

- Engineered petunias with CHS or DFR
  - Thought that this would enhance color production, but instead it stopped all color formation
  - This turning off of a plant gene by an engineered copy of the same gene is called co-suppression
  - This can be temporally and spatially variable
  - Thought to be due to antisense produced by readthrough of gene caused by a selectable marker placed in opposite orientation:



Grierson et al., 1991

- All of this has implications for a breeding program. Originally thought that elite cultivars would each be engineered for specific genes. However:

  1) Regeneration and engineering appears to be genotype-dependent, so not every cultivar is amenable to current engineering/regeneration technology. In general, transformation ability is correlated with agronomic quality. E.g., cotton.

  2) Because expression varies so much between independent transformation events, it has turned out to be more efficient to engineer one cultivar that is easily engineerable, find one individual with a high level of transgene expression, then backcross the transgene into other cultivars. This is as opposed to indiviudally engineering each genotype.

- Engineered plants nearing the market place

'Bromotal'	cotton	Calgene	resistance to bromoxynil
	cotton	Monsanto	Bt resistance to insects
	soybean	Monsanto	
	Cantaloupe	UpJohn	Resistance to papaya ringspot virus, watermelon mosaic virus -2, and zuchinni yellows virus

SOMACLONAL VARIATION:

- Plants recovered from culture usually are different in many ways from the elite cultivar that went into culture. These differences are referred to as <u>somaclonal variation</u>

- Then some somaclones were reported to be superior to the cultivar they came from, and the

craze was on. Somaclonal variation was then viewed as a way to increase the genetic diversity of plants.

- Callus, and especially protoplasts, are most susceptible to somaclonal variation.

- As time in culture increases, so does the incidence of somaclonal variation

- **Types of somaclonal variation:**

  - Point mutations
  - Aneuploidy
  - Chromosomal rearrangements
  - Changes in ploidy
  - Changes in gene copy number
  - Activation of transposable elements
  - Cytoplasmic mutations
  - Chromosome substitution
  - Chromosome breakage
  - Changes in DNA content

Embarrassing moments at gene parties

- **Causes of somaclonal variation:**

1) Preexisting variability in the form of accumulated mutations in cells derived from the zygote.

2) Mutations induced by media (Hoffman et al., 1982)
   - doubled haploids from microspore culture → have heterozygous loci

3) Genomic shock (McClintock, 1984)
   - Late-replicating heterochromatin in corn, rye, and oats
   - Nucleotide pool imbalances
   - Mitotic recombination

- **Breeding via somaclonal variation**

WITH SELECTION . In the early days of tissue culture, the thought was that bacterial selection systems could be applied to plant cells. Plant cells from elite cultivars would be exposed to a mutagen or selection agent, and plants with the new trait regenerated.

- Mechanism of resistance must be the same at the cell level as at the whole plant level

    -Eg., corn hybrids resistant to imidazolinone herbicides

- Van den Bulk, 1991: Review of somaclones obtained using selection:

    - Alfalfa resistant to *Fusarium*
    - Barley resistant to *Helminthosporium*
    - Eggplant resistant to a mycloplasma-like organism
    - Oats resistant to *Helminthosporium*
    - Rape resistant to *Phoma lingam*
    - Tobacco resistant to *Pseudomonas* and TMV
    - Tomato resistant to *Fusarium* and TMV
    - Wheat resistant to *Helminthosporium*

****The vast majority of reports do not have progeny data to test the stability and heritability of the trait.****

WITHOUT SELECTION

- Rapeseed resistant to *Phoma lingam*
- Tomato resistant to TMV and *Fusarium*
- Celery resistant to *Fusarium*

- In practice, somaclonal variation has not lived up to its potential. Most changes that are useful are not novel, that is, they represent mutations already existing in the gene pool.

- Some plant breeders argue that variability is not the greatest limiting step. The greatest limiting step for them is *screening* of germplasm.

- Many of the changes called somaclonal variation are epigenetic in nature, and disappear after one or more seed generations. Altered methylation patterns are the most likely reason.

- The net result is that somaclonal variation is not the most efficient way to go after variability. It is expensive, the results are random, and they may only be epigenetic. Conventional mutagenesis may be a more economical and efficient option.

- Nevertheless, there are some cultivars on the market derived from somaclonal variation:

    - DNAP 9, a tomato with resistance to *Fusarium*.
    - DNAP 17, a tomato with increased soluble solids.
    - Bell Sweet, a bell pepper with very few seeds.

OTHER USES

- The large amount of chromosome breakage and deletions that occur are a way to transfer alien chromosome segments containing desired traits from one species to another:
    - The chromosomes do not pair in the F_1, making introgression through crossing over and recombination impossible.
    - For example, resistance to cereal cyst nematode from rye and to barley yellow dwarf from *Thinopyrum* are being introgressed into wheat this way.

LAWYERS, GUNS, AND MONEY:

Effects on intellectual property rights

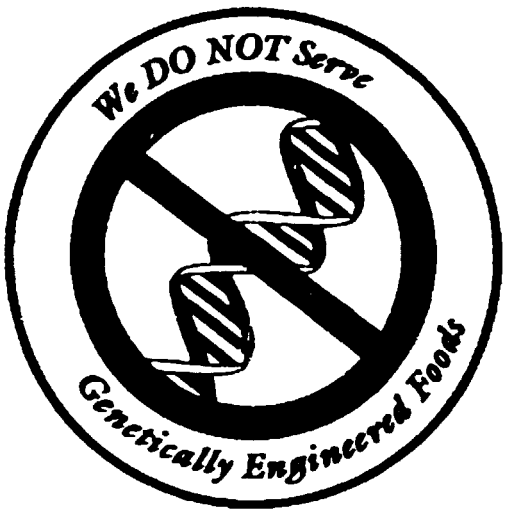
- Traditionally, exchange of plant germplasm has been free and unrestricted.
- Now, individual gene transfer technology, genes, and individual components have been patented.
- The overwhelming majority are owned by large companies:
    - Gun technology belongs to DuPont
    - *Agrobacterium* technology belongs to Monsanto
    - Hygromycin resistance belongs to DowElanco
    - Soybean regeneration belongs to Agrigenetics
    - Bt genes belong to Plant Genetic Systems
    - Cotton transformation belongs to Agracetus
    - Coat protein technology belongs to Monsanto
    - Antifreeze belongs to DNAP
    - Chalcone synthase belongs to DNAP (pat no. 5,034,323)
    - Chitinase belongs to Mogen
- Biotechnology is so expensive that patents are the only way companies have to recover their costs
    - The International Biodiversity treaty recently signed by Clinton limits patent rights for bioteccompanies, and could have adverse effects on biotechnology research
- The net result is that extensive negotiations and legal contracts are necessary for anything to be done in this area:
    - Can involve "technology access fees".
    - All involve royalty payments.
    - All involve restrictions on use.
- The release of a single engineered cultivar can involve contract negotiations with several companies.

Effect of regulations

- Regulated by EPA, USDA-APHIS, and FDA

- Regulations subject to public perception

  - Foundation for Economic Trends (Reremy Rifkin) - opposed to any form of genetic engineering as being dangerous
  - Pure Foods campaign -- organizing a national boycott
  - Environmental Defense Fund, Council for Responsible Genetics



- Public and press are generally too poorly educated to understand issues and technology involved

  Have mistaken impressions of what is and isn't possible with genetic engineering

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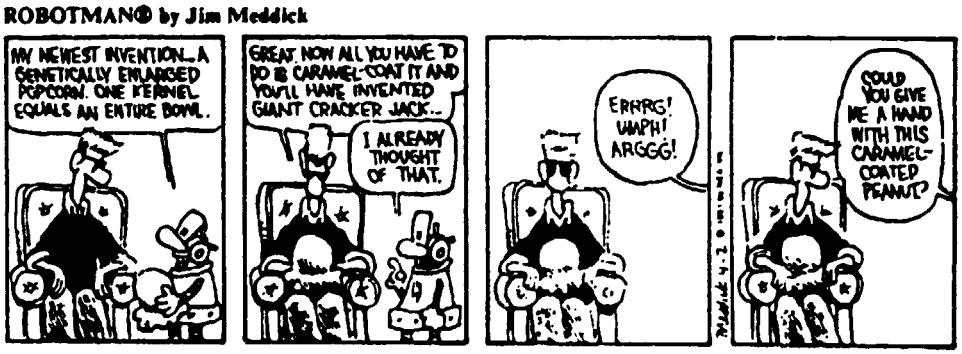
ROBOTMAN® by Jim Meddick



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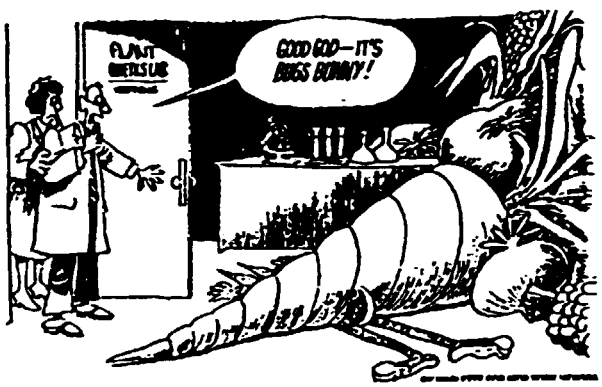
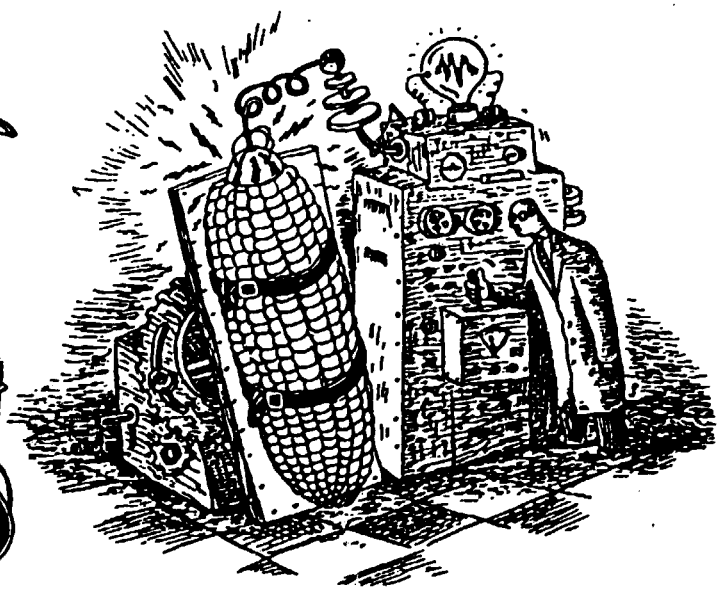


Irresponsible press

New York Times, June 1, 1992



Barron's, June 1, 1992

AGY/BOT 890

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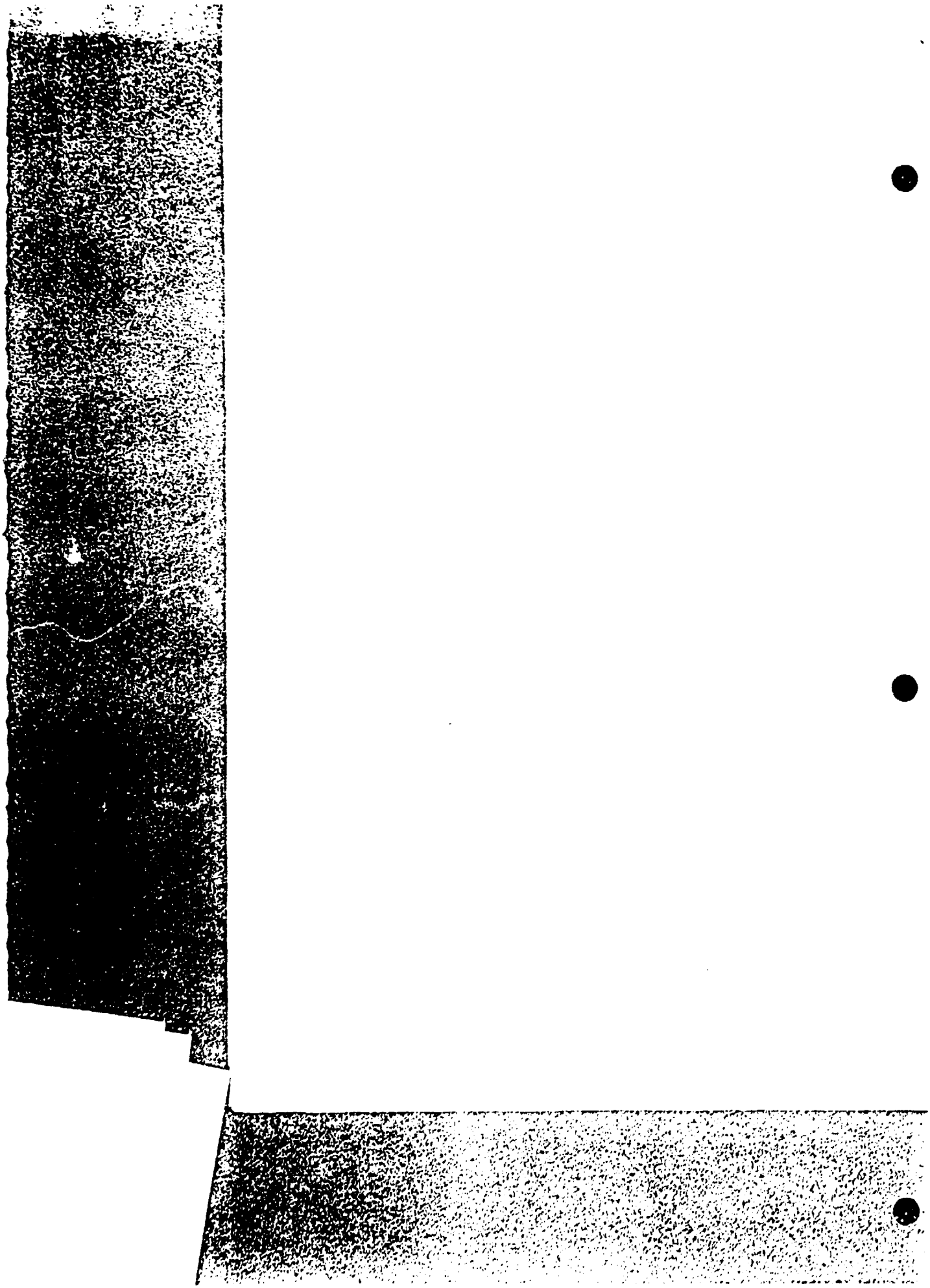

Chapter 7
Somatic Embryogenesis: Potential for Use in Propagation and Gene Transfer Systems

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Introduction

Since 1958 when the first plant embryos were obtained from somatic tissues of carrot (*Daucus carota*) cultured *in vitro* (Reinert, 1958; Steward, 1958), ever increasing numbers of species and tissues have been induced to form somatic embryos. In the process, a greater understanding of the phenomenon has been achieved, and the ability to exploit it has increased. Although somatic embryos are derived from somatic cells, they closely resemble their sexual counterparts and presumably result from expression of genes regulating the same developmental pathway. The evolution of this pathway must date back at least to the evolution of seed plants, and its basic features appear to be highly conserved. As a result, it is possible to apply the most basic principles to somatic embryogenesis across the range of seed-bearing plants.

A wide range of plant tissues has been used as explant sources from which to obtain somatic embryos, with tissues derived from immature embryos being especially amenable to induced embryogenesis. Responding species are as diverse as wheat, *Triticum aestivum* (Ozias-Akins & Vasil, 1982), and soybean, *Glycine max* (Lippmann & Lippmann, 1984) (Fig. 7.1). The use of zygotic embryos as an explant source is normally not a limitation, except in the case of cross-pollinated species, in which case a zygotic embryo (or seedling) represents an unknown genotype.

Cells within a zygotic embryo are believed to express the genes necessary for the embryogenic developmental programme. In simplest terms, they need only to become independent from positional constraints imposed by

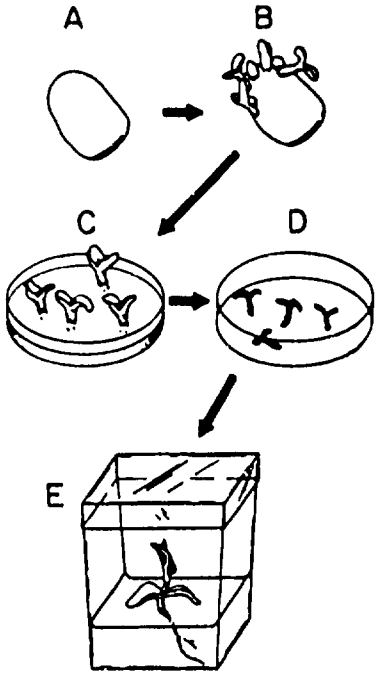

Fig. 7.1. Basic regeneration protocol for soybean (based on Lazzeri *et al.*, 1985; Parrott *et al.*, 1988). A). Immature cotyledons, with the embryonic axes removed, are exposed to 10 mg/l of α-naphthalene acetic acid (NAA) for 7–10 days, and then transferred to hormone-free basal medium. B) Somatic embryos develop around the distal periphery of the cotyledon. After 20 days on hormone-free basal medium, the somatic embryos are removed from the explant tissue and transferred to hormone-free basal medium. C) Somatic embryos are allowed to mature for at least one month on basal medium. D) Somatic embryos are desiccated for one week. E) Desiccated embryos germinate when transferred to basal medium. (Artwork by J. Adang.)

other cells within the tissue. Consequently, the disruption of such tissues alone can be sufficient in some species to permit the development of somatic embryos on hormone-free media, as has been observed with East Indies walnut (*Albizia lebbeck*) and carrot (Gharyal & Maheshwari, 1981; Smith & Krikorian, 1989). In recognition of the existence of an embryogenic state within cells prior to the formation of somatic embryos, the term pre-embryogenic determined cell (PEDC) was coined (Sharp *et al.*, 1982). Because PEDCs can divide to form an embryo directly, the process has been referred to as direct somatic embryogenesis.

Embryogenic developmental programmes are not expressed in other, more differentiated plant tissues that give rise to somatic embryos, and several cell division cycles in the presence of an auxin are normally required before the embryogenic developmental pathway is once again expressed. The term induced embryogenic determined cell (IEDC) is used to identify cells that acquire embryogenic potential in culture (Sharp *et al.*, 1982). The various cell generations that intervene between the original explant and the formation of somatic embryos are manifested as a callus. In this case, somatic embryogenesis is said to be indirect. Once formed, an IEDC and a PEDC are functionally equivalent, and the term embryogenic cell (EC) could be considered to be a better descriptor.

The terms PEDC and IEDC provide convenient ways to classify tissues, but in reality represent extremes of a continuum. Apparently, the embryogenic developmental programme is not terminated abruptly and replaced by another, but alters gradually over a number of cell cycles. The net result is that increasingly greater amounts of reprogramming are necessary to form ECs as the cells diverge in time and space from the original embryogenic condition. The observation that older cells from immature cotyledons of soybean form more abnormal somatic embryos than do younger cells (Hartweck *et al.*, 1988) fits this model of somatic embryogenesis. Such gradients in the morphology of somatic embryos have also been observed in flax (*Linum usitatissimum*) by Pretova & Williams (1986b) and in tomato (*Lycopersicon*) species by Young *et al.* (1987). In addition, explants derived from tissues associated with reproduction (e.g. nucellar or anther tissues) and hypocotyls or cotyledons of young seedlings tend to be more easily induced to an embryogenic state than do cells from mature tissues (Williams *et al.*, 1990).

When a tissue consists of ECs, the stimulation of cell division may be all that is necessary to perpetuate the embryogenic state and form somatic embryos. This could explain the formation of somatic embryos following the exposure of immature zygotic embryos to low concentrations of cytokinin in oilseed rape, *Brassica napus* ssp. *oleifera* (Pretova & Williams, 1986a), flax (Pretova & Williams, 1986b), alfalfa (*Medicago sativa*) and various species of clover (*Trifolium*) (Maheswaran & Williams, 1984, 1985, 1986a, b). The perpetuation of cycles of repetitive embryogenesis in microspore-derived somatic embryos of oilseed rape on hormone-free medium has been attributed to high concentrations of endogenous cytokinins within the ECs of the somatic embryos (Loh *et al.*, 1983). In this particular case, repetitive embryogenesis is stopped by exposure to exogenous cytokinin, presumably because exogenous cytokinins are able to activate the metabolic machinery that ordinarily maintains low concentrations of internal cytokinins.

Whenever an IEDC must be produced from a non-embryogenic cell, exposure to an auxin is necessary, and exposure to a cytokinin can be detrimental to the process (e.g. Wenck *et al.*, 1988). In fact, high concentrations of endogenous cytokinins have been associated with lack of embryogenic capacity in Napiergrass, *Pennisetum purpureum*, and orchardgrass, *Dactylis glomerata* (Rajasekaran *et al.*, 1987; Wenck *et al.*, 1988). Alternatively, in A188, an embryogenic genotype of maize (*Zea mays*), endogenous auxin concentrations in the ovule are 16–20 times lower than those in non-embryogenic genotypes (Carnes & Wright, 1988). The mode of action of auxin in the redetermination of an IEDC remains unknown, and is probably not due to a single factor. On exposure to auxin, substantial DNA methylation occurs (LoSchiavo *et al.*, 1989), which may stop or hinder the expression of existing developmental programmes within the cell. Stress may also have the same effect (Kamada *et al.*, 1989). Isolation of cells and disruption of tissue may also play a role in the redetermination of cells. This factor has been reviewed extensively by Williams & Maheswaran (1986) and by Smith & Krikorian (1989). Although factors as diverse as plasmolysis (Wetherell, 1984) and tissue necrosis (Hartweck *et al.*, 1988; Trigiano *et al.*, 1989) obviously lead to isolation of cells from the rest of a tissue, auxins themselves induce the property of friability, which is caused by rapid cell separation (Evans *et al.*, 1981). Finally, once a cell or small group of cells has been isolated, an auxin may help establish polarity. Polarity has been found to precede and accompany differentiation in cell aggregates of carrot (Brawley *et al.*, 1984; Nomura & Komamine, 1986; Gorst *et al.*, 1987; Rathore *et al.*, 1988; Timmers *et al.*, 1989) and sweet potato, *Ipomoea batatas* (Chée & Cantliffe, 1989). Application of a low-voltage field greatly increased the frequency of somatic embryos formed on callus of alfalfa (Dijak *et al.*, 1986), plausibly in response to polarity imposed by the electrical field.

Once an EC has been obtained, the continued presence of an auxin can be detrimental to normal development of somatic embryos (Halperin & Wetherell, 1964; Parrott *et al.*, 1988). The strikingly negative effect that 2,4-D can have on the quality of somatic embryos was illustrated by Kamada *et al.* (1989). Carrot somatic embryos were induced by treatment with high concentrations of sucrose, cadmium ions, sodium hypochlorite, or 2,4-D, and encapsulated in calcium alginate to produce synthetic seeds. For synthetic seeds containing a single large embryo, 30–50% of encapsulated embryos from cadmium treatments developed both a radicle and a green bud, whereas only 15% of embryos derived from 2,4-D treatment did so. The best time to withdraw auxin from the medium is not clear, but probably corresponds to the time that auxin contents drop naturally in ovules developing *in planta* (Carnes & Wright, 1988; Carman, 1989).

If the concentration of auxin is high enough, instead of proceeding to the next stage of its ontogeny, a somatic embryo may instead give rise to new somatic embryos. Such a process has been described at various times as secondary, recurrent or repetitive embryogenesis (Fig. 7.2). Depending on the species and the culture system in use, the repetitive cycle may be expressed as continuous propagation of various embryonic stages, including proembryogenic masses (PEMs) as in carrot (e.g. Halperin & Wetherell, 1965), globular embryos as in *Citrus* (e.g. Button *et al.*, 1974) and soybean (Finer, 1988; Finer & Nagasawa, 1988), or even cotyledonary stage embryos, as in alfalfa (Lupotto, 1983, 1986). Other factors that can influence the proliferation of somatic embryos include the ammonium to nitrate ratio in the medium (Smith & Krikorian, 1989) and a low pH (4.0) in the medium (Smith & Krikorian, 1990). Such cycles of repetitive embryogenesis can be broken by the removal or reduction of auxin in the culture medium, permitting the development of mature embryos. It is the ability of repetitive embryogenesis to perpetuate the embryogenic state indefinitely and produce large numbers of embryos that makes somatic embryogenesis a

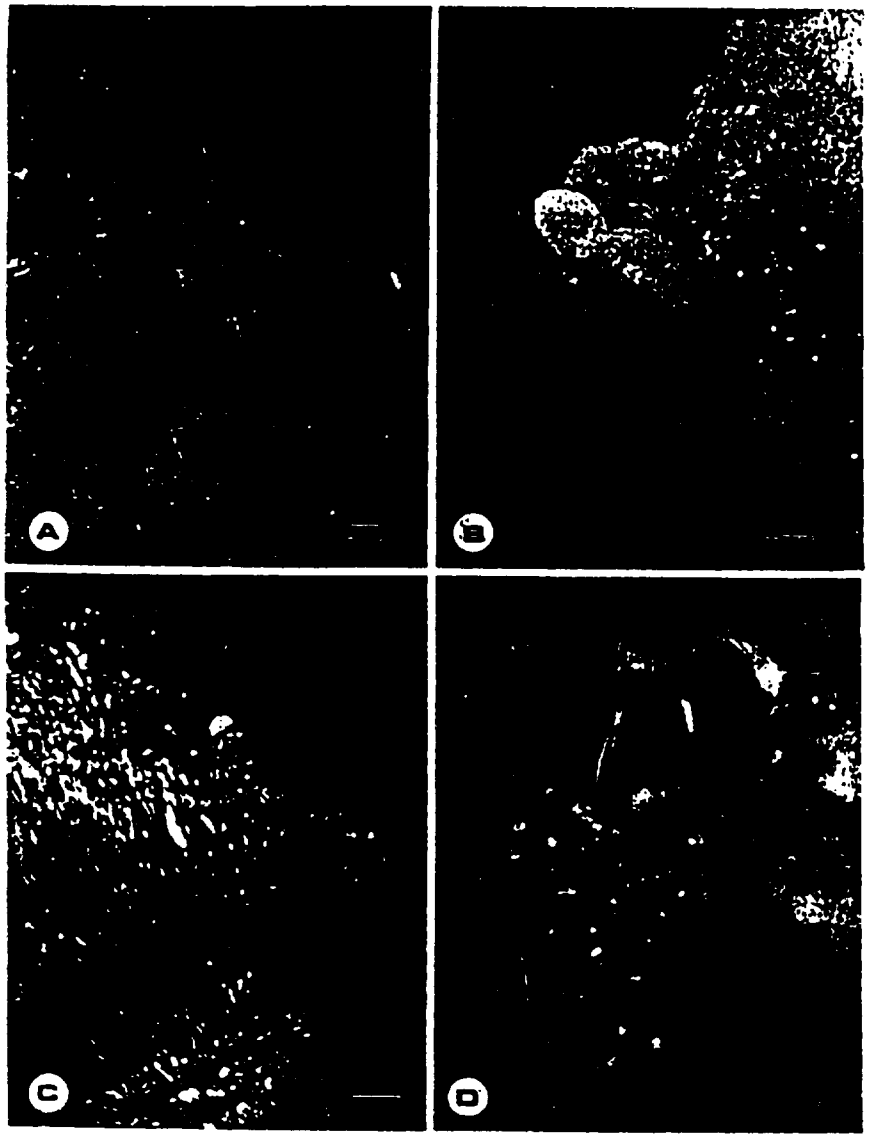

Fig. 7.2. Repetitive somatic embryogenesis. A) B) Globular somatic embryos of soybean giving rise to further globular embryos. C) Globular and D) torpedo-stage embryos that have formed along the hypocotyl of larger, cotyledonary-stage somatic embryos of alfalfa.

powerful tool capable of being exploited for diverse goals such as mass propagation and production of transgenic plants.

Factors affecting the application of embryogenic cultures and somatic embryos

Genetic control of regeneration

Biotechnology has been expected to assist plant breeding programmes, but applications of biotechnology to plant breeding have frequently been limited by two major factors: the lack of regeneration capacity in desired tissues, cultivars or crops, and the lack for most plant species of a simple reliable method for genetic transformation with defined DNA sequences. The advent of biolistic technology has removed a major barrier for the delivery of exogenous DNA into plant cells, leaving the recovery of plants from transgenic cells as a major limitation. It may be that before biotechnology can make a significant contribution to the improvement of many crops, it will be necessary to breed for increased regeneration capacity. As an example, the argument for breeding for regeneration has been made for potato (*Solanum tuberosum*), in which the ability to form somatic embryos in cultured anthers is a heritable trait (Sonnino *et al.*, 1989), and crosses between parents with low embryogenic capacity can result in progeny with much higher regeneration capacities (Jacobsen & Sopory, 1978).

The ability to form somatic embryos is, in most cases, not merely an intrinsic property of a species. Instead, it is a property under genetic control, such that individual genotypes within a species can differ in their ability to undergo somatic embryogenesis. This phenomenon has been widely documented in several species, including red clover, *Trifolium pratense* (MacLean & Nowak, 1989), alfalfa (Mitten *et al.*, 1984; Brown & Atanassov, 1985; Meijer & Brown, 1985; Chen & Marowitch, 1987; Chen *et al.*, 1987; Bianchi *et al.*, 1988; Walton & Brown, 1988), soybean (Komatsuda & Ohyama, 1988; Parrott *et al.*, 1989), cotton (Trolinder & Xhixian, 1989), maize (Green & Phillips, 1975; Bartkowiak, 1982; Lu *et al.*, 1983a; Beckert & Qing, 1984; Duncan *et al.*, 1985; Hodges *et al.*, 1986), rice, *Oryza sativa* (Abe & Futsuhara, 1986), barley, *Hordeum vulgare* (Hanzel *et al.*, 1984; Lührs & Lörz, 1987; Ohkoshi *et al.*, 1987) and wheat (Sears & Deckard, 1982; Lazar *et al.*, 1983; Maddock *et al.*, 1983; He *et al.*, 1988).

The most complete definition of the genetic control of regeneration has been accomplished in alfalfa. Two dominant genes, named *Rn1* and *Rn2*, were initially found to be necessary for a high frequency of regeneration to occur (Reisch & Bingham, 1980). Two dominant genes were also found to be required for regeneration in other populations of alfalfa. These were named *Rn3* and *Rn4* by Wan *et al.* (1988) and *Rna* and *Rnb* by Hernández-Fernández and Christie (1989). Although given different names, these three gene pairs have not been tested for allelism, making it conceivable that they are allelic in all populations. In cucumber, two dominant genes are necessary for an intermediate frequency of somatic embryogenesis, while the p

sence of a third dominant gene provides a high frequency of regeneration. A single dominant gene conditions for embryogenesis from leaf tissues of orchardgrass (Gavin *et al.*, 1989). Only in red clover has regeneration capacity been attributed to a recessive gene, *rg*, while the ability to form callus prior to the initiation of somatic embryos is due to a single dominant gene, *C* (Broda, 1984).

Wheat, although a hexaploid species, makes an excellent organism in which to study the genetic control of regeneration, as a wide range of cytogenetic stocks are available. Using these stocks, a gene (or genes) on chromosome 4B has been found to be important for regeneration (Mathias & Fukui, 1986; Higgins & Mathias, 1987). In addition, a gene with major influence on regeneration capacity is located on the long arm of chromosome 2D, and genes with smaller influences have been located to the long arm of chromosome 2A and the short arm of chromosome 2B. These three genes appear to be regulated by a gene on the long arm of chromosome 2B (Kaleikau *et al.*, 1989). Finally, an example of the role cytoplasm may have on regeneration capacity is found in wheat. A rearrangement of the mitochondrial genome has been found in cultures of the cultivar Chinese Spring which have lost their embryogenic capacity. This rearrangement is like the arrangement normally found in the cultivars Talent, Captitole and Thésée, which all lack regeneration capacity (Rode *et al.*, 1988).

In most other species in which the genetic control of regeneration has been studied, it has not been possible thus far to identify individual genes, and investigators have resorted to the evaluation of regeneration capacity in quantitative terms. This is partly due to the large environmental influence on somatic embryogenesis. In a study of regeneration capacity in Brussels sprouts (*Brassica oleracea* var. *gemmifera*), genetic effects accounted for only 50% of the observed variability in somatic embryogenesis from microspores. Partial dominance was observed for regeneration capacity, and narrow-sense heritability was estimated at 0.48 (Ockendon & Sutherland, 1987).

One of the earliest attempts at a quantitative study of embryogenic capacity was conducted on red clover, in which additive genetic effects were found to be the most important determinants of the potential for somatic embryogenesis (Keyes *et al.*, 1980). This observation has held with almost all other species studied.

In an investigation of five maize inbreds differing in ability to form embryogenic callus, and F_1 and F_2 plants derived from a partial diallel, additive genetic effects explained 70% of observed variability for regeneration capacity, which was highly heritable (Tomes & Smith, 1985). Cytoplasmic effects were significant in depressing embryogenic capacity. In a separate set of crosses between A188, a genotype with a high regeneration capacity, and the widely used inbreds, B73 and Mo17 (Willman *et al.*, 1989), additive effects were more important than dominance effects in determining both the percentage of immature embryos forming somatic embryos and the number of plants regenerated per explant. Differences in regeneration from reciprocal crosses suggested that cytoplasmic or other maternal effects influence regeneration capacity. Based on analysis of genetic variances, the authors suggested that at least one gene or one group of linked genes was responsible for determining the frequency of somatic embryogenesis.

Analysis of a diallel between four genotypes of rice (Peng & Hodges, 1989) suggested that additive genetic effects (general combining ability) explained 49% of the variability observed for the number of plants regenerated, while dominance genetic effects (specific combining ability) and cytoplasmic effects accounted for 40% and 90%, respectively, of the observed variation. When frequency of explants undergoing regeneration was the parameter evaluated, additive, dominance and cytoplasmic effects accounted for 61%, 29% and 10% of the observed variation, respectively.

Diallel analysis of five winter wheat cultivars showed that additive, non-additive and cytoplasmic effects all influenced regeneration, with additive effects being the most important. Cytoplasmic effects are nevertheless sufficient to necessitate careful selection of maternal parents to ensure regeneration success (Ou *et al.*, 1989). Only in barley have dominance genetic effects been found to be more important than additive effects, as determined from the analysis of a diallel between seven genotypes (Komatsuda *et al.*, 1989). Nevertheless, broad-sense heritability of regeneration was estimated at 0.86, while narrow-sense heritability was estimated to be 0.72.

As with any trait under genetic control, it is possible to breed for the capacity to regenerate via somatic embryogenesis. The earliest success was in the breeding of tetraploid alfalfa for regeneration capacity, and resulted in Regen-S, a genotype with a high frequency of somatic embryo formation (Bingham *et al.*, 1975). The availability of Regen-S has facilitated a broad range of studies, including:

1. physiological aspects of somatic embryogenesis (Walker *et al.*, 1979; Walker & Sato, 1981; Stuart & Strickland, 1984a, b);
2. *in vitro* selection and subsequent recovery of plants resistant to the toxin produced by *Fusarium oxysporum f.* sp. *medicaginensis* (Hartman *et al.*, 1984);
3. regeneration of plants from protoplasts (Johnson *et al.*, 1981; Dijak *et al.*, 1986);
4. genetic transformation of alfalfa (D'Halluin *et al.*, 1990).

More recently, diploid alfalfa has also been bred for regeneration capacity (Ray & Bingham, 1989).

In addition to the breeding efforts in alfalfa, breeding and selection have increased the capacity for somatic embryo formation from microspores in maize (Petolino *et al.*, 1988) and rapid-cycling *Brassica campestris* (Aslam *et al.*, 1990). In maize, only 3.5% of F_1 plants from a three-way cross were

embryogenic, while this percentage increased to 23.4% of embryogenic S_1 plants after the first *in vitro* selection cycle. In *Brassica*, only 46% of plants in the parental population were embryogenic. This frequency increased to include 96% of the plants in the S_3 generation.

Obtaining plants from somatic embryos

It is self-evident that recovery of plants from somatic embryos, a process referred to as conversion, is essential for the ultimate success of any system based on somatic embryogenesis. Ease with which somatic embryos convert to plants differs widely across genotypes, species and culture systems, representing the entire continuum from the readily accomplishable to the currently impossible.

It is difficult to evaluate the comparative conversion efficiencies of various species and culture systems, as this statistic is seldom reported in the literature. Many authors have limited their reporting to the successful recovery of plants, providing little or no information on conversion rates. Many studies have not been designed to provide this information. For some species, the absolute conversion rate may not be a critical factor. For example, recovery of plants from somatic embryos of carrot and alfalfa, and microspores of oilseed rape is generally not considered limiting, although this is not necessarily due to high conversion rates. Instead, since large numbers of somatic embryos are readily obtainable, a low conversion rate becomes inconsequential. On the other hand, low numbers of somatic embryos coupled with a low conversion rate make it very difficult, if not impossible, to recover plants. For example, there are no published reports of plants recovered from somatic embryos of bean, *Phaseolus vulgaris* (Martins & Sondahl, 1984), and recovery of plants from somatic embryos of red bud, *Cercis canadensis*, has been very limited (Trigiano *et al.*, 1988).

Several factors probably contribute to lack of conversion capacity in somatic embryos. Just as the formation of somatic embryos is under genetic control, there is some evidence that conversion ability is also under genetic control, e.g. in alfalfa (Seitz Kris & Bingham, 1988) and soybean (Komatsuda & Ohyama, 1988). This control is separate from that controlling embryogenesis. In triticale (*X. triticosecale*), the formation of somatic embryos from microspores is under the control of nuclear genes with additive action, while conversion of these embryos is influenced by additive, non-additive and cytoplasmic effects (Charmet & Bernard, 1984). To the degree that they can be manipulated *in vitro*, simulation of the events that occur *in planta* is probably the best way to optimize both the maturation of somatic embryos and their conversion to plantlets. Timing of growth regulator applications to match those that occur *in planta*, use of low oxygen tensions and desiccation of mature embryos have all been suggested as treatments that can increase the efficiency of plantlet production (Carman, 1988, 1989).

Auxin

Although auxin is used to induce somatic embryogenesis, continued exposure to auxin has long been known to have a detrimental effect on the development of the apical meristem of somatic embryos (Halperin & Wetherell, 1964). Previous discussion in this chapter centred on continued exposure to auxin as a means of locking somatic embryos into a given developmental phase and establishing cultures undergoing repetitive embryogenesis. Continuous exposure can, however, be detrimental to normal development once ECs have been induced and the resulting somatic embryos have completed the initial stages of development. Exposing the explant tissue to an auxin for a short period followed by transfer to hormone-free basal medium has led to the recovery of somatic embryos and plants from alfalfa (Walker & Sato, 1981), pecan, *Carya illinoensis* (Wetzstein *et al.*, 1989), and black locust, *Robinia pseudoacacia* (Merkle & Wiecko, 1989). Other examples of the transfer of young proembryos to low- or no-auxin medium for their development include carrot (Gorst *et al.*, 1987) and *Eucalyptus citriodora*. Work in soybean (Parrott *et al.*, 1988) showed that only one week's exposure of explant tissues to auxin was necessary to obtain somatic embryos, and that increasingly longer exposures resulted in decreased development of the shoot meristem in the somatic embryos subsequently formed. In certain systems, auxin effects may persist for some time after removal of exogenous auxin. Activated charcoal has been added to the culture medium to further remove auxin from tissues (Buchheim *et al.*, 1989).

Desiccation

Desiccation, another characteristic of zygotic embryogenesis *in planta*, may play a role in terminating embryogenic developmental processes and triggering the germination and seedling developmental programmes (Rosenberg & Rinne, 1986, 1987, 1988). In those instances where high sucrose concentrations in the medium have been used to achieve conversion competence in somatic embryos (e.g. Lee & Thomas, 1985; Janick, 1986; Buchheim *et al.*, 1989; Carman, 1989), osmotic desiccation is a plausible explanation for the success of the treatment.

The imposition of an outright desiccation step has assisted in the conversion of somatic embryos of some species. Initial attempts at the desiccation of somatic embryos, coupled with the successful recovery of plants, were carried out in orchardgrass, and resulted in a 4% conversion rate following 3 weeks of storage (Gray *et al.*, 1987). Twenty percent of grape (*Vitis longii*) somatic embryos converted after desiccation, as opposed to only 5% conversion of somatic embryos that had not been desiccated (Gray, 1987). A subsequent study identified one genotype of grape that produced well-

developed embryos and responded well to desiccation, resulting in a 34% conversion rate. Conversion for this genotype was higher following desiccation than following treatments with benzyladenine (BA), gibberellin (GA) or abscisic acid (ABA) (Gray, 1989). Soybean embryos matured for 4 weeks on a basal medium survived desiccation and converted into plants at ten times the frequency of their non-desiccated counterparts (Hammatt & Davey, 1987; Parrott *et al.*, 1988). Similarly, a 5-day desiccation of pecan somatic embryos also raised germination rates (Wetzstein *et al.*, 1989). Finally, desiccation of interior spruce somatic embryos under high relative humidity resulted in a very high, uniform germination, and a conversion rate of 50%. In contrast, only 5% of embryos that were not desiccated converted into plants (Roberts *et al.*, 1990b).

Zygotic embryos developing *in planta* undergo a period during which they contain high concentrations of ABA. The role of ABA during embryogenesis is not completely clear, but there is evidence that ABA helps regulate both the accumulation of storage proteins and proteins that may help to protect the embryos during desiccation (Galau *et al.*, 1986, 1990; Hughes & Galau, 1989). The addition of 10^{-7} M ABA to developing somatic embryos promoted the development of mature, well-formed embryos of caraway (Ammirato, 1974) and carrot (Ammirato, 1983). In some conifers, including spruce (*Picea*) species and hybrids, exogenous ABA is critical for normal accumulation of storage reserves and subsequent viability of somatic embryos (Roberts *et al.*, 1990a). Application of ABA has also been associated with acquisition of desiccation tolerance in somatic embryos of alfalfa, such that 60% of somatic embryos exposed to ABA survived desiccation treatment (Senaratna *et al.*, 1989, 1990).

Mass propagation systems

Mass propagation of embryos

The fact that many embryogenic systems can be perpetuated via repetitive embryogenesis makes them potentially attractive for mass production of clonal plantlets. Theoretically, a culture initiated from a single explant can produce an unlimited number of embryos. This potential for virtually unlimited multiplication gives somatic embryogenesis a huge advantage over conventional vegetative propagation systems such as rooted cuttings, which are limited to the amount of material that can be harvested from the mother plant. Multiplication rates from embryogenic cultures also generally exceed those attainable with other tissue culture regeneration systems such as shoot micropropagation. A high multiplication rate, however, is only the first of several potential advantages offered by somatic embryogenesis in comparison with other vegetative propagation systems. For many species, particularly those characterized by the production of PEMs, both the proliferation of embryogenic cells and the development of individual somatic embryos can be accomplished in liquid medium, making possible the manipulation of very large numbers of propagules with a minimum of labour. For example, Drew (1980) estimated that a single litre of an embryogenic carrot suspension culture contained 1.35 million somatic embryos. If this capacity for liquid culture can be combined with automated, continuous culture technologies, there is the potential for even greater economies of scale.

Besides the features of embryogenic cultures which make them directly adaptable to large-scale production of plants, somatic embryos themselves have several desirable characteristics as propagules. Chief among these is the fact that the product of somatic embryogenesis is an embryo, in many cases very similar to the embryo in a seed of the same species, and potentially able to be exploited in the same way as a complete propagule comprising embryonic shoot and root tissues. Even more important than these physical attributes, however, is the fact that a somatic embryo carries the 'program' to make a complete plant, and is capable of doing so with very little labour input. Unlike other vegetative propagation systems, there is no requirement for separate shoot growth and rooting steps to obtain complete plantlets. Furthermore, in contrast to regeneration systems that rely on organogenesis or axillary branching, many embryogenic systems are capable of producing individual embryos, unattached to either mother tissue or other embryos. No additional labour input is required to obtain individual propagules before further manipulation. Thus, embryogenic cultures produce propagules that are not only complete, but also discrete as well. The combination of these two properties gives somatic embryos potential for direct delivery to the greenhouse or field as components of artificial seeds or in fluid drilling systems. Such applications have been the subject of a number of studies over the past five years.

Somatic embryos do differ from their sexual counterparts in that they have bypassed genetic segregation and recombination. Consequently they represent a method of clonal propagation, maintaining the genotype of the plant from which the explant tissues came, and subject only to the natural mutation rate and mutations that may be induced *in vitro*. The latter mutation rate has never been strictly quantified, but is lower than that incurred by organogenic regeneration systems (Hanna *et al.*, 1984). Since somatic embryos must express many more developmental genes in order for ontogeny to be completed successfully, they may be less tolerant of mutations and epigenetic changes than organogenic cultures (Ozias-Akins & Vasil, 1988). Hence, there is the potential for lower variability among plantlets derived via embryogenesis than among those produced via organogenesis. This may be important to plant propagators interested in maintaining fidelity among regenerants.

For at least one plantation species, oil palm (*Elaeis guineensis*), the large-scale production of somatic embryos to provide clonal material is already a reality. Since 1983, over 280 ha of experimental oil palm plantations have been established in Ivory Coast, using somatic embryo-derived plants. Recently, the first performance assessments of these plants showed that the clones provided the desired homogeneity for such characters as percentage of oil per mesocarp. Although a few abnormalities were observed in floral morphology, these apparently did not affect overall oil yield (Durand-Gasselin *et al.*, 1990).

Two features mentioned in this section which may be of special significance for commercialization purposes are the potential for somatic embryos to be grown in large volumes in continuous liquid culture and the potential for these embryos to be used as directly delivered propagules. Detailed discussions of these areas of research follow.

Scale-up potential

As noted earlier, an important advantage of many embryogenic cultures with respect to mass propagation is the ability not only to maintain the cultures in suspension, but to induce embryo development in this state as well. Over the past decade, researchers have developed several techniques to obtain large numbers of well-developed somatic embryos from suspension cultures. Although the primary aim of these early experiments was elucidation of the process involved in embryo development, the application of these techniques to mass propagation is now apparent. Fujimura & Komamine (1975) reported that high rates of synchronous embryo formation could be obtained in embryogenic carrot suspension cultures by thorough removal of auxin from the culture medium, combined with sieving the suspensions through nylon screens to obtain cell clusters of uniform size. Later this method was refined by subjecting the fractionated cell clusters obtained by sieving to density-gradient centrifugation in Ficoll solutions. This step was followed by repeated centrifugation of the heaviest fraction of cell clusters at low speed for very short periods in culture medium (Fujimura & Komamine, 1979). The density-gradient fractionation step effectively removed larger vacuolated cells from the suspension, resulting in synchronous embryo formation, with over 90% of the initial cell clusters forming embryos. The synchronous embryo populations were subsequently employed in studies designed to define the morphological and biochemical stages of embryo development (Fujimura & Komamine, 1980; Fujimura *et al.*, 1980, 1981). Nomura & Komamine (1985) extended the fractionation of embryogenic suspension cultures to single cells, which could be separated by density-gradient centrifugation in Percoll solutions. Embryogenic frequencies of cell populations obtained in this manner were as high as 30%, and the frequency could be increased to 90% by manual selection of single spherical cells.

Populations of suspension-cultured somatic embryos separated by developmental stage have also been purified without the necessity of density-gradient centrifugation. Giuliano *et al.* (1983) fractionated carrot PEMs on nylon sieves, cultured the PEMs in basal medium for 6–8 days and fractionated the developing embryos on a second set of sieves. Differential sedimentation of early embryo stages and undifferentiated cells in the liquid medium following fractionation made it possible to further purify the embryos.

While fractionation using sieves and/or density-gradient centrifugation has been sufficient to produce synchronous populations of embryos in carrot, there have not been corresponding reports of similar success with embryogenic suspensions of other species. It is likely that other systems require different physical or chemical conditions to promote synchrony. As previously discussed in the section on plantlet production from somatic embryos, Ammirato (1974) showed that 10^{-7} M ABA promoted the production of relatively synchronous populations of mature somatic embryos of caraway (*Carum carvi*), preventing precocious germination and the production of multiple embryo clusters. Later, when he reported that the same level of ABA had a similar effect on suspension-cultured carrot somatic embryos (Ammirato, 1983), he proposed that regulation of embryo maturation by ABA might be used to facilitate large-scale batch cultures of somatic embryos for applied purposes. Nadel *et al.* (1990) combined ABA and sieving on metal mesh screens to obtain synchronous populations of celery (*Apium graveolens*) somatic embryos. These authors stressed that, while the effect of ABA was significant in synchronization of the embryos, it did not improve singularization or maturation of the embryos.

The promising experimental results noted above, obtained using model systems of embryogenic cells and somatic embryos grown in liquid medium, have prompted researchers to test the application of engineering technology to these cultures. The ultimate goal of this research is the development of large-scale mechanized or automated culture systems for commercialization. Such systems have the potential to generate huge numbers of embryos with low labour inputs, decreasing the costs per propagule to the point where, depending on the crop, they may be competitive with sexual seeds. Furthermore, in combination with value-added inputs to the propagules made possible by hybridity or by genetic engineering, such large-scale clonal multiplication systems may someday be the preferred means of production for some specialized crops (see sections on economics and gene transfer).

Although the idea of applying scale-up technologies to somatic embryo production has been discussed for some time, few results from the actual testing of model systems have been reported to date. An early effort at large-scale culture of carrot cells in 20-litre carboys was reported by Backs-Hüsemann & Reinert (1970). However, few embryos were formed. Over the past 20 years, one system which has been repeatedly tested for use with plant cell cultures is the stirred-tank bioreactor (Wilson *et al.*, 1971; Ma[illegible].

1980; Kurz & Constabel, 1981), an apparatus originally designed for microbial fermentations. Most efforts to adapt this system to plant suspension cultures have had the goal of harvesting plant cell by-products (e.g. Ten Hoopen *et al.*, 1990), not propagules. Regardless of the goal, however, those employing these bioreactors with plant cells have struggled with the problem of high shear forces generated by stirring (Fowler, 1987; Leckie *et al.*, 1990). As a result, many researchers have concentrated on alternative bioreactor designs using means of agitation which produce less shear. Air-driven bioreactors are one such alternative, and have been shown to support growth of several plant cell types (Fowler, 1984). When adapted to the production of somatic embryos, air-lift bioreactors have for the most part given disappointing results. Stuart *et al.* (1987) reported that air-lift bioreactors could produce large numbers of alfalfa somatic embryos, but conversion rates of these embryos were disappointing. Walker (1989) tested relative embryo production by suspension cultures of Norway spruce (*Picea abies*) grown in air-lift bioreactors versus shaken flasks. Although conversion was not tested, somatic embryos were produced at a higher frequency in the shaken flasks.

The most efficient bioreactor designs for growth of plant cells are those allowing continuous culture, as opposed to batch culture (Styer, 1985). In a continuous-culture bioreactor, the tank is initially filled and inoculated as with a batch culture. Then, as the culture grows into the log phase, fresh medium is introduced at a low rate while the same volume of spent medium and cells is removed. Those attempting to adapt this bioreactor design to production of somatic embryos are faced with the problem of spent-medium removal without lowering the concentration of cells needed to perpetuate embryo production. Styer (1987) reported that use of a spin filter allowed removal of spent medium from the bioreactor without loss of suspension-cultured carrot cells, enabling maintenance of cell populations at the desired density. Furthermore, replacement of cell-proliferation medium with embryo-differentiation medium in this bioreactor produced a constant number of PEMs, each of which increased in cell number.

An automated system for large-scale commercial plant propagation, incorporating a bioreactor, has been developed by Plant Biotech Industries, Ltd. This system is apparently capable of handling somatic embryos, as well as other propagules such as microtubers and bulblets (Levin *et al.*, 1988; Levin & Vasil, 1989). A bioprocessor controls separation, sizing and distribution of propagules into a culture vessel, and the system is linked to an automated transplanting machine capable of transferring 8000 plantlets per hour to potting mix in greenhouse trays. Levin *et al.* (1988) claimed that compared with conventional tissue culture propagation techniques, their bioreactor-based system provided substantial savings in space, time and labour, as well as lower contamination rates, together with accurate monitoring and control of temperature, pH and gas concentrations.

Despite promising results reported in the last few years, bioreactor technology has yet to meet its apparent potential to produce somatic embryos capable of growing into plantlets on a scale that would make them economically competitive with true seedlings. Recently, Cazzulino *et al.* (1990) reported on a systematic method to improve the capability of bioreactors to produce competent embryos, by applying a kinetic model of carrot somatic embryo development in suspension culture. The model was developed by monitoring substrate utilization, culture growth and embryo development over time in an embryogenic culture. The kinetic model will be employed to optimize bioreactor conditions for high-frequency production of mature somatic embryos capable of growing into plants.

It is probably only a matter of time until the performance of a number of embryogenic suspension culture systems will be improved to the point where they can be combined with bioreactor technology for economic, large-scale plantlet production. As mentioned earlier, however, it should be remembered that some embryogenic systems are much more amenable to suspension culture than others. For example, systems such as soybean develop in liquid medium not as true cell suspensions or PEMs, but as adherent clumps of proliferating globular embryos (Finer & Nagasawa, 1988), making procedures such as synchronization by sieving very difficult with current techniques. Clearly, if large-scale soybean somatic embryo production is to be accomplished, means of manipulating these cultures will have to be developed that are very different from those described above for carrot. Even some embryogenic systems which appear to respond well in suspension culture have not met expectations when tested for productivity of mature embryos capable of conversion to plantlets. For example, Stuart *et al.* (1987) reported that the conversion percentage of alfalfa somatic embryos grown on semisolid medium was three times higher than that of embryos grown in liquid medium in shaker flasks, and 30 times higher than that of embryos grown in bioreactors. Researchers working with alfalfa have resorted, therefore, to a modified protocol in which embryogenic cell clusters are removed from suspension before development into mature embryos. Large numbers of roughly synchronous somatic embryos are obtained by sieving embryogenic suspensions on nylon mesh and immediately placing the desired fraction of cell clusters, with the mesh, on to semisolid basal medium (McKersie *et al.*, 1989; Senaratna *et al.*, 1989, 1990).

As with alfalfa, embryogenic suspension cultures of yellow poplar (*Liriodendron tulipifera*) are very prolific with respect to embryo production, but embryos allowed to mature in liquid medium convert poorly compared with embryos developing from PEMs on semisolid medium. Therefore, as illustrated in Fig. 7.3, embryogenic suspensions of this species were fractionated by sieving and the desired fraction of PEMs was backwashed from the screen on to a disc of filter paper in a Buchner funnel. After the liquid medium was drawn off under low vacuum, the filter paper with PEMs was

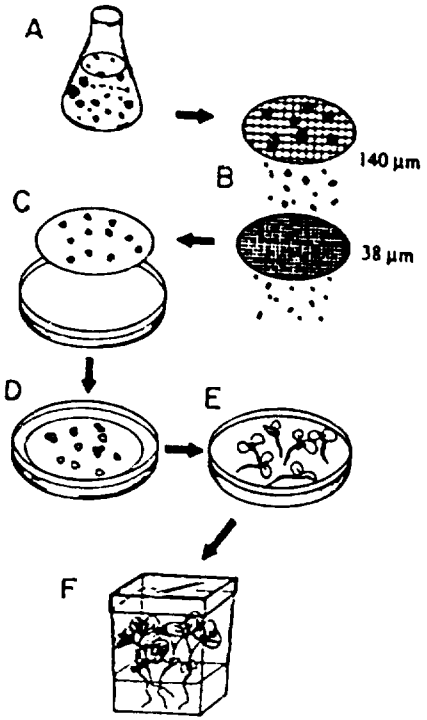

Fig. 7.3. Synchronization by sieving of yellow-poplar embryogenic suspension cultures. A) Embryogenic cells are grown in a liquid induction medium. B) Sieving the suspension through stainless steel mesh isolates PEMs, which are retained on the 38 µm sieve. C) The isolated PEMs are placed on filter paper and plated on semisolid basal medium. D) Mature embryos develop synchronously over the course of 12–14 days. E) Transfer of the mature somatic embryos to basal medium without filter paper permits germination. F) Plantlets are ready to transfer to a soil mix 6–8 weeks after transfer to plant development medium. (Artwork by J. Adang.)

placed on semisolid basal medium. This protocol of fractionation and plating, combining the high proliferation of the embryogenic suspension culture with the favourable maturation conditions of semisolid medium, resulted in production of hundreds of roughly synchronous, well-formed embryos within a few weeks (Fig. 7.4). Over 70% of the embryos obtained in this manner were capable of converting to plantlets (Merkle & Wiecko, 1990).

As illustrated by the examples above, relatively large-scale production of somatic embryos from suspension cultures is possible, even without a complete understanding of the factors controlling embryo development in liquid medium. In alfalfa and yellow poplar, problems arose from a differential ability of somatic embryos to mature correctly in liquid medium as compared with semisolid medium. The immediate solution was simply to avoid having maturation occur in liquid medium. Certainly for experimental purposes, such systems can produce adequate numbers of embryos. For mass production of propagules, however, economic application of embryogenic suspension cultures appears to depend on the ability to combine these systems with bioreactor technology and still achieve efficient production of consistent embryos. If all the steps of somatic embryo proliferation development and maturation are to be accomplished in the bioreactor, the conditions controlling these stages in liquid culture must be defined.

Delivery systems

Encapsulation of embryos

In many instances, the only major differences between somatic embryos and their zygotic counterparts are the lack of storage reserves and the absence of a seed coat. Seed storage reserves are normally present either in the form of endosperm (megagametophyte in the case of conifer embryos) or storage cotyledons. For somatic embryos, endosperm and megagametophyte are

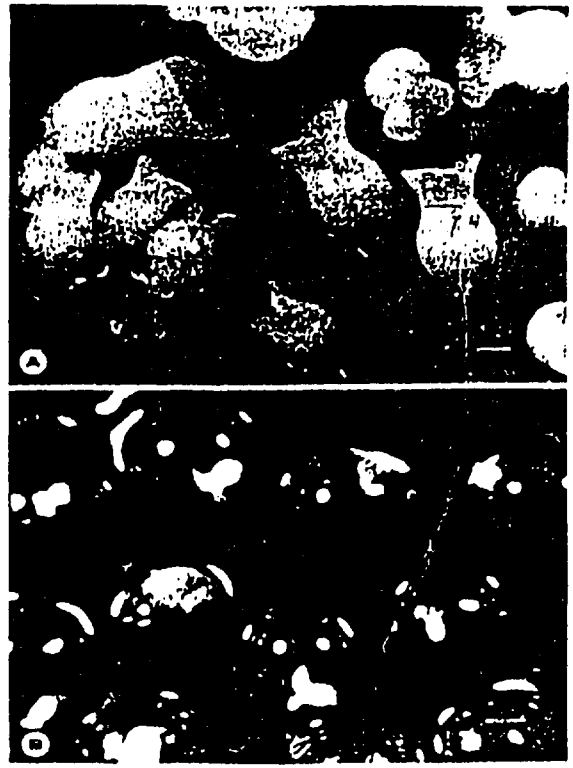

Fig. 7.4. Synchronized somatic embryos and their use as synthetic seeds. A) Mature somatic embryos of yellow poplar two weeks after sieving and plating on filter paper. B) Calcium alginate-encapsulated somatic embryos of yellow poplar. (Bar = 500 µm.)

absent, and, even in species where reserves are stored in the cotyledons, these organs do not generally achieve normal storage capacity in culture. Because of these missing seed components, additional protection and nutrition must be supplied in order for somatic embryos to be used as directly delivered propagules. Systems are needed which are capable of protecting the embryos until they are delivered to the desired growing locations, keeping them viable until conditions are favourable for plantlet survival, and supplying them with the nutrients and other compounds needed to promote early growth. The similarity of somatic embryos to zygotic embryos suggests a number of ways in which delivery systems may be developed to handle them. Basically three delivery methods have been tested over the past eight years: encapsulation to produce artificial seeds, fluid drilling, and use of desiccated, uncoated somatic embryos. Artificial seeds tested to date can be further classified into two subgroups: desiccated somatic embryos encapsulated in a water-soluble resin and hydrated somatic embryos encapsulated in hydrated gels.

The concept of a synthetic seed made by encapsulating individual somatic embryos was first presented by Murashige (1978), but the required properties of a synthetic seed coat were first clearly defined by Redenbaugh *et al.* (1986, 1987a). According to these authors, the encapsulation material must both protect the embryo, being sufficiently durable to withstand handling during transport and planting, and at the same time allow for germination and conversion. It should also be able to hold and deliver nutrients, other chemical factors and even micro-organisms to promote germination and early plant growth. In addition, if a synthetic seed is to be used by current growers, it must be sowable using existing greenhouse and farm machinery.

Encapsulation of somatic embryos was first reported by Kitto & Janick (1985a, b), who applied synthetic seed coats to clumps of carrot somatic embryos by mixing equal volumes of embryo suspension and a 5% solution of polyethylene oxide (Polyox WSR N-750), a water-soluble resin, which subsequently dried to form polyembryonic wafers. Embryo 'hardening' treatments, including treatments with 12% sucrose or 10^{-6} M ABA, chilling and high inoculum density, increased survival of encapsulated and desiccated somatic embryos to as high as 58%. Kim & Janick (1989) also reported survival for up to 9 days of desiccated somatic embryo of celery encapsulated in Polyox, while non-encapsulated embryos did not survive beyond 4 days of desiccation. The authors hypothesized that survival enhancement by Polyox encapsulation may have been due to slowed drying brought about by high relative humidity inside the wafers. Although Polyox-encapsulated, desiccated somatic embryos were demonstrated to produce plantlets, conversion frequencies were not reported for either the carrot or celery embryos.

The first work on development of artificial seeds using individually encapsulated somatic embryos was reported by Redenbaugh *et al.* (1984, 1986, 1987a, b), who encapsulated somatic embryos of alfalfa, celery and cauliflower (*Brassica oleracea*) by mixing them with sodium alginate and dropping them into a solution of calcium chloride to form calcium alginate beads via an ion exchange reaction (Fig. 7.4). Other hydrogels were tested as possible encapsulating agents, but were not found to be as useful. Redenbaugh *et al.* (1986) found that the encapsulated embryos germinated and formed seedling-quality plants *in vitro* at the rate of 29% for randomly picked alfalfa somatic embryos and 55% for celery somatic embryos that had been selected for high quality. Encapsulated alfalfa and celery embryos sown in sand or peat plugs converted at 7% and 10%, respectively. Various problems with encapsulation in alginate were identified, including rapid leaching of water-soluble nutrients out of the capsules, obstruction of root and shoot emergence, rapid drying of capsules when exposed to air and tackiness of the capsules, which caused difficulty with handling and machine planting (Redenbaugh *et al.*, 1987a). Some of these problems were resolved. For example, root and shoot penetration of the capsule was facilitated by controlling the alginate concentration and complexing time, and a coating compound prevented the capsules from drying too rapidly and lowered the tackiness of the capsules to the point where they could be machine planted. The authors emphasized, however, in this and later studies (Redenbaugh *et al.*, 1987b; Fujii *et al.*, 1989; Redenbaugh & Ruzin, 1989; Redenbaugh, 1990), that the success of an encapsulation system is determined not so much by the ability to successfully encapsulate the embryos, but by the quality of the embryos themselves, since, with or without encapsulation, conversion to plantlets is controlled by this variable. Lutz *et al.* (1985), who tested encapsulation of individual carrot somatic embryos in a gel matrix, also concluded that embryo quality was the limiting factor in developing a delivery system, although they did not report conversion frequencies. Alginate encapsulation has also been tested with loblolly pine (*Pinus taeda*) somatic embryos, which survived encapsulation and storage at 4°C for 4 months, but did not convert to plantlets (Gupta & Durzan, 1987).

In recent work, Fujii *et al.* (1989) employed selected embryos with *in vitro* conversion frequencies of 70–90% to test the effects of embryo maturation treatments on conversion frequency of alfalfa artificial seeds on potting mix. Embryo maturation with ABA at 1–5 μM resulted in greenhouse conversion rates of up to 64% when a humidity-tent watering system was used to prevent soil surface drying. Attempts to replace ABA with 8% mannitol during maturation did not result in similarly high conversion rates.

Fluid drilling

Another potential delivery system for somatic embryos is fluid drilling, in which embryos are suspended in a viscous carrier gel which is extruded into the soil. Originally, this technique was applied as a means of sowing pre-

germinated seeds in order to improve seedling emergence and the uniformity of the crop stand (Currah *et al.*, 1974). The primary problem with application of fluid drilling to carrot somatic embryos was found to be that embryos at a developmental stage early enough to bulk-handle for fluid drilling would not continue development and convert to autotrophic plantlets without the addition of sucrose to the gel. The sucrose level necessary to permit conversion, however, would also be expected to promote rapid growth of contaminating micro-organisms in this non-aseptic system (Drew, 1979; Lutz *et al.*, 1985). Baker (1985) conditioned carrot somatic embryos with sucrose and ABA prior to sowing them into gel supplemented with growth regulators, sucrose and nutrients, on top of soil mix in the greenhouse. Embryo survival, however, was only 4% and no embryos converted to plantlets. Schultheis *et al.* (1986) tested different gels supplemented with nutrients, vitamins and sucrose for their effect on survival and growth of sweet potato somatic embryos. While embryos did not survive beyond 6 days in some gels, one product (Natrosol 250HHR) allowed embryo growth. Schultheis & Cantliffe (1988) subsequently found that 20–25% of sweet potato somatic embryos formed plants when placed in the same gel containing MS medium supplemented with sucrose, maltose or glucose.

One concept for the generation of artificial seeds from somatic embryos does not rely on any coating or matrix to maintain embryo viability during storage or delivery. Instead, it makes use of the fact that zygotic embryos typically cease growth, lose water and become dormant or quiescent until a signal for germination is received. As discussed earlier, this natural desiccation pattern can be used to greatly enhance conversion of somatic embryos of a number of species. In addition to promoting conversion, dehydration is an alternative method for inducing somatic embryo quiescence for storage and handling (Gray, 1987). Gray (1989) also emphasized the potential role of uncoated dehydrated somatic embryos in germplasm conservation, where the primary goal is long-term storage of relatively small numbers of propagules. However, as development of propagules for commercial planting was not an objective of his study, he did not test conversion of his dehydrated grape somatic embryos under greenhouse conditions or discuss the encapsulation that presumably would be necessary for large-scale sowing.

Economics of mass propagation via somatic embryogenesis

There is general agreement that somatic embryogenesis and in particular its application as artificial seeds offer the best potential of any tissue culture regeneration system for economic mass propagation of many crop species. Most plants produced by current organogenic *in vitro* methods cannot profitably be sold for less than about 25 cents per plant, and 40–60% of this production cost is accounted for by labour (Sluis & Walker, 1985). It is also generally accepted, however, that much higher production efficiency must be achieved before somatic embryos can compete with seeds of most species on a commercial basis. For example, Redenbaugh *et al.* (1987a) estimated the cost of alfalfa artificial seeds to be 0.026 cents each, including labour, materials and overhead, compared with 0.00066 cents per true alfalfa seed at that time. Nevertheless, if artificial seeds with modest unit costs can be developed for crops such as hybrid vegetables and flowers, they may be competitive with the relatively expensive natural seeds of these plants. Furthermore, value-added components such as clonal uniformity and epistatic interactions that are not normally heritable through sexual propagation may justify substantially higher costs for somatic embryos than for natural seeds of the same species (Redenbaugh *et al.*, 1987a).

Use in gene transfer systems

Prerequisites for a gene transfer system

Several systems have been devised that are capable of introducing foreign DNA into plant cells. These include microinjection of protoplasts (Crossway *et al.*, 1986; Mathias, 1987) and electroporation of DNA into protoplasts (Horn *et al.*, 1988; Toriyama *et al.*, 1988; Chupeau *et al.*, 1989; Chapter 4), two processes that are limited by the range of species for which plants can be efficiently regenerated from protoplasts. The domestication of the crown-gall pathogen, *Agrobacterium tumefaciens*, coupled with regeneration from leaf tissues (Horsch *et al.*, 1985), has made possible the transformation of those crops that are susceptible to *Agrobacterium* and regenerate via organogenesis, especially solanaceous or cruciferous species (Chapter 3). In such a system, *Agrobacterium* inserts into individual plant cells a gene that confers resistance to an antibiotic or herbicide, along with a gene of economic or academic importance. When the corresponding antibiotic or herbicide is incorporated into a medium that would otherwise permit callus formation, only those cells that have acquired the resistance gene from *Agrobacterium* are able to divide, effectively sorting non-transformed from transformed cells. Plants regenerated from transformed cells are themselves transgenic. Many plants have been transformed using this method, but most agriculturally important plants are not included in the list (Gasser & Fraley, 1989; Table 3.2).

Implicit in any gene transfer system is the requirement that, regardless of the methods by which DNA is inserted into a plant cell, the recipient cell must be totipotent. In *Agrobacterium*-mediated transformation systems, not only must the recipient cell be totipotent, but it must also be susceptible to *Agrobacterium*, a trait that varies among species and among tissues within a plant (Matthysse & Gurlitz, 1982). Lack of totipotency associated

protoplasts of several species is perhaps the factor most limiting to their use in transformation via microinjection or electroporation. For this reason, much attention has been given to the potential for isolation and culture of protoplasts from embryogenic cells. Theoretically, since the cells from which the protoplasts were isolated are known to be regenerable, the protoplasts would be expected to retain this property and yield cultures capable of forming whole plants (Shillito *et al.*, 1989). Thus, if embryogenic protoplasts are used in gene transfer protocols such as electroporation, microinjection or polyethylene glycol-mediated DNA uptake, there should be a high likelihood of recovering transgenic plants. In three groups of plants, embryogenic cultures have proven to be especially valuable in providing a source of totipotent protoplasts. These are the graminaceous species, citrus species, and forest trees, especially coniferous species.

Among graminaceous species, neither mesophyll-derived protoplasts nor protoplasts derived from non-morphogenic cell suspensions were found to be capable of regenerating whole plants (Vasil, 1985). However, when Vasil & Vasil (1980) isolated and cultured protoplasts from embryogenic cultures of pearl millet (*Pennisetum glaucum*), they gave rise to cell clusters from which embryos and plantlets were regenerated. Since this report, embryogenic suspension cultures have been used as sources of regenerable protoplasts of guinea-grass (*Panicum maximum*; Lu *et al.*, 1983a), Napiergrass (Vasil *et al.*, 1983), rice (Fujimura *et al.*, 1985; Abdullah *et al.*, 1986; Toriyama *et al.*, 1986; Yamada *et al.*, 1986; Kyozuka *et al.*, 1987), sugarcane (*Saccharum officinarum*; Srinivasan & Vasil, 1986), perennial ryegrass (*Lolium perenne*; Dalton, 1988), tall fescue (*Festuca arundinacea*; Dalton, 1988) and orchardgrass (Horn *et al.*, 1988). Protoplasts derived from embryogenic cultures of maize were used to regenerate somatic embryos (Kamo *et al.*, 1988; Vasil & Vasil, 1987) and plantlets which turned out to be sterile (Rhodes *et al.*, 1988). More recently, Shillito *et al.* (1989) and Prioli & Sondahl (1989) reported regeneration of fertile plants of élite inbred maize lines from protoplasts derived from embryogenic suspensions. Already, embryogenic suspension-derived protoplasts have proven useful for production of transgenic plants of graminaceous species. Transgenic (but sterile) maize plants were obtained following electroporation of embryogenic suspension-derived protoplasts (Rhodes *et al.*, 1988) and protoplasts from embryo-derived callus of rice (Shimamoto *et al.*, 1989). In these two examples, the tissues used to obtain protoplasts could be classified as consisting of IEDCs and PEDCs, respectively. It is clear that the embryogenic state of such cells facilitates the recovery of somatic embryos from cell colonies derived from protoplasts.

Among citrus species, Vardi *et al.* (1982) first reported regeneration of plantlets from protoplasts isolated from embryogenic nucellar callus of orange, mandarin and grapefruit cultivars. Since this report, plant regeneration has been achieved from embryogenic suspension-derived protoplasts of a number of citrus species and cultivars (Vardi & Galun, 1988). Although we are not aware of reports of the use of these protoplast cultures for regeneration of transgenic plants, they have made possible the production of interspecific and even intergeneric somatic hybrids via protoplast fusion (Ohgawara *et al.*, 1985; Grosser *et al.*, 1988a, b, 1989; Kobayashi & Ohgawara, 1988; Kobayashi *et al.*, 1988).

As for graminaceous species, the key to successful regeneration from protoplasts of coniferous species proved to be the isolation of protoplasts from embryogenic callus and suspension cultures. Given that the first reports of somatic embryogenesis in conifers did not appear until 1985 (Hakman *et al.*, 1985; Nagmani & Bonga, 1985), the application of embryogenic cultures to protoplast studies has had a large impact in a short period of time. To date, regeneration of somatic embryos from embryogenic suspension-derived protoplasts has been reported for loblolly pine (*Pinus taeda*; Gupta & Durzan, 1987), white spruce (*Picea glauca*; Attree *et al.*, 1987, 1989a; Bekkaoui *et al.*, 1987), Douglas-fir (*Pseudotsuga menziesii*; Gupta *et al.*, 1988), European silver fir (*Abies alba*; Lang & Kohlenbach, 1989) and black spruce (*Picea mariana*; Tautorus *et al.*, 1990). In addition, plantlet production from protoplast-derived somatic embryos has been reported for white spruce (Attree *et al.*, 1989b) and hybrid larch (*Larix×eurolepis*; Klimaszewska, 1989).

Among hardwood forest tree species, another group for which successful protoplast culture has lagged, embryogenic suspension cultures have also been shown to be a valuable source of highly regenerable protoplasts. Two hardwood species for which embryogenic suspensions have been used as sources of protoplasts capable of yielding somatic embryos and plantlets are sandalwood (*Santalum album*; Rao & Ozias-Akins, 1985) and yellow-poplar (Merkle & Sommer, 1987).

Despite the success with which embryogenic suspension cultures have been applied to protoplast culture in forest tree species, there have been no reports to date of their use for stable integration of foreign DNA into somatic embryos or plantlets. There have, however, been reports of transient expression of DNA electroporated into embryogenic suspension-derived protoplasts of loblolly pine (Gupta *et al.*, 1988), Douglas fir (Gupta *et al.*, 1988) and white spruce (Bekkaoui *et al.*, 1987). Those species whose protoplasts are totipotent are also amenable to transformation technologies less tedious than regeneration from protoplasts.

Gene transfer in indirect somatic embryogenesis systems

Regeneration via indirect somatic embryogenesis, characterized by the presence of a callus phase prior to the formation of somatic embryos, is as amenable to *Agrobacterium*-mediated transformation as an organogenic system. As described previously, transformed cells are selectively multiplied

to form a callus, but an embryo, not a shoot, is ultimately formed by the callus. Plants that have been transformed via indirect somatic embryogenesis include alfalfa (Deak *et al.*, 1986; Shahin *et al.*, 1986; Chabaud *et al.*, 1988; D'Halluin *et al.*, 1990); cotton, *Gossypium hirsutum* (Firoozabady *et al.*, 1987; Umbeck *et al.*, 1987); carrot (Thomas *et al.*, 1989); eggplant, *Solanum melongena* (Filippone & Lurquin, 1989); and sunflower, *Helianthus annuus* (Everett *et al.*, 1987).

A related technique involves the use of *Agrobacterium rhizogenes*, the causal agent of hairy root disease, instead of *A. tumefaciens*. In this instance, the callus phase is replaced by proliferation of roots from transformed cells, and somatic embryos develop directly from transformed root tissues. This technique has been successfully used with both cucumber, *Cucumis sativus* (Trulson *et al.*, 1986), and alfalfa (Spano *et al.*, 1987; Sukhapinda *et al.*, 1987). There is a drawback in that growth regulators produced by the *A. rhizogenes*-derived genes responsible for rhizogenesis can alter the morphology of regenerated plants (see Chapter 3).

A compelling advantage of adapting *Agrobacterium*-mediated transformation to indirect embryogenic systems is that, once obtained, transformed calli or roots can be propagated continuously to produce large numbers of embryos. For this method to be effective, however, not only must a plant species be amenable to *Agrobacterium*, but it must also have the ability to form somatic embryos from callus. At present, only a limited number of species fulfil both of these requirements. As discussed previously in this chapter, even within a species, genotypes differ in their ability to form somatic embryos. Consequently, preselection for an embryogenic genotype may be necessary for the efficient recovery of transformants.

Gene transfer in direct embryogenic systems

A large number of species are amenable to regeneration via direct somatic embryogenesis, especially from explants derived from immature zygotic embryos. Some species produce somatic embryos by this technique alone. The combination of transformation with direct embryogenesis therefore represents the greatest potential for transformation of a number of important species.

In principle, the recovery of transgenic plants from a species that regenerates via direct somatic embryogenesis should be possible as long as three conditions are met. Firstly, somatic embryos must have a unicellular origin. Otherwise, embryos originating from multiple cells will be chimeric in nature, with transformed and non-transformed sectors arising from transformed and non-transformed cells within the original cell mass. Secondly, large numbers of single cells must regenerate; and, thirdly, the transformation system must transform large numbers of cells. The last two conditions are necessary to ensure a reasonable probability that a given cell will be transformed and will be recovered as a somatic embryo. For example, if in a given explant only 1 in 10^5 cells become embryogenic, and the available transformation technique transforms only 1 in 10^6 cells, the probability of transforming a cell that will become embryogenic is the product of the two, or 1 in 10^{11}.

In practice, the two critical properties of regeneration (from single cells at high frequency) do not commonly occur together. Nevertheless, an example of such a system is found in oilseed rape, individual microspores of which can be induced to become embryogenic. Microspores and proembryos derived from microspores were exposed to *Agrobacterium*, and transgenic plants subsequently recovered (Pechan, 1989). Embryos were originally believed not to have the necessary cell wall sites for infective attachment of *Agrobacterium* (Lippincott & Lippincott, 1978; Matthysse & Gurlitz, 1982; Sequeira, 1984), but the successful transformation of embryogenic cultures shows that this is not an absolute limitation.

Repetitive embryogenesis and plant transformation

An obvious limitation to plant transformation via direct somatic embryogenesis is that somatic embryos arising directly from an explant can have multicellular origins (reviewed by Williams & Maheswaran, 1986), while available gene transfer techniques transform only individual cells. Furthermore, the lack of a callus phase precludes the opportunity to preferentially propagate transformed cells before formation of somatic embryos. The result is the development of chimeric somatic embryos comprising transformed and non-transformed sectors.

The process of repetitive somatic embryogenesis circumvents the problem of chimeric embryos, by allowing recovery of completely transformed secondary embryos from transformed sectors within a primary somatic embryo. Repetitive cycles of direct embryogenesis effectively substitute for the callus phase found in indirect embryogenic systems, and make repetitive embryogenesis a powerful method by which to obtain a wide range of transgenic plants.

As embryos formed during repetitive embryogenesis can originate from single cells (e.g. Haccius, 1977; Polito *et al.*, 1989), the number of transformed cells in the original embryo need not be high. This principle was first used for *Agrobacterium*-mediated transformation of embryogenic suspensions of carrot (Scott & Draper, 1987), and was later applied to embryogenic cultures of English walnut, *Juglans regia* (McGranahan *et al.*, 1988, 1990). Even if chimeric embryos are still recovered from the first cycle of repetitive embryogenesis, continued cycling in the presence of a selective agent eventually results in embryos consisting entirely of transformed cells. Since the transformation of walnut was first reported, this technique has also been used to transform secondary embryogenic cultures of oilseed rape originally derived from microspores (Swanson & Erickson, 1989).

Microprojectile transformation of repetitively embryogenic systems

As described above, *Agrobacterium*-mediated transformation combined with repetitive direct embryogenesis may be an efficient method by which to obtain transgenic plants, as long as the species or genotype in question is susceptible to *Agrobacterium*. This requirement, however, immediately excludes most monocotyledonous and some dicotyledonous plants of

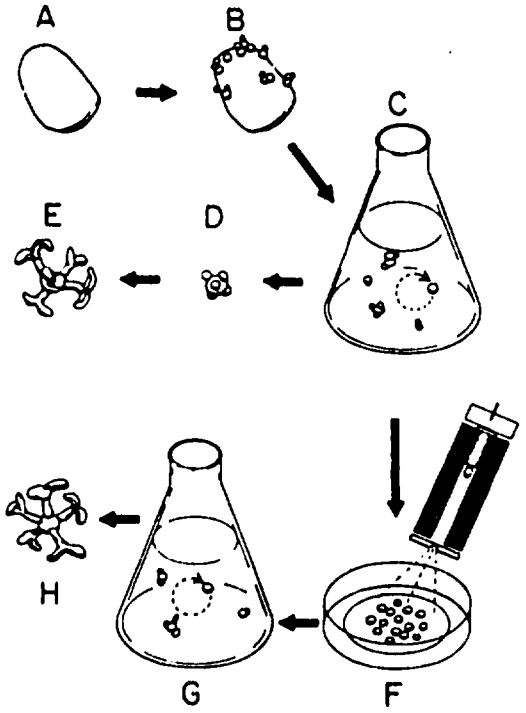

Fig. 7.5. Embryogenic suspensions of soybean, and their use in genetic transformation (based on Finer & Nagasawa, 1988; McMullen & Finer, 1990). A) Immature cotyledons, with the embryonic axes removed, are exposed to 40 mg/l of 2,4-dichlorophenoxyacetic acid (2,4-D) for 30 days. B) Somatic embryos form but their development is arrested at the globular stage. Globular-stage embryos give rise to secondary somatic embryos. C) Somatic embryos are placed in liquid culture, where the globular-stage embryos are propagated indefinitely. D) The repetitive cycle is broken by removing the embryogenic clusters from liquid culture and placing them on hormone-free basal medium. E) Globular embryos develop to the cotyledonary stage, at which time they can be separated, matured, desiccated and germinated to give plantlets. F) Embryogenic clusters growing in suspension can be subjected to microprojectile bombardment. G) Following bombardment, the embryogenic clumps are returned to liquid culture and grown in the presence of a selective agent. H) Recovery of transgenic embryos. (Artwork by J. Adang.)

agricultural importance (Chapter 3). Limitations imposed on plant transformation by the host range of *Agrobacterium* are being overcome by use of microprojectile bombardment, also known as particle-gun or gene-gun systems, to propel DNA-coated heavy metal particles into plant cells (Klein *et al.*, 1987; Sanford, 1988; Chapter 5). When applied to indirect or repetitively embryogenic systems, the technique has the advantage that, once transformed embryogenic cell lines are obtained, the capacity to produce somatic embryos is virtually unlimited.

The first to report the use of microprojectile bombardment for the transformation of an embryogenic suspension were Finer & McMullen (1990). Proliferation of a cotton embryogenic suspension after bombardment resulted in the production of transformed embryos, from which plants were subsequently obtained. McMullen & Finer (1990) later extended this technique to include the transformation of embryogenic suspensions of soybean (Fig. 7.5). The transformation of soybean is especially significant, as this species has been extremely difficult to transform using *Agrobacterium*. Although transformation of soybean following bombardment of meristematic tissues has also been reported (Christou *et al.*, 1989), this technique produces chimeric plants and lacks the potential for mass propagation associated with embryogenic systems. More recently, the bombardment of embryogenic cultures has been used to recover fertile transgenic maize plants (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990), using herbicide resistance as a selectable marker. Transgenic plants of the forest tree, yellow poplar, have also been obtained following microprojectile bombardment of PEMs isolated from embryogenic suspensions (cover photograph; H. D. Wilde, S. A. Merkle & R. B. Meagher, submitted).

Conclusions

Although present systems for commercial micropropagation and experimental transformation are based almost exclusively on shoot organogenesis, there are definite indications that somatic embryogenesis will contribute improved methods for future biotechnological applications. The process of somatic embryogenesis offers a means for propagating essentially limitless numbers of discrete propagules, functionally similar to seed embryos, in large-scale or continuous cultures. At present, therefore, it represents the greatest potential for scaling up volume and reducing the labour costs of mass propagation. The most immediate problems are to define the conditions required for normal development and maturation of embryos of a variety of species in liquid culture, and to develop more reliable methods for direct delivery of propagules to the greenhouse or field.

Looking further ahead, somatic embryogenesis also offers potential for efficient production of transgenic populations in a range of plant species.

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Chapter 4

APPLICATIONS OF SOMATIC EMBRYOGENESIS AND EMBRYO CLONING

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I. INTRODUCTION

Somatic embryogenesis is the process by which somatic cells develop through the stages of embryogeny to give whole plants without gamete fusion. Although a number of specialized examples of somatic embryogenesis have been reported to occur in vivo, the process is best known as a pathway for regeneration in vitro. Somatic embryogenesis may be "direct", with embryonic cells developing directly from explant cells, or it may be "indirect", with a number of unorganized, non-embryonic mitotic cycles interposed between differentiated explant tissue and recognizable embryonic structures. The linguistic distinction can be misleading. In practice these terms define opposite ends of a continuum whose intermediate regions may be difficult to quantify. To distinguish patterns of embryogenesis as direct or indirect based simply on intercalation of mitotic cycles between explant and embryo organization is, in physiological terms, an oversimplification. The most meaningful way to define "direct" and "indirect" appears to be with reference to the epigenetic state of explant cells. Thus, somatic cells which are themselves embryonic, or not far removed from embryonic, are generally more easily induced to undergo somatic embryogenesis than differentiated vegetative cells. Highly differentiated cells appear to require major epigenetic changes, making the initiation of embryogenesis less direct. In these terms, the directness of embryogenesis is measured as epigenetic "distance" of explant cells from the embryonic state. This concept was recognized by Sharp et al. (1980, 1982) and Evans et al. (1981) who classified cells undergoing somatic embryogenesis as either "pre-embryogenic determined cells" (PEDCs) or "induced embryogenic determined cells" (IEDCs). PEDCs are epigenetically embryonic at explanting, e.g., cells of a zygotic embryo, whereas IEDCs are the product of an epigenetic switch to the embryonic state in culture. Once induced, IEDCs are functionally equivalent to PEDCs, and both can be maintained and multiplied in the embryonic state under appropriate culture conditions. Such cultures consist of proliferating globular proembryos or proembryogenic masses (PEMs). The critical difference between direct and indirect embryogenesis is therefore the distinction between PEDCs and IEDCs. Direct and indirect embryogenesis have distinct advantages and limitations with respect to particular applications.

Somatic embryos have been induced from a variety of plant tissues, most frequently from zygotic embryos, germinating seedlings, shoot meristems, and young inflorescences. In certain species, nucellus (Kochba, 1972, Srinivasan and Mullins, 1980), leaf (Conger et al., 1983), anther filament (Jorgensen, 1989), or root (Chang and Hsing, 1980) have also given rise to embryos. In addition, the production of haploid plants from cultured anthers or microspores involves a switch of young microspores to the embryogenic state and their development into haploid somatic embryos [See Williams and Maheswaran (1986) for a summary of explant types giving rise to embryos]. Because of the broad spectrum of conditions and applications associated with direct plus indirect embryogenesis, we will emphasize systems based on zygotic embryo or primary somatic embryo explants, the process also known as embryo cloning. We assume that these explants are composed predominantly of PEDCs or cells requiring relatively minor epigenetic reprogramming to express the embryogenic state. Our main concerns will be with potential applications of embryo cloning, and with the factors that limit this potential. We will, however, draw on results from other embryogenic systems whenever these are relevant to the discussion. Cellular, biochemical, and molecular aspects of somatic embryogenesis have been discussed by Terzi and LoSchiavo in this book. An additional relevant discussion of gene activity during embryogenesis has been presented by Hughes and Galau (1989). Potential applications of somatic embryogenesis in agriculture, horticulture, and forestry have been reviewed by Day (1980) and Dunstan (1988).

2 THE PROCESS OF SOMATIC EMBRYOGENESIS - RECENT PROGRESS

2.1 *Induction of the embryogenic state*

Induction of the embryogenic state in differentiated explants often requires extensive proliferation through unorganized callus cycles, death or disruption of surrounding explant cells, and/or high levels of a synthetic auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) or picloram. Plasmolysis of explant cells was also shown by Wetherell (1984) to enhance somatic embryogenesis in wild carrot (*Daucus carota*). These factors are presumed to alter the epigenetic state of cells, and may be related in their ability to disrupt the cell-cell interactions required to maintain coordinated patterns of development. The role of cell isolation in induction of somatic embryogenesis was reviewed by Williams and Maheswaran (1986) and has been discussed more recently by Smith and Krikorian (1989). These latter authors showed that breakage or wounding of zygotic embryos at explanting led to formation of somatic embryos on a hormone-free medium. Constituent cells of the damaged tissues were presumably released from positional or chemical restraints and were thus able to express their innate embryogenic potential on a medium permissive for growth. A similar observation was made for *Dyosma pleiantha* by Chuang and Chang (1987). Embryogenic callus was induced from wounded zygotic embryos, while intact embryos failed to respond. Cell isolation is manifest early in somatic embryogenesis by the formation of a cuticle. In embryogenic cultures, each proembryonic group of cells becomes separated from surrounding cells by thickened, cutinized walls on the outer surface (Williams and Maheswaran, 1986). An early biochemical indicator of somatic embryogenesis is, therefore, an up-regulation of the enzyme acetyl CoA carboxylase, representing an increase in lipid synthesis (Nikolau et al., 1987).

The developmental stage attained by a zygotic embryo at explanting generally governs the response obtained *in vitro*. Direct somatic embryogenesis, especially in the absence of exogenous auxin, is normally associated with a relatively brief developmental period between the time of cotyledon initiation and the beginning of seed maturation (Maheswaran and Williams, 1986b). During this time, embryonic cell division appears to directly clone the existing early embryonic epigenetic state. Even within this developmental window, some differentiation away from the embryogenic state may be inferred from the nature of structures produced by cellular proliferation. In flax (*Linum usitatissimum*), late cotyledonary stage embryos produced numerous accessory cotyledons in addition to well formed somatic embryos (Pretova and Williams, 1986a). A similar observation was made by Young et al. (1987) for *Lycopersicon*. Older immature zygotic embryos gave rise to accessory cotyledons and shoot apices rather than recognizable somatic embryos. Developmental gradients within a zygotic embryo are also evident by their response to different auxins. For example, in soybean (*Glycine max*) the tissue that preferentially produces somatic embryos in response to the auxin α-naphthaleneacetic acid (NAA) is a submarginal crescent around the distal edge of cotyledons excised from immature embryos 3-5 mm in length (Lazzeri et al., 1985, Hartweck et al., 1988). This crescent of cells appears to represent the latest maturing region of the cotyledons, which has just completed cell division but has not yet started to accumulate storage reserves. When the stronger 2,4-D is used as the inducing auxin, somatic embryos are initiated from a more extensive subset of cotyledon cells (Hartweck et al., 1988).

Starting with a culture of embryogenic cells in the form of proliferating proembryonic masses, the process of regeneration requires, first, the initiation of bipolar differentiation to produce cotyledons, shoot apex and root pole, and then, in sequence, maturation of embryos, germination or conversion to plantlets, and transfer of plants out of culture into soil. At each of these steps, losses and limitations reduce the potential numbers of regenerated plants.

2.2 *Recurrent embryogenesis*

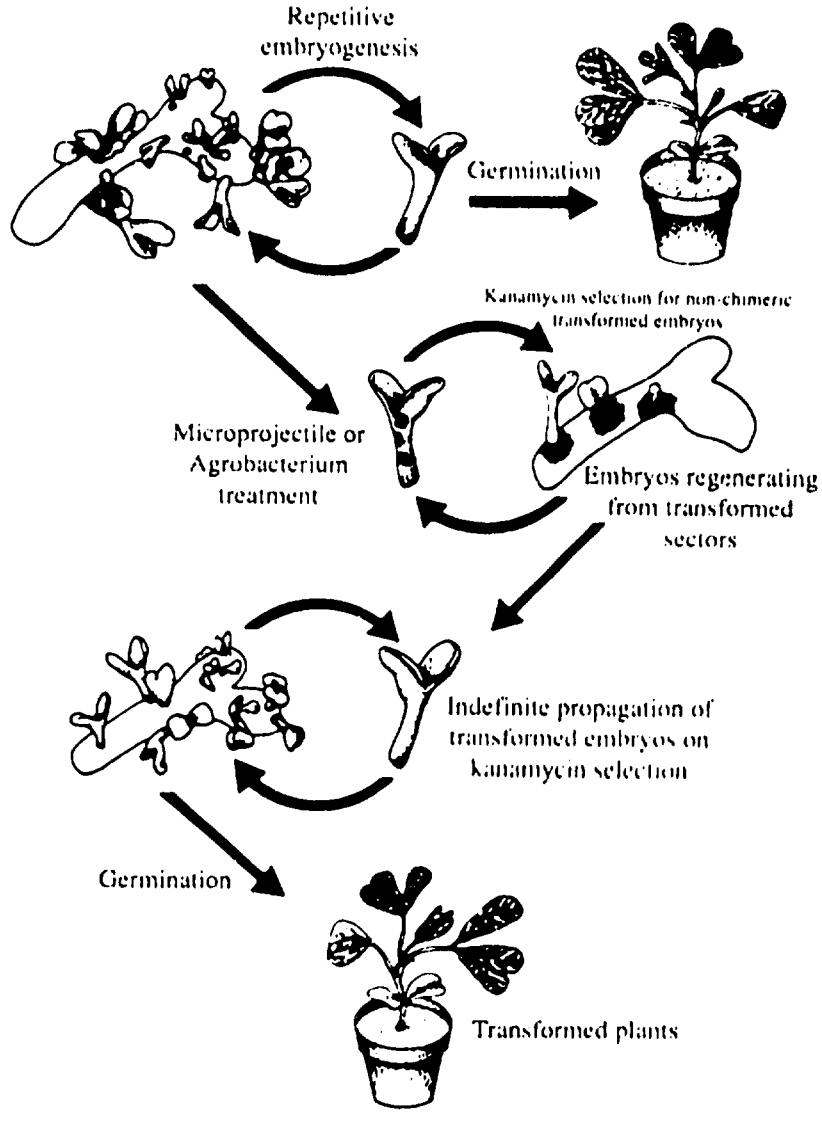
The power of embryo cloning techniques and their exploitation for mass propagation, metabolite production, or genetic transformation have recurrent embryogenesis as their basis. Recurrent embryogenesis, also termed repetitive, accessory, proliferative, or secondary embryogenesis, occurs when primary somatic embryos fail to mature normally into plantlets and instead give rise to successive cycles of embryos, most commonly from superficial cells of the cotyledons or hypocotyl (Fig. 1). The process is probably homologous with the proliferation of globular proembryos in standard embryogenic cultures, differing only with

respect to the stage at which integrated control of development is lost. Expressions of recurrent embryogenesis are best viewed as a continuum, with proliferation of globular PEMs or early globular stages (Finer, 1988) at one extreme, and the development of early embryogenic stages on bipolar embryos or germinating plantlets (Lupotto, 1983, 1986) at the other extreme. Recurrent embryogenesis may become a problem if it cannot be controlled when germination and normal growth are required. Where it can be stimulated or prevented at will, it offers the advantage of greatly facilitating mass propagation.

The maintenance of recurrent cycles of somatic embryogenesis can be spontaneous as is the case with alfalfa (*Medicago sativa* L., Lupotto, 1983, 1986), in which recurrent cycles are maintained in the absence of growth regulators. When similar cultures were started in the laboratory of W.A. Parrott and monitored for 1 year, each somatic embryo would give rise to an average of approximately 30 new somatic embryos per month (Fig. 2). At this rate, one somatic embryo could be multiplied into 2.37×10^{29} embryos in a single year, a figure that underscores the propagative potential of recurrent embryogenesis.

More frequently, however, the initiation of recurrent cultures requires that the developing embryos be locked into a developmental stage beyond which they cannot proceed, thereby repeating a cycle. This can be achieved by initial exposure to a very high auxin concentration such as 40 mg/l of 2,4-D, followed by maintenance of the recurrent system using a lower level of auxin, such as 5 mg/l of 2,4-D (Finer and Nagasawa, 1988), which prevents the transition from preembryonic to embryonic development. In other species, recurrent embryogenesis may be maintained by the same 2,4-D level used to induce somatic embryogenesis in first place (Merkle and Sommer, 1986). The PEMs of proliferating embryogenic culture, such as the scutellar "e" or "type 2 callus" of maize (*Zea mays* L.)and other cereals, appear to represent globular somatic embryos which are unable to initiate normal bipolar growth. Instead they eventually lose intercellular coordination, allowing a new cycle of globular proembryos to develop independently from superficial cells. To obtain a differentiating embryo, bipolar growth must be initiated, and all cells of the embryo must continue to develop coordinately as a group. In many systems, complete or partial removal of auxin is sufficient to break the cycle of recurrent embryogenesis and allow bipolar embryos to form in large numbers (Thorpe, 1988).

Fig. 1. Recurrent embryogenesis and its use in genetic transformation and mass propagation. Although new embryos can form from older embryos at any stage of development, this example depicts recurrent embryogenesis occurring from cotyledonary stage embryos. Developing embryos can be exposed to *Agrobacterium* or bombarded with microprojectiles, transforming individual epidermal cells. As the embryo continues to grow and develop, transformed cells give rise to patches of transformed tissue from which transformed embryos develop on selection medium. In this example, kanamycin is used as the selection agent, but several other agents could be used, depending on the vector used in transformation. As long as the cycles of recurrent embryogenesis are maintained, transformed or nontransformed embryos can be propagated indefinitely.



2.3 *Auxin vs cytokinin for induction of embryogenesis*

Although a majority of studies have employed auxins, particularly 2,4-D and NAA, for the induction of somatic embryogenesis from immature embryonic explants, cytokinins have also been used as inducing agents in some instances. Typical of these two distinct regimes are the induction by 2,4-D and NAA of somatic embryos on immature cotyledons of soybean (Lazzeri et al., 1985; Ranch et al., 1985; Barwale et al., 1986;) and the stimulation of direct somatic embryogenesis on immature hypocotyls of various clovers (*Trifolium* spp.), flax, and oilseed rape (*Brassica napus*) by BAP in the presence of yeast extract (Maheswaran and Williams, 1984, 1985, 1986a, b; Pretova and Williams, 1986a,b). When young cotyledonary embryos of white clover (*Trifolium repens*) are explanted and exposed to the cytokinin BAP (6-benzylamino purine), the hypocotyl responds with the formation of somatic embryos (Maheswaran and Williams, 1984). When 2,4-D is used instead of BA, it is the cotyledons that form somatic embryos (Parrott, unpublished). Likewise, pea (*Pisum sativum*) shows a positive response from hypocotyl cells in the presence of cytokinin (J. R. Myers, pers. comm.), but it is the cotyledon cells that respond in the presence of auxin (Kysely et al., 1987).

Genotype, tissue type, and developmental stage may all be determining factors in the comparative ability to respond to auxin or cytokinin. When used alone, the power of BAP to induce somatic embryogenesis appears to be limited to PEDCs along the hypocotyl of very immature embryos. In contrast, auxins are effective in inducing somatic embryogenesis from a much wider range of tissues and developmental states. Auxins alone appear to have the potential to generate IEDCs from nonembryogenic tissue. Several authors (Tulecke and McGranahan, 1985) have used a combination of both auxins and cytokinins to induce somatic embryogenesis. In these instances, it is not clear which role, if any, was played by the cytokinin in the induction of somatic embryos.

2.4 *Obtaining embryos from embryogenic cultures*

As stated earlier, a removal or decrease of the auxin concentration in the growth medium can break the cycle of continuous proliferation of PEMs, and permit embryos to develop to maturity. The removal of auxin could plausibly exert its effect by lowering friability to enhance cell-cell contacts and permit an increased expression of polarity present within embryogenic cell clusters. Except for single cells in suspension in the dark, cells in culture have inherent polarity imposed by attachment to neighboring cells, orientation on a semi-solid medium, and possibly even the direction of illumination. In somatic embryogenesis, electrical polarity has been found to precede the formation of bipolar embryos. Brawley et al. (1984) showed that in globular somatic proembryos of carrot (*Daucus carota*), ionic currents flowed inward at the site of the future shoot and out at the site of the future root pole. These currents were identified as being largely a K^+ influx and H^+ efflux (Rathore et al., 1988). Similar ionic currents have been detected around haploid embryos forming from immature pollen grains of tobacco (*Nicotiana tabacum*; Overall and Wernicke, 1986). In

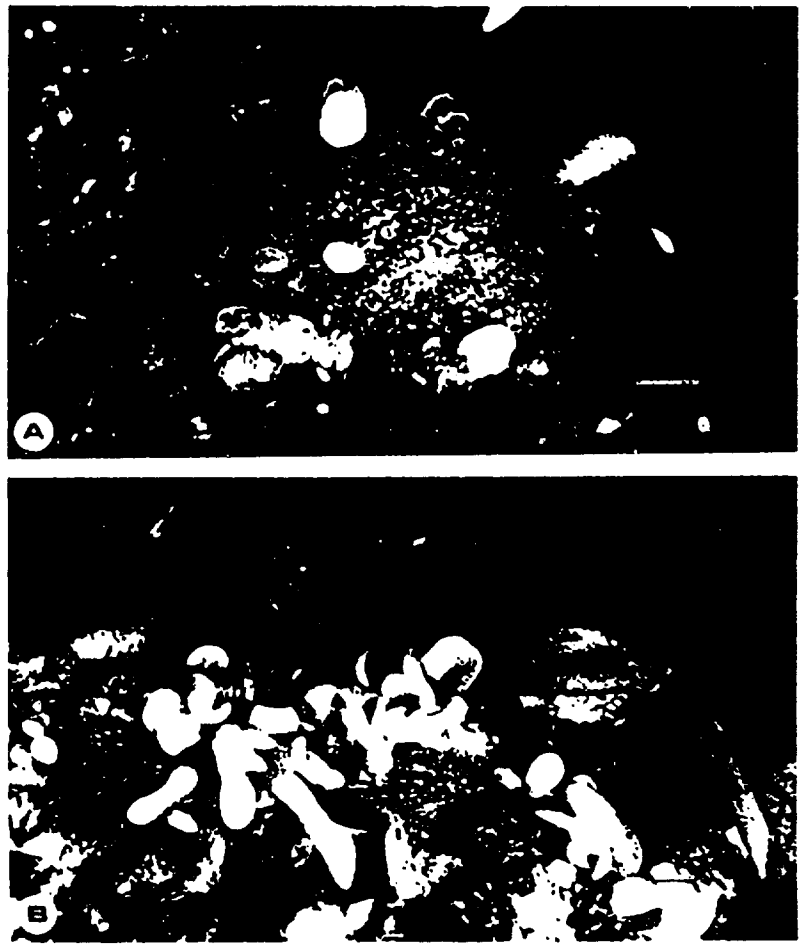


Fig. 2. Recurrent somatic embryogenesis in alfalfa. A. Globular to heart stage embryos developing on a cotyledon of a somatic embryo (bar = 500 μm). B. Cotyledonary stage somatic embryos on the hypocotyl of an older somatic embryo. These new embryos will soon give rise to a new cycle of somatic embryos (bar = 500 μm).

carrot somatic embryogenesis, electrical polarity is accompanied by asymmetry in the distribution of activated calmodulin. Using fluphenazine fluorescence, Timmers et al. (1989) showed that polarity in the distribution of activated calmodulin already exists in the globular proembryogenic masses before morphological polarity is visible. Activated calmodulin concentration is higher in the region of the root pole. During the later stages of bipolar development, fluphenazine fluorescence is also strong in the region of the forming shoot apex. Polarity in development of somatic embryos has also been detected as asymmetry in DNA synthesis by Nomura and Komamine (1986).

In direct somatic embryogenesis from immature zygotic embryos, suppression of normal polarity may play a role in embryo induction. Zygotic embryos in which growth of the main axis is weak or suppressed are more likely to give rise to somatic embryos from superficial cells (Ho and Sussex, 1971; Maheswaran and Williams, 1986b, Pretova and Williams, 1986a, Muralidharan and Mascarenhas, 1987). Weakening of the factors maintaining polarity may be associated with failing growth of the main axis, allowing individual cells to escape from coordinated control. The ultimate disruption of the main axis would be maceration of the zygotic embryo tissue (Smith and Krikorian, 1989). Weakening of intrinsic polarity factors may also explain the success or failure of explant tissue to become embryogenic depending on its orientation on the growth medium (Hartweck et al., 1988).

If formation of somatic embryos follows the establishment of polarity, then the deliberate imposition of polarity may act to induce embryos in undifferentiated cell cultures. In certain instances, agents that enhance polarity have been found to enhance the differentiation of embryos from PLMs. Dijak et al. (1986) found that somatic embryogenesis from mesophyll protoplasts of alfalfa was enhanced by exposure of newly isolated protoplasts to low-voltage electrical fields. An alternative means of imposing polarity is the attachment of cells to a substrate. The production of embryos of sweet potato (*Ipomoea batatas*) from liquid culture was improved by anchoring pre-embryogenic aggregates to alginate beads to maintain a physiological polarity (Chee and Cantliffe, 1989). This phenomenon might potentially be exploited by deliberate imposition of polarity to help synchronize embryogenic cultures for improvement of mass propagation systems.

2.5 *Maturation of embryos and the development of germinability*

In order for germination to occur, embryos must have functional shoot and root apices capable of meristematic growth. High auxin levels can inhibit development and growth of the shoot meristem if young proembryos are not transferred to a low-auxin or zero-auxin medium after induction (Muralidharan and Mascarenhas, 1987; Gorst et al., 1987; Parrott et al., 1988). It may even be necessary to add activated charcoal to the medium to remove as much auxin as possible from the somatic embryos (Buchheim et al., 1989). At low auxin levels, shoot meristem formation is generally achieved early after the initiation of cotyledons, so that under inappropriate culture conditions, germination can occur prematurely to give

weak or inviable plantlets (Merkle and Wiecko, 1989). To produce vigorous plantlets, a period of embryonic growth and maturation is required before germination. This is normally achieved by culturing at sucrose levels of 3 to 6 %, although progressively increasing levels up to 40% have been used for some species (Lee and Thomas, 1985, Janick, 1986; Buchheim et al., 1989). Where progressively increasing sucrose levels are used to achieve maturation, osmotic desiccation by the high-sucrose medium is likely to be involved.

For some species, efficient conversion to plantlets also requires the imposition of temporary desiccation before germination. This procedure, which mimics seed maturation in vivo, may be necessary to trigger metabolic processes needed for germination and seedling growth (Rosenberg and Rinne, 1988). Mature soybean somatic embryos, desiccated in empty petri dishes until they shriveled to 40-50% of their original volume, rapidly imbibed water following transfer to medium, and converted to plantlets with at least seven times the frequency of non-desiccated embryos (Hammatt and Davey, 1987). Similarly, Parrott et al. (1988) found that desiccation in empty petri dishes promoted rapid and uniform germination of soybean somatic embryos, if they were first allowed to mature to a minimum age on basal medium.

Gray et al. (1987) found that somatic embryos of orchardgrass (*Dactylis glomerata*) became quiescent when desiccated to 13% water by incubating them in empty plastic petri dishes at 70% relative humidity at 23°C. When desiccated embryos were stored for 21 days and rehydrated in vitro, 8% germinated and 4% produced viable plantlets. Fifty-eight per cent of grape (*Vitis longii 'Prince'*) somatic embryos treated similarly germinated after 21 days of storage and 20% produced rooted green plants (Gray, 1987). It is notable that only 5% of control grape somatic embryos never subjected to dehydration produced green plants, apparently owing to a dormancy requirement. Therefore, Gray (1989) concluded that nondehydrated grape embryos were dormant whereas dehydrated embryos were quiescent since they germinated directly after imbibition. Desiccation has also been shown to promote conversion in pecan (*Carya illinoensis*). Desiccation of mature pecan somatic embryos for 5 days in 4-compartment petri dishes, with one compartment containing distilled water, resulted in up to 70% of the embryos producing roots following transfer to germination medium (Wetzstein et al., 1989).

Carman (1989) reported that gradual reduction in osmotic potential through desiccation of mature somatic embryos of wheat (*Triticum aestivum*) improved germination percentages. Senaratna et al. (1989, 1990) were able to confer desiccation tolerance on alfalfa somatic embryos by treating them with ABA at the torpedo to cotyledonary stages of their development. Over 60% of the ABA-treated alfalfa embryos survived desiccation to 10-15% moisture and converted to plantlets when placed on moist filter paper or sown directly onto sterile soil. Furthermore, the vigor of the plantlets from dried somatic embryos was greater than that of plantlets derived from embryos which had not been dried, although lower than that of true seedlings. Heat shock treatment induced a degree of desiccation tolerance in

alfalfa somatic embryos that was equivalent to that conferred by ABA treatment but did not have detrimental effects on subsequent growth of plantlets.

Ammirato (1974) showed that ABA at 10^{-1} M prevented precocious germination of somatic embryos of caraway (*Carum carvi*) in suspension. In addition, ABA promoted the development of well-formed cotyledons and suppressed production of multiple-embryo clusters. Ammirato (1983) later reported that the same level of ABA had a similar effect on suspension-cultured carrot somatic embryos, producing embryos more similar to their zygotic counterparts than those grown without ABA. Based on these results, he proposed that regulation of embryo maturation by ABA might be used to facilitate large-scale batch cultures, mechanized planting, artificial induction of dormancy and incorporation into artificial seeds. It has also been shown that the presence of ABA in the medium is critical to obtain normal somatic embryos of some conifers (Becwar et al., 1987; von Arnold and Hakman, 1988; Roberts et al., 1990).

In planta, ABA levels are highest during early maturation of dicotyledonous embryos, and induce the accumulation a group of hydrophilic proteins thought to act as desiccation protectants. The role of ABA in initiating the accumulation of storage reserves has not been ruled out, especially as the initiation of reserve accumulation coincides with the highest levels of endogenous ABA (Hughes and Galau, 1989; Galau et al., 1990). Recent evidence supporting the role of ABA in the initiation of reserve accumulation was provided by Roberts et al. (1990), who found the presence of ABA essential for the stimulation of storage protein accumulation in somatic embryos of interior spruce (mixtures of *Picea glauca*, and *P. engelmannii* and their hybrids). The application of exogenous ABA to somatic embryos at a stage corresponding with high levels of ABA *in planta* may aid recovery of more normal somatic embryos.

There has been a tendency in reported literature of exposing somatic embryos to auxins and/or cytokinins during their enlargement, maturation, and germination. The rationale or physiological justification for this exposure is not immediately obvious, as the developing embryo should be autonomous for these growth regulators. In fact, it has long been evident that the presence of any auxin in the medium can lead to poorly developed apical meristems (Halperin and Wetherell, 1964), which can in turn be a factor contributing to low conversion rates. Once the initial induction of embryogenesis has occurred following exposure to an auxin, the most successful protocols can be expected to be those that parallel *in vitro* the events that occur *in vivo*, permitting the somatic embryos to complete their cycle of development as normally and with as little interference from external factors as possible. This is perhaps best illustrated by Smith and Krikorian (1989) who used tissue disruption instead of exogenous growth regulators to induce somatic embryogenesis. Furthermore, no exogenous growth regulators were necessary at any stage of somatic embryogenesis.

2.6 *Acclimatization of plantlets*

Plantlets grown in vitro in a water saturated atmosphere show reduced development of cuticular waxes and abnormal stomatal function (Wetzstein and Sommer, 1983, Blanke and Belcher, 1989). On removal from culture, losses of such plantlets can be high if they are not protected from transpirational water loss while roots and normal leaves are developing. Acclimatization of culture-grown plantlets remains a problem in commercial micropropagation, since plantlets must usually be subjected to progressively reduced humidity over a period of weeks. Somatic embryogenesis offers some hope of avoiding or minimizing acclimatization problems if embryos can be removed from culture at physiological maturity and germinated under normal growing conditions (e.g., Pretova and Williams, 1986b).

2.7 *Genetic control of embryo cloning*

While genotype has been acknowledged for some time as a factor that influences regeneration from cell culture, very little is known about the genetic components of somatic embryogenesis from immature zygotic embryos. Nevertheless, some information is available on genetic effects on embryo cloning in soybean and in cereal grass species. Although the genetics of regeneration of alfalfa is especially well documented and individual genes have been identified (Reisch and Bingham, 1980) and named (Wan et al., 1988, Hernández-Fernández and Christie, 1989), that information is not presented here, as the studied protocols have involved regeneration from callus formed on explants from seedlings or mature plants rather than embryos.

Genetic variability for regeneration via somatic embryogenesis has been documented for a wide variety of species, including soybean (Komatsuda and Ohyama, 1988; Parrott et al., 1989b), maize (Duncan et al., 1985; Hodges et al., 1986), rice (*Oryza sativa*; Abe and Futsuhara, 1986), barley (*Hordeum vulgare*; Hanzel et al., 1984, Ohkoshi et al., 1987), and wheat (Lazar et al., 1983, He et al., 1988). Currently it has not been possible to identify individual genes in any of these species. The available evidence does suggest that genetic control of regeneration capacity is largely additive and highly heritable in maize (Tomes and Smith, 1985; Willman et al., 1989), rice (Peng and Hodges, 1989), and wheat (Ou et al., 1989). Only in barley have dominance effects been reported to be of larger magnitude than additive genetic effects (Komatsuda et al., 1989).

Cytoplasmic effects have also been important in maize (Tomes and Smith, 1985; Willman et al., 1989), and rice (Peng and Hodges, 1989). In these crops, as in wheat, cytoplasmic effects are sufficient to necessitate careful selection of maternal parents to ensure regeneration success (Ou et al., 1989). The role of cytoplasm in conferring regeneration capacity in wheat has been examined further by Rode et al. (1988). Non-embryogenic callus can be derived from embryogenic callus initiated from immature zygotic embryos of the cultivar Chinese Spring. The mitochondrial DNA of the non-embryogenic callus has undergone rearrangements relative to that of Chinese Spring, and these rearrangements are

like those found in the cultivars Talent, Thésée, and Capitole, which lack embryogenic capacity. This suggests that certain mitochondrial genotypes are essential for regeneration.

The use of defined cytogenetic stocks has made it possible to further elucidate the nature of the genetic control of regeneration in wheat. The use of substitution lines for Group 4 homoeologous chromosomes has identified Chromosome 4B as being important for regeneration (e.g., Mathias and Fukui, 1986; Higgins and Mathias, 1987). Because of additional evidence implicating Group 2 chromosomes in regeneration capacity, Kaleikau et al. (1989) used ditelosomic and nullisomic-tetrasomic lines to further study the effects of the Group 2 chromosomes on regeneration capacity. A factor with major influence on regeneration capacity was identified on the long arm of Chromosome 2D, while minor response factors were identified on the long arm of Chromosome 2A and the short arm of Chromosome 2B. Finally, a major regulatory gene controlling expression of the regeneration factors was located on the long arm of Chromosome 2B. Interestingly, a series of dwarf genes, which affect the metabolism of indoleacetic acid and gibberellic acid, and genes affecting photoperiodicity, are also located on Groups 2 and 4 chromosomes. Regeneration genes may then be genes that affect hormonal metabolism at the cellular level (Kaleikau et al., 1989).

In summary, the capacity to undergo somatic embryogenesis from immature zygotic embryos is under some degree of genetic control. This control appears to be the result of a low number of genes, and is therefore highly heritable and amenable to selection. Consistent with the observation that low numbers of genes are responsible for regeneration capacity, regeneration capacity appears to consist of two major parameters which are commonly measured in the literature. The first is the frequency of explants which regenerate, and the second is the average number of somatic embryos formed per regenerating explant. These parameters are likely to be substantially independent, and can be selected for individually. In the future, strategies for the mass propagation and genetic transformation of crops that have been recalcitrant thus far will most likely depend increasingly on the breeding and development of germplasms with a high capacity to undergo recurrent somatic embryogenesis. Once capacity for regeneration has been backcrossed into elite lines and agronomically superior cultivars, embryo cloning techniques will finally be sufficiently efficient to play an important role.

3 APPLICATIONS

3.1 *Mass Propagation*

The high-volume multiplication of embryonic propagules is the most commercially attractive application of in vitro somatic embryogenesis. As commercially conceived, the system involves harvesting maturing embryos from a continuously proliferating embryogenic culture of elite genotype, and converting the harvested clonal embryos to "seedling" transplants or synthetic seeds for delivery to the grower. Although the induction of normal embryo physiology, scale-up of culture volume, and design of field delivery systems have so

far prevented industrial applications, recent intensive work on synthetic seed systems shows commercial promise.

For many applications, somatic embryos have powerful advantages for mass propagation in comparison to both conventional clonal propagation methods (e.g., rooted cuttings, grafting) and other in vitro regeneration systems (e.g., micropropagation). One advantage of propagation via somatic embryogenesis is the very high multiplication rates possible with many embryogenic systems. Depending on the plant species, virtually unlimited numbers of embryos can be generated from a single explant. In comparison, multiplication by rooted cuttings is limited to the amount of material available from the mother plant, and, for most species, micropropagation also is characterized by relatively low multiplication rates. A second advantage of somatic embryogenesis is that, for many species, both growth of the embryogenic tissue and development of the somatic embryos can be carried out in liquid medium, making possible the handling of enormous numbers of embryos at one time. Drew (1980) estimated that one liter of a carrot suspension culture contained 1.35 million somatic embryos. Thus, in comparison to rooted cuttings and micropropagation, somatic embryos offer the potential for high volume, large-scale propagation systems that can be translated into significant labor savings. Even greater economies of scale may be possible if bioreactor and continuous culture technologies can be applied to embryogenic systems. Experiments aimed at such scale-up of somatic embryo production are discussed later in this section. In addition, the observation has been made that plants derived from somatic embryos are less variable than those derived via organogenesis. This may reflect an intolerance of somatic embryos to mutations in any of the numerous genes that must be necessary for ontogeny to be successfully completed (Ozias-Akins and Vasil, 1988). In contrast, vegetative meristems may be more tolerant to mutations and epigenetic changes.

Probably the most obvious advantage of somatic embryogenesis in comparison to other clonal propagation methods is the fact that the product is an embryo. The morphological and physiological similarity of somatic embryos to zygotic embryos means that they are almost complete propagules in themselves, with embryonic roots, shoots and leaves (or at least cotyledons) and, most importantly, the "program" to make a complete plant. Thus, unlike other clonal propagation systems, no separate shoot growth or rooting steps are required for plantlet production, again providing savings in labor. Furthermore, unlike organogenic or axillary branching systems, many embryogenic systems produce discrete embryos, and thus require no physical separation from mother tissue or other embryos in order to be handled, which once again means savings in labor. These last two features of somatic embryos —the fact that they are complete and discrete propagules— gives them one more potential advantage that has received intensive investigation in the past 5 years: the potential for direct delivery to the greenhouse or field, thereby eliminating the need for transplanting and lowering the cost per plantlet.

Over the past few years, some of these special characteristics of somatic embryogenesis have been examined for possible commercialization purposes. The potential for somatic

embryos to be grown in large volumes in continuous culture and employed as direct-delivered propagules has received much attention. These technologies are discussed in detail below.

Scale up potential. The fact that both the growth of embryogenic cells and subsequent development of somatic embryos can be carried out in liquid medium gives somatic embryogenesis the potential to be combined with engineering technology to create large-scale mechanized or automated culture systems. Such systems are capable of producing huge numbers of propagules with low labor inputs. With the application of this technology, costs per propagule have the potential to be reduced to the point where they may be competitive with seed-derived plants, depending on the crop.

Although much has been written about the possibilities of applying such scale-up technologies to somatic embryogenesis, little actual testing of model systems has been reported to date. The first report of large-scale embryogenic cultures described an attempt to grow carrot cells in 20-liter carboys, which resulted in the formation of few embryos (Backs-Husemann and Reinert, 1970). The biological/mechanical system most often described for application to embryogenic systems is the stirred-tank bioreactor, a mass culture system originally developed for microbial fermentations, but more recently adapted for growing plant cells on a large scale (Wilson et al., 1971; Martin, 1980; Kurz and Constabel, 1981). A major problem with adapting these bioreactor designs for use with plant cells is the high shear that stirring generates in these systems (Fowler 1987). Air driven bioreactors, with lower shear levels, have been tested as possible alternatives to the stirred-tank design, and have supported successful growth of a number of plant cell types (Fowler 1984). Styer (1987) reviewed bioreactor designs suitable for plant cell culture and concluded that although cells could be grown as batch, semi-continuous or continuous cultures, the highest efficiency can be obtained by continuous culture. In a continuous culture bioreactor, following the initial filling, inoculation and growth into log phase, fresh medium is introduced at a low rate while the same volume of spent medium and cells is removed. However, the constant removal of cells characteristic of most bioreactor designs precludes the maintenance of high cell populations. Styer (1985), working with embryogenic carrot cell suspension cultures, showed that use of a spin filter allowed removal of spent medium from the bioreactor without cell loss, thus enabling maintenance of high populations of cells at the desired stage. Replacement of cell proliferation medium with embryo differentiation medium in the spin filter bioreactor resulted in a constant number of PEMs, each of which continued to increase in cell number. Stuart et al. (1987) found that air-lift bioreactors gave slightly higher yields of alfalfa somatic embryos compared to propeller-stirred bioreactors or cultures grown in flasks on a shaker. However, they reported that conversion of bioreactor-produced alfalfa somatic embryos was extremely low compared to that of embryos produced in flasks or on agar-based culture systems. Walker (1989) also found that embryogenic suspension cultures of Norway spruce (*Picea abies*) grown in an air-lift bioreactor did not produce somatic embryos at as high a frequency as the same lines grown in shaker flasks.

Plant Biotech Industries, Ltd. has developed an automated system for large-scale commercial propagation of plants, which makes use of somatic embryos as well as other propagules such as microtubers and bulblets (Levin et al., 1988; Levin and Vasil, 1989). The system integrates a bioreactor with a bioprocessor in a closed system for separation, sizing and distribution of propagules into a culture vessel, and even employs an automated transplanting machine which transfers plantlets to soil mix in greenhouse trays at the rate of 8000 per hour. The authors claimed that their bioreactor-based system could cut production costs of plantlets by as much as 60% compared with conventional tissue culture propagation methods. Other benefits of bioreactor technology listed by Levin et al. (1988) include lower contamination rates, savings in space, time and labor, accurate monitoring and control of temperature, pH, and gasses.

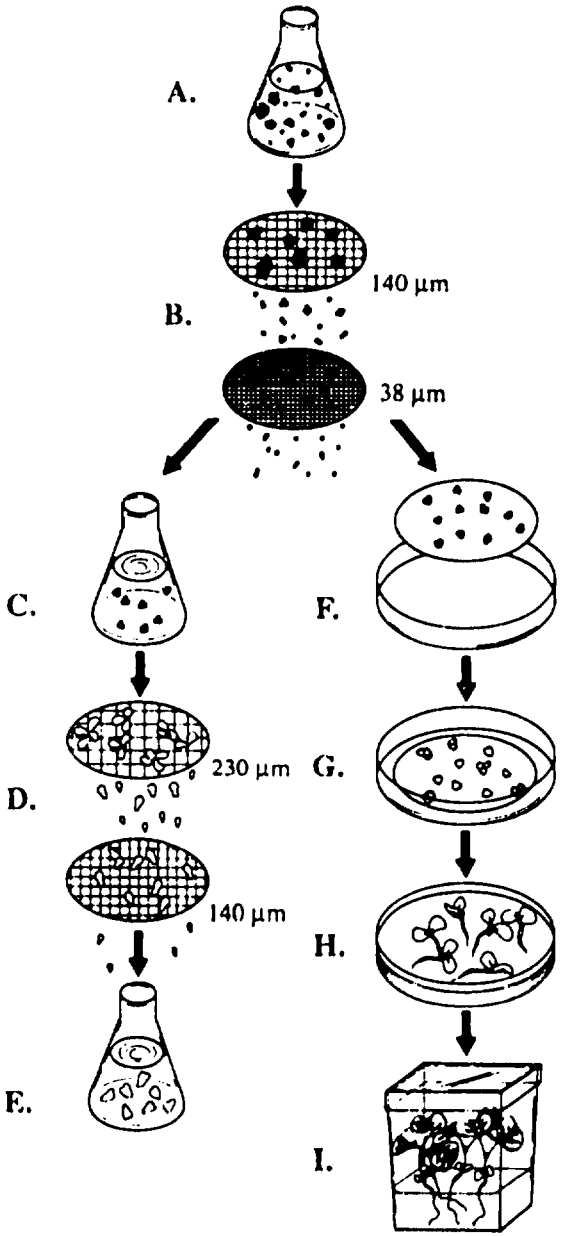
To date, the application of bioreactor technology has apparently not met its potential to produce hundreds of thousands of clonal embryos capable of growing into plants. To improve the capability of bioreactors to produce competent embryos, a group of researchers has recently developed a kinetic model of carrot somatic embryo development in suspension culture by monitoring substrate utilization, culture growth and embryo development over the time course of an embryogenic culture (Cazzulino et al., 1990). They intend to apply the model in optimizing bioreactor conditions for the production of somatic embryos mature enough to grow into plants.

Since the concept of somatic embryo production by continuous culture in bioreactors has yet to realize its potential, researchers desiring to obtain large numbers of well developed somatic embryos for experimental purposes have had to find other methods for producing these embryo populations. Fujimura and Komamine (1975) found that they could obtain a very high rate of embryo formation in embryogenic carrot suspension cultures when the size of cell clusters was made uniform by sieving with nylon screens and when auxin was thoroughly removed from the culture medium. The authors later refined this method by subjecting the fraction obtained by sieving to density gradient centrifugation in Ficoll solutions, followed by repeated centrifugation of the heaviest fraction of cell clusters at low speed for very short periods in culture medium (Fujimura and Komamine, 1979). By thoroughly removing the vacuolated cells from the suspension, this procedure resulted in synchronous embryo formation, with over 90% of the initial cell clusters forming embryos. The authors went on to employ the synchronous populations of embryos for morphological and biochemical definition of the stages of embryo development (Fujimura and Komamine, 1980; Fujimura et al., 1980; 1981). Later, Nomura and Komamine (1985) fractionated populations of single suspension-cultured carrot cells by sieving on nylon screens followed by density gradient centrifugation in Percoll solutions to obtain embryogenic frequencies of up to 30%. By manual selection of spherical single cells, the frequency of embryogenesis, which apparently proceeded in a roughly synchronous manner, could be increased to 90%. A method for synchronization of embryogenic carrot suspension cultures which avoided density gradient centrifugation was devised by Giuliano et al. (1983). In their method,

purification of the different stages of carrot somatic embryos was obtained by fractionating PEMs on nylon sieves, culturing the developing embryos in basal medium for 6-8 days, and fractionating the developing embryos on a second set of sieves. Differential sedimentation of early embryo stages and undifferentiated cells in the liquid medium following fractionation made it possible to draw off undifferentiated cells with a capillarized Pasteur pipette, allowing further purification of embryos.

As is to be expected, some embryogenic systems are more amenable to suspension culture than others, and those which respond well in suspension culture will more easily be manipulated for large-scale production of somatic embryos. However, even some species which grow very well in suspension have proven recalcitrant for high-frequency production of mature embryos capable of conversion to plantlets, when compared to the same materials grown on semisolid medium. For example, Stuart et al. (1987) found that the conversion percentage of alfalfa somatic embryos grown on semisolid medium was three times higher than that of embryos grown in liquid media in shaker flasks, and 30 times higher than for that of embryos grown in bioreactors. For alfalfa, this problem has been at least partially overcome by sieving suspension cultures on nylon mesh and immediately placing the desired fraction of cell clusters, with the mesh, onto semisolid basal medium for embryo development. This treatment resulted in production of large numbers of roughly synchronous somatic embryos (McKersie et al., 1989, Senaratna et al., 1989; 1990). Embryogenic suspension cultures of yellow-poplar (*Liriodendron tulipifera*) could be synchronized by a combination of fractionation on stainless steel sieves and culturing in medium supplemented with 5×10^{-5} M ABA (Figs. 3, 4A, B), but these embryos failed to convert when transferred to semisolid medium (Merkle et al., 1989; Merkle, 1990). As an alternative [illegible] yellow-poplar suspensions were fractionated and the desired fraction of PEMs was immediately plated by backwashing PEMs from the mesh onto filter paper, which was [illegible] semisolid medium (Fig 3). Within 2 weeks, hundreds of roughly synchronous, [illegible]ed embryos developed directly on the filter paper (Figs. 4C, D). Mature embryos obtained in this manner converted to plantlets at a frequencies over 70% (Figs. 5A D),

Fig. 3. Fractionation/synchronization of embryogenic yellow-poplar suspension cultures for mass propagation. A. Embryogenic suspension cultures are grown in shaken flasks of liquid induction medium. B. PEMs are fractionated on stainless steel sieves, saving the fraction that passes through 140 µm, but not 38 µm mesh. C. Saved fraction is cultured for one week in liquid basal medium. D. Globular stage embryos are fractionated again to eliminate clusters and free cells, saving the fraction that passes through 230 µm, but not 140 µm mesh. E. Globular stage embryos are cultured an additional 7-10 days in basal medium to obtain synchronous heart-torpedo stage embryos. Alternatively, following the first fractionation, F. PEMs are immediately placed on filter paper, which is then plated on semisolid basal medium. G. PEMs are cultured on filter paper/basal medium for 12-14 days to obtain synchronous, mature embryos. H. Mature embryos are transferred to basal medium without filter paper to promote germination. I. Germinants are transferred to plantlet development medium, from which plantlets are ready for transfer to soil mix in 6-8 weeks.



Merkle, 1990). Until the factors limiting the performance of somatic embryos grown in continuous culture relative to that of embryos grown in liquid batch culture or on semi-solid medium are understood, the use of bioreactors for mass propagation via somatic embryogenesis will be limited. In the meantime, further modification of the fractionation/plating procedures outlined here may help provide large numbers of readily-convertible somatic embryos for research purposes or artificial seeds.

3.2 *Protoplast Culture*

Embryogenic callus and suspension cultures, as well as somatic embryos themselves have been employed as a source of protoplasts for a range of species. The logic of this approach is that isolation of protoplasts from cells or tissues that are themselves regenerable will likely yield protoplast cultures capable of forming whole plants (Shillito et al., 1989). Earliest application of the regenerative potential of protoplasts isolated from embryogenic material was made with embryogenic carrot suspension cultures (Grambow et al., 1972). Since then in three groups of plant species, viz. graminaceous species, citrus species, and forest trees (especially conifers), embryogenic cultures have proven to be especially valuable in providing a source of regenerable protoplasts.

In the Gramineae, regeneration of callus or even sustained cell divisions in mesophyll-derived protoplasts could not be achieved following methods that had previously proven successful with mesophyll protoplasts of solanaceous species (Vasil, 1985). Although there were many reports of sustained cell divisions in protoplasts isolated from nonmorphogenic cell suspension cultures of the Gramineae, the protoplast-derived calli failed to undergo morphogenesis. Therefore, Vasil and Vasil (1980) turned to embryogenic cultures derived from immature embryos of pearl millet (*Pennisetum glaucum*) as a source of protoplasts. These protoplasts could be cultured to give rise to cell masses, from which embryoids and eventually plantlets could be regenerated. Similar success was subsequently reported using embryogenic suspensions of several other graminaceous species.

Embryogenic citrus suspension cultures have not only provided a source of regenerable protoplasts, but also made possible the production of interspecific and even intergeneric somatic hybrid plants. Interspecific somatic hybridization in citrus was first achieved by Kobayashi et al. (1988), who fused protoplasts isolated from an embryogenic suspension

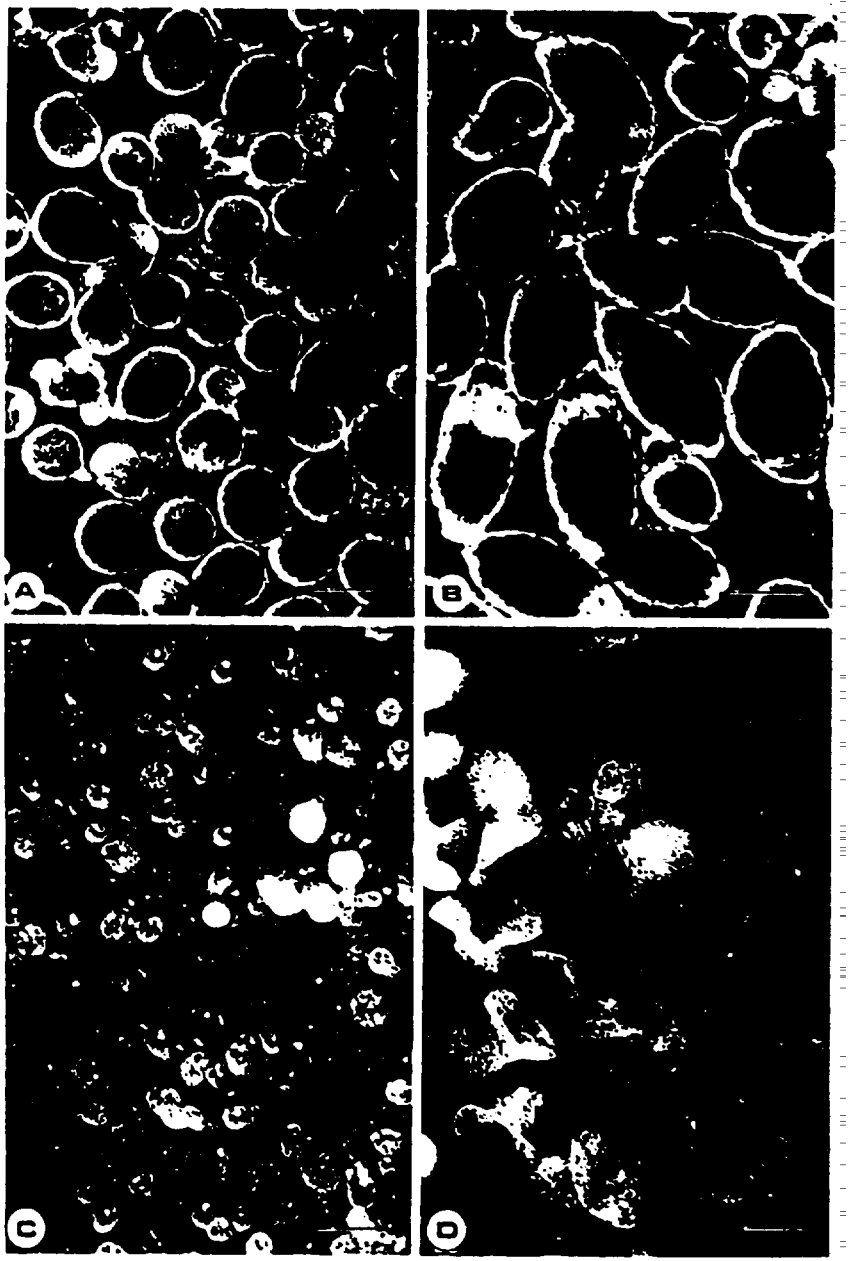


Fig. 4. Synchronous yellow-poplar somatic embryo populations obtained by fractionation of PEMs on stainless steel sieves (see Fig. 3 for protocol). A. Globular-stage yellow-poplar somatic embryos derived from fractionated PEMs 3 days after second sieving (bar = 500 µm). B. Early torpedo-stage yellow-poplar somatic embryos derived from fractionated PEMs 10 days after second sieving (bar = 500 µm). C. Roughly synchronous population of yellow poplar somatic embryos 6 days after fractionation of PEMs and plating on filter paper placed on semisolid medium (bar = 500 µm). D. Mature yellow-poplar somatic embryos 14 days after fractionation and plating on filter paper placed on semisolid medium (bar = 500 µm).

culture of navel orange (*C. sinensis*) cultivar Washington with leaf protoplasts of satsuma mandarin (*C. unshiu*) cultivar Hayashi. Interspecific somatic hybrids have since been produced between a number of citrus species (Kobayahsi and Ohgawara, 1988; Grosser et al., 1989) using similar techniques. The first intergeneric somatic hybrid made with protoplasts isolated from an embryogenic citrus suspension culture was reported by Ohgawara et al. (1985), who fused embryogenic protoplasts of *C. sinensis* cultivar Trovita with leaf protoplasts of a sexually compatible species, trifoliate orange (*Poncirus trifoliata*). Other intergeneric hybrids derived from embryogenic citrus protoplasts have since been reported (Kobayashi and Ohgawara 1988; Grosser et al., 1988a), including hybrids between sexually incompatible genera. Grosser et al. (1988b) fused protoplasts isolated from embryogenic cultures of *C. sinensis* with protoplasts isolated from epicotyl-derived callus of Philipine box orange (*Severina disticha*). Hybrid plants were regenerated from the fusion products. Thus, the ability to fuse protoplasts isolated from embryogenic citrus cultures with protoplasts isolated from other sources has allowed breeders to bypass barriers to sexual hybridization in this group of plants.

The ability to isolate protoplasts from embryogenic cultures of forest trees has had a large impact on regeneration studies for this group of plants, in particular coniferous species. Although a few researchers reported the growth of protoplasts isolated from conifer cotyledons, leaves, or suspension cultures to the colony or even callus/suspension stage (Kirby and Cheng, 1979; Hakman and von Arnold, 1983; David et al., 1986), achieving morphogenesis in the resulting cultures remained a barrier. The development of embryogenic callus and suspension cultures (Hakman et al., 1985; Nagmani and Bonga, 1985; Hakman and Fowke, 1987; Gupta and Durzan, 1986, 1987a; Durzan and Gupta, 1987; Attree et al., 1989a; Schuller et al., 1989; Tautorus et al., 1990) proved to be the key to the production of morphogenic protoplasts in coniferous tree species. Attree et al. (1989b) and Klimaszewska (1989) have recently reported the regeneration of plantlets from protoplasts isolated from embryogenic cultures of white spruce and hybrid larch (*Larix x eurolepis*), respectively.

Embryogenic cultures have also been shown to be a valuable source of regenerable protoplasts in some hardwood forest tree species. Rao and Ozias-Akins (1985) isolated protoplasts from embryogenic cell suspension cultures derived from proliferating shoot segments of a 20-year-old sandalwood tree (*Santalum album*). The protoplasts could be cultured to form embryogenic cell aggregates, somatic embryos and eventually plantlets.

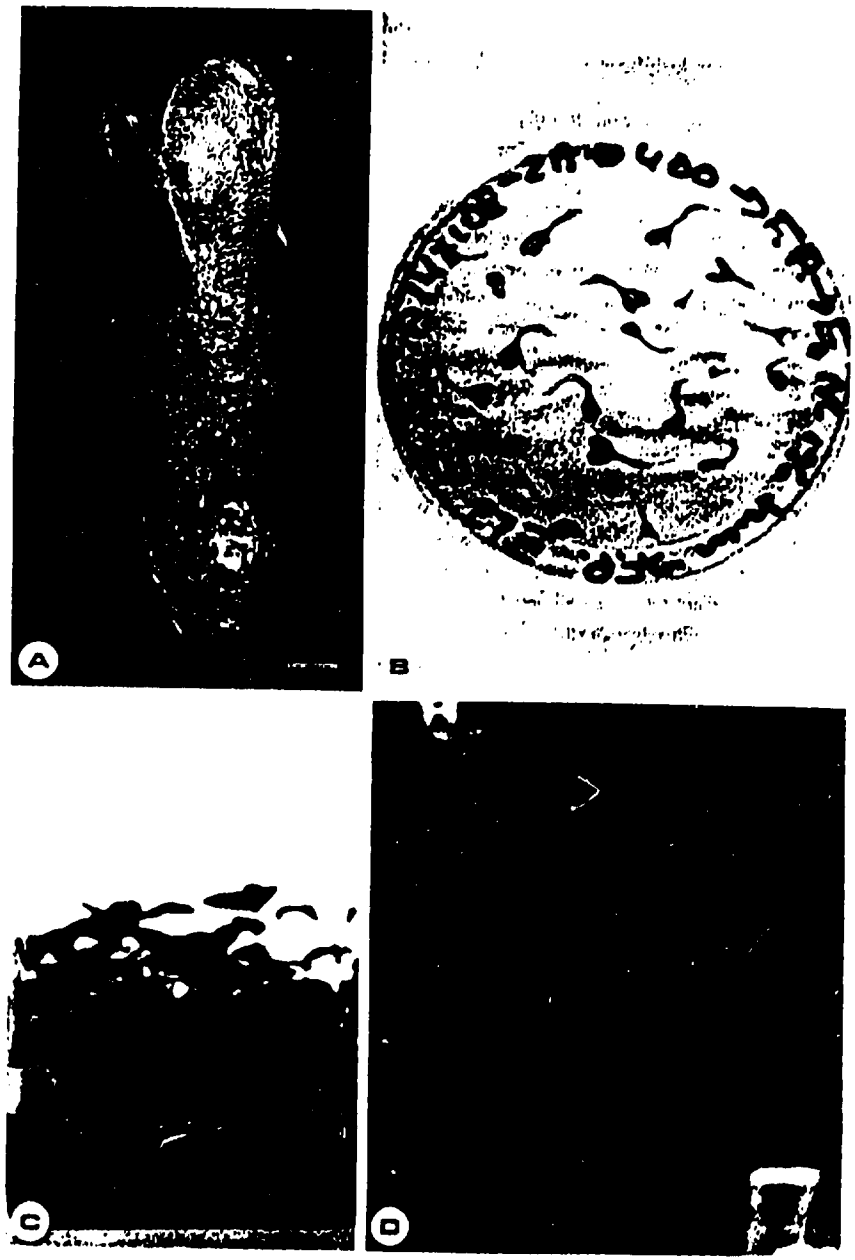


Fig. 5. Yellow-poplar and magnolia somatic embryo maturation, conversion, and plantlet acclimatization. A. Mature yellow-poplar somatic embryo, at the onset of germination, obtained from fractionation and plating (bar = 200 µm). B. Germinating yellow-poplar somatic embryos obtained from fractionation and plating of PEMs, following transfer from filter paper to fresh medium. C. Yellow-poplar plantlets derived from somatic embryos. D. Sweetbay magnolia (*Magnolia virginiana* L.) plantlets derived from somatic embryos following transfer to soil mix in a humidifying chamber.

Similarly, embryogenic suspension cultures of yellow-poplar provided protoplasts capable of regenerating whole plants via embryogenesis (Merkle and Sommer, 1987a, b).

3.3 *Embryo cloning and gene transfer*

Regeneration in several species, especially trees and large-seeded legumes, is limited to regeneration via direct somatic embryogenesis from immature zygotic embryos (Tulecke and McGranahan, 1985; Trigiano et al., 1988). The embryos form directly on the original explant tissue. If callus is present, it grows concomitantly with the somatic embryos. The embryos do not originate from it, thereby bypassing any opportunity that a callus phase provides to sort transformed cells from non-transformed cells. Confounding the problem is the fact that these somatic embryos may have originated from groups of cells rather than from single cells within the explant (Williams and Maheswaran, 1986; Hartweck et al., 1988). As current gene transfer techniques transform single cells, not clumps of cells, the recovery of chimeric embryos consisting of transformed and non transformed tissues is virtually assured (Parrott et al., 1989a). Whereas the absence of a callus phase is the main factor that has limited genetic transformation in these species, this barrier has been overcome by the use of recurrent embryogenesis. Scott and Draper (1987) successfully transformed carrot by exposing proembryogenic suspensions to *Agrobacterium*. McGranahan and colleagues (1988, 1990) were also able to use cultures of walnut (*Juglans regia*) undergoing recurrent embryogenesis to obtain transgenic plants. In recurrent embryogenesis, a cycle is initiated whereby somatic embryos continuously proliferate from previously existing somatic embryos. The cycles of embryo proliferation effectively substitute for a callus phase. Even if a chimeric embryo is obtained in the first cycle of regeneration, it becomes possible to obtain a non-chimeric embryo from the patch of transformed tissue on the original embryo (Fig. 1). The recurrent embryos appear to have an epidermal or subepidermal origin (Finer and Nagasawa, 1988), which allows embryogenic tissues to readily be exposed to *Agrobacterium*. There is also evidence that recurrent embryos have single cell origins (Polito et al., 1989). Consequently, if a transformation technique is applied to a primary somatic embryo instead of a zygotic embryo, it should become possible to obtain totally transgenic somatic embryos, and this has, in fact, been observed for walnut (McGranahan et al., 1990).

The nature of recurrent embryogenesis also makes it ideally suited to particle gun-mediated transformation (Klein et al., 1987). Instead of relying on *Agrobacterium* to mediate the transfer of genes into plant cells, the particle gun literally shoots into plant cells DNA that has been precipitated onto particles of a heavy metal. Embryogenic suspension cultures of cotton (Finer and McMullen, 1990) yielded an average of 30 stably transformed cell lines following each firing of the gun. This represents the stable transformation of approximately 0.7% of the cells present at the time of bombardment. Similar results have been obtained following bombardment of embryogenic suspensions of soybean (McMullen and Finer, 1990) and maize (Gordon-Kamm et al., 1990). These transformed embryogenic cell lines can, theoretically, give rise to an unlimited number of somatic embryos. Furthermore, the plant species which should be transformable by this procedure should not be limited to those within the host range of *Agrobacterium tumefaciens*.

3.4 *Embryo cloning and metabolite production*

Recurrent embryogenesis offers potential for *in vitro* production of embryo metabolites, such as lipids and seed storage proteins. Although this technology is not yet commercially viable, in that production is still more costly than extraction from natural seeds, the absence of seed tissues surrounding somatic embryos may prove eventually to be a significant advantage. Few examples are currently available, but borage (*Borago officinalis*) would be one such candidate. Borage seeds contain high levels of γ-linolenic acid, which can be used as a precursor for prostaglandins or in the treatment of atopic eczema (Quinn et al., 1989). Somatic embryos of borage produce γ-linolenic acid like their zygotic counterparts, yet are easier to manage because borage suffers from indeterminate growth and seed shattering in the field. Such a system could also produce a continuous supply of the metabolite throughout the year, as embryo production would not be limited to the growing season.

Jojoba (*Simmondsia chinensis*) is a source of high-quality industrial lubricants extracted from the seed, and somatic embryos derived from zygotic embryos of jojoba have waxes identical to those of the zygotic embryos (Lee and Thomas, 1985). The advent of recurrent embryogenic systems initiated from immature embryos, makes jojoba another candidate for metabolite production *in vitro* (Wang and Janick, 1986). The same would be true for cacao (*Theobroma cacao*), whose somatic embryos obtained from zygotic embryos accumulate lipids similar to those of zygotic embryos, including oleo-palmitostearin, the major ingredient of cocoa butter (Janick et al., 1982). As cacao embryos can be proliferated indefinitely (Pence et al., 1980), they could be a feasible source of cocoa butter.

3.5 *In vitro screening and selection*

The embryogenic process appears to be very sensitive to exogenous chemical compounds. Concentrations of the antibiotics kanamycin (10 mg/l) and hygromycin (2 mg/l), which are not high enough to affect soybean callus growth, will completely inhibit soybean somatic embryogenesis (Parrott, unpublished results). This phenomenon is apparently not limited to soybean. Five Mm ethanol will decrease somatic embryogenesis in carrot by 50%, but 20 Mm ethanol is required to obtain the same inhibition of carrot cells in culture (Perata et al., 1988). The ethylene inhibitors aminoethoxyvinylglycine, amino-oxyacetic acid, 2,4-dinitrophenol, and salicylic acid will inhibit somatic embryogenesis of alfalfa at concentrations too low to inhibit tissue growth (Meijer and Brown, 1988).

High sensitivity to compounds that disturb metabolic processes may be an intrinsic characteristic of somatic embryos, which are basically organisms undergoing a delicate differentiation process. This sensitivity could potentially be exploited to identify plant genotypes resistant to certain factors, such as aluminum toxicity or toxins produced by pathogens. In one attempt (MacDonald and Ingram, 1986), haploid lines of winter oilseed

rape (*Brassica napus* ssp. *oleifera*) which were undergoing recurrent cycles of embryogenesis were exposed to the toxin produced by the fungus, *Alternaria brassicicola* (Schw.) Wilts. Some of the regenerated plants were more resistant to the fungus than the control plants, but there was no relationship between *in vitro* sensitivity to the toxin and resistance at the whole plant level. An attempt to repeat the experiments using culture filtrates of *Leptosphaeria maculans* (also known as *Phoma lingam*), the causal organism of stem canker, was not successful (Newsholme et al., 1989). Embryogenic lines were selected which continued to proliferate in the presence of the toxin, but the resistance expressed by the embryogenic cultures did not manifest itself in the form of increased resistance at the whole plant level. One possible explanation for the lack of resistance in regenerated plants is that stable somatic mutations did not occur in the secondary embryos, or if they did occur, they were at a frequency too low to be of value in selection. If the frequency of somatic mutations is indeed too low, they could be increased by the presence of a mutagen in the selection medium. Thus, the use of such a mutagen could potentially explain the success of Sacristan (1982) in obtaining heritable resistance to stem canker following the selection of embryogenic lines exposed to the toxin. (Some of the plants regenerated in the absence of the toxin were also found to have acquired resistance to the fungus.) Another possibility is that since the rapeseed tested was already relatively resistant to the toxin, new mutations would probably confer decreased, rather than increased, resistance to the toxin. A final possibility explaining the lack of resistance in regenerated plants is that the mode of action of the toxin may be different at the embryo level compared with non-embryogenic cells or the whole plant. In one extreme example (Yu et al., 1990) filtrates of *Verticillium albo-atrum* added to embryogenic cultures of alfalfa actually promoted somatic embryogenesis while having a detrimental effect on the growth of non-embryogenic cells.

Nevertheless, the *in vitro* screening of somatic embryos would be especially useful to a breeder trying to introgress a resistance trait into adapted germplasm. Rather than screening large numbers of F_2 progeny for the presence of the desired trait, immature F_2 seed or embryos could be placed on regeneration medium containing the selective agent (i.e., high levels of aluminum or a fungal toxin). Only those zygotic embryos containing the desired resistance would proliferate, while those not resistant would not, thereby eliminating them from the population.

4 LIMITATIONS

Some of the problems associated with somatic embryogenesis are mentioned in Section 2. Besides the limitations imposed by the genotypes of individuals within a given species, there are species, genera, and families that have been more amenable to somatic embryogenesis than others. Raghavan (1986) listed the Umbelliferae, Rutaceae, Ranunculaceae, and Solanaceae as being especially amenable to somatic embryogenesis. Since then there has been a proliferation of reports of somatic embryogenesis across a wide range of gymnosperms and angiosperms. Eventually, it should become possible to regenerate

all species via somatic embryogenesis provided that the right genotype, explant, and concentration of inducing auxin are selected.

Another limitation is that the embryogenic potential of a culture may decline over time until regeneration is no longer possible under previously permissive conditions. This may represent inadvertant selection for non-embryogenic cell types which were present at low frequencies in the original culture. An example would be embryogenic suspension cultures (Finer and Nagasawa, 1988) whose success is density-dependent. Low densities favor the growth of embryogenic cells while high densities favor the growth of nonembryogenic cells. Alternatively, the embryogenic cells may lose their regenerative capacity through mutation or regulatory (epigenetic) modifications. Since there have been numerous reports of prolonged or indefinite retention of embryogenic potential in a number of species, this type of loss of totipotency appears not to be a necessary consequence of culture, but is more likely to reflect suboptimal growth conditions for PEDCs.

Plants regenerated from somatic embryos or embryogenic suspensions have in some instances been sterile or shown reduced fertility, a problem to which maize (Gordon-Kamm et al., 1990) is particularly sensitive. Oil palm (*Elaeis guineensis*) plants obtained from somatic embryos have been sterile upon reaching reproductive age (Florkowski and Purcell, 1989), a problem attributed to high concentrations of hormones in the culture medium. Epigenetic changes in the explant tissue (root) or somaclonal changes occurring in culture may also be contributing factors.

Although zygotic embryos are the desirable initiating tissue where the genotype to be multiplied is a heterotic F_1 hybrid or the progeny of selected parents, in many instances the aim is to clone a mature plant after performance evaluation. In these instances, indirect embryogenesis from mature plant parts is necessary, since zygotic embryos represent new, untested variability. This is especially important for trees and outcrossing species. The following discussion, however, is relevant to embryogenic cultures from a variety of sources, and composed of either PEDCs or IEDCs.

5 CONCLUSIONS

Embryogenesis is the result of long-term evolutionary processes, and must be highly conserved among higher plants. All plants that reproduce by seed must have all the necessary genes for embryogenesis. Theoretically, for somatic embryogenesis to occur, it is simply necessary to activate the necessary genes at the appropriate time and in the necessary sequence. Individual plants with the capacity to undergo somatic embryogenesis could conceivably represent mutations in the promoter regions of key genes that start the embryogenic process, or alternatively, that permit factors to be repressed which would otherwise repress the process. Such mutations would permit the activation of embryogenesis by stimuli other than gametic fusion. Somatic embryogenesis is now sufficiently prevalent and widespread that observations across a very wide range of species reveal striking similarities and patterns, presumably reflecting the fundamental biological phenomena that are

intrinsic to the process. Not only should this facilitate the development of strategies to regenerate any given species via somatic embryogenesis, but it should also make possible the development of a unified view of somatic embryogenesis.

Although a multitude of potential applications of somatic embryogenesis are already under development, technology to exploit this process is now only in its infancy, and new applications are still being developed. For example, recurrent embryogenesis has been proposed as a way to increase the number of difficult-to-obtain embryos derived from interspecific crosses (Ozias-Akins, 1989). Most recently the power of recurrent embryogenesis has been harnessed by Kato (1989) for efficient colchicine doubling of the chromosome number of camellia (*Camellia japonica*). In the future, edible nuts, such as pecans or walnuts, matured from somatic embryos in a bioreactor may have more consistent quality than those harvested off trees. Their production may even be cost effective as the shelling process would be bypassed altogther.

There is no doubt that plant cell totipotency, expressed as somatic embryogenesis and the development of entire plants from such embryos, is a powerful tool we can apply to plant propagation and improvement. As our understanding of the process continues to increase, we will be able to make efficient and economical use of this tool. We believe that many or all of the limitations discussed in this review will be overcome, and plant cloning by somatic embryogenesis will ultimately have a dramatic impact on plant breeding and propagation.

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SOMATIC EMBRYOGENESIS IN LEGUMES

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1. Introduction

1.1 Importance and distribution of legumes: Legumes are members of the third largest family of flowering plants, and as such, are globally distributed, with as many as 19,000 species. Several species have the ability to undergo a symbiotic association with nitrogen-fixing bacteria of the genera *Rhizobium* or *Bradyrhizobium*. Consequently, legumes tend to have high protein contents, and are an essential source of food, feed, and forages. They are also used as ornamentals, or valued for green manure, timber, gums, and other compounds. Only the grasses are of greater economic importance (Allen and Allen 1981).

1.2 Significance of somatic embryogenesis in legumes: Despite their widespread use and importance, legumes have traditionally been difficult to regenerate from cell culture (McHughen and Swartz 1984; Huyghe 1990). Although not yet as common in legumes as organogenesis, regeneration via somatic embryogenesis offers great potential for use in mass propagation or non conventional genetic improvement programs, including transformation. Unlike shoots derived via organogenesis, somatic embryos consist of complete propagules that do not require a separate rooting regime. As technology develops further, it may become possible to grow large numbers of somatic embryos in bioreactors for use as artificial seed to propagate elite or hybrid genotypes.

Over the past five years, great progress has been made in the development of regeneration systems for legumes, and many legumes are now amenable to regeneration via somatic embryogenesis. The purpose of this review is to focus on patterns of regeneration that are becoming evident across species. The study of these patterns and their application could help facilitate both a fundamental understanding of somatic embryogenesis and the development or refinement of regeneration systems for most legumes.

1.3 Review of previous work: A previous review found that almost all reports of regeneration in legumes have been confined to members of the Papilionoideae, the subfamily of greatest economic importance. Plants had been regenerated via organogenesis from 54 different legume species, while only 39 species had been regenerated from somatic embryos (Parrott et al. 1992). However, organogenic and embryogenic protocols have not been applied to these species with equal frequency. Consequently, these figures are a more accurate reflection of the popularity of organogenic protocols than of the leguminous species with embryogenic potential. A listing of species currently regenerated from somatic embryos, already numbering 50, may be found in Table 1. In addition, reports of somatic embryo formation without plant recovery may be found in Table 2. In some of these instances, plant recovery was never an objective. For the others, plants should be recoverable as regeneration protocols become more refined.

2. Somatic embryogenesis

2.1 Induction of an embryogenic state: At first glance, somatic embryogenesis in legumes appears to be a collection of diverse mechanisms. Some legumes, such as clovers (*Trifolium* spp.), have been reported to form somatic embryos (Figure 1A) upon exposure to cytokinins (e.g. Maheswaran and Williams 1984). Others, such as soybean (*Glycine max*), form somatic embryos in response to auxins (Figure 1B), and regenerate directly from zygotic embryo explant tissues without an intervening callus phase (Lazzeri et al. 1985). Finally, some legumes (Figure 1C), notably alfalfa (*Medicago sativa*), regenerate well from callus (Bingham et al. 1988). The use of auxins and cytokinins to induce embryogenesis, as well as the presence or absence of a callus phase, can be explained collectively under the concepts put forth by Sharp et al. (1982). These authors used the term PEDC (pre-embryogenic determined cell) to describe cells within very immature zygotic embryos, recognizing that these are expressing an embryogenic developmental program. In such tissues, the presence of a cytokinin to

stimulate cell division is all that is required to propagate the embryogenic state and to form new somatic embryos (Figure 1A). As zygotic embryos mature, the original embryogenic program is replaced by the expression of other developmental programs. In this case, the presence of an auxin is required to return the tissues to their original embryogenic state (Figure 1C). Once the tissues have been returned to the original embryogenic state, they consist of induced embryogenic cells, or IEDCs (Sharp et al. 1982). An IEDC and a PEDC should be functionally equivalent.

Tissues at different stages of development require different numbers of mitotic divisions in the presence of an auxin before becoming embryogenically determined. These intervening cell divisions are manifested as a callus phase. When few, if any, mitotic divisions are required for embryogenesis, such as that which occurs from zygotic cotyledons, embryogenesis is said to be direct (Figure 1A & B). When many divisions are required, such as regeneration from leaf-derived callus, somatic embryogenesis is said to be indirect (Figure 1C). Tissues associated with reproduction, such as ovary or anther tissue, or tissue from young seedlings, may be more easily returned to an embryogenic state than more mature tissue (Williams et al. 1990). Occasionally, some legumes, such as *Trifolium rubens*, can be identified which retain the capacity for direct embryogenesis from mature tissues (Cui et al. 1988). The ability to induce somatic embryogenesis in these plants without an intervening callus phase may make such species ideal for further investigations of the induction process.

The role of auxin for induction of an embryogenic state is not clear. Some evidence from pea (*Pisum sativum*) suggests that soluble auxin-binding proteins play a role in somatic embryogenesis. Picloram (4-amino-3,5,6-trichloropicolinic acid), which does not affect cell wall elongation in pea, but does induce somatic embryogenesis, only binds to cytoplasmic receptors. Furthermore, soluble auxin-binding proteins are found in zygotic embryos only at the developmental stage which will give rise to somatic embryos, and are limited to the axis, the region from which somatic embryos originate (Jacobsen 1991).

Little additional research on the induction of an embryogenic state has been done in legumes. Nevertheless, this process has been reviewed extensively using evidence from families of plants other than legumes (Merkle et al. 1990; Parrott et al. 1991). Briefly, DNA methylation, which may terminate or alter existing gene expression programs, occurs in the presence of auxins. In addition, cells may become isolated from their neighbors through the severance of plasmodesmata or necrosis of surrounding tissues, interrupting cell-cell interactions, which in turn, can reinforce the termination of existing gene expression programs and the reexpression of an embryogenic development program. Embryogenically determined cells of soybean have been found to have ultrastructural features in common with megagametophytes (Bonnelle et al. 1990), highlighting the degree of intracellular changes which may occur during the induction of embryogenesis. Another function of auxins may be the subsequent imposition of polarity within an embryogenic cell or cells. The role of polarity has also been reviewed (Merkle et al. 1990; Parrott et al. 1991), and may help establish the identity and coordinate the behaviour of embryogenic cells separately from that of surrounding tissues (Williams and Maheswaran 1986).

If these steps are correct, then other factors that alter gene expression programs (e.g., stress) or disrupt cell-cell interactions (physical disruption of the tissue) should lead to the formation of somatic embryos, and such examples are available from other plant families. Within the legumes, hypocotyl segments of the the East Indies walnut, *Albizia lebbeck*, (Gharyal and Maheshwari 1983) and of kardofan, *Clitoria ternatea*, (Dhanalakshmi and Lakshmanan 1992) form somatic embryos on growth-regulator-free medium. The application of a low-voltage field to protoplast-derived callus of alfalfa stimulates the formation of somatic embryos, perhaps in response to polarity imposed by the electrical field (Dijak et al. 1986).

The auxin most commonly used to induce embryogenesis is 2,4-dichlorophenoxyacetic acid (2,4-D), but examples may be found where the use of 2,4-D is ineffective (Greinwald and Czygan 1991). Other auxins, such as α-naphthaleneacetic acid (NAA) and picloram are also used. Legumes differ in

response to various auxins. For example, soybean responds to both NAA and 2,4-D, with NAA giving fewer but more normal embryos (Lazzeri et al. 1987a). NAA induces somatic embryos from only the distal perimeter of cotyledon explants, while 2,4-D induces somatic embryos from most of the epidermal surface of the cotyledon explant (Hartweck et al. 1988). In contrast, white clover (*Trifolium repens*) responds well to 2,4-D but poorly to NAA (Parrott 1991). The use of 2,4-D in lupins (*Lupinus* spp.) stimulates more embryo formation than the use of NAA, while the use of indole-3-acetic acid (IAA) and indole-3-butyric acid was not effective (Nadolska-Orczyk 1992).

The role of exogenous cytokinins during the induction phase is difficult to assess, as published studies almost never include the appropriate control, namely an auxin treatment without any cytokinin. The role of a cytokinin may depend on whether somatic embryogenesis is direct or indirect. When somatic embryogenesis is possible from callus, such as in alfalfa (Saunders and Bingham 1975) or in lentil, *Lens culinaris* (Saxena and King 1987), the frequency of somatic embryo formation is apparently enhanced by the presence of cytokinins in the callus induction medium. However, in direct systems, such as in crownvetch, *Coronilla varia*, in which somatic embryos formed directly on roots developed from callus (Arcioni and Mariotti 1982), or in pea (Kysely and Jacobsen 1990) and soybean (Lippmann and Lippmann 1984; Lazzeri et al. 1987a), in which somatic embryos form directly from immature zygotic embryos, addition of cytokinin reduces the frequency of embryo formation. In lupins, addition of a cytokinin does not affect the frequency of somatic embryogenesis if 2,4-D is the inducing auxin, but lowers the frequency if NAA is the inducing auxin (Nadolska-Orczyk 1992).

Some legume species, such as soybean, have not responded to conventional auxin-cytokinin treatments to form somatic embryos from callus tissue. The cocultivation of soybean callus with *Pseudomonas maltophilia* to induce somatic embryo formation (Yang et al. 1991) is evidence that it may eventually be possible to regenerate more species from callus tissue. The role of the *P. maltophilia* in embryo induction is unknown, but raises the intriguing possibility that unidentified compounds exist with the ability to turn on the gene(s) necessary to initiate the embryogenic pathway.

Somatic embryogenesis is influenced by explant source and genotype. The most responsive explant type is species-specific. For those species which form somatic embryos from callus, the choice of explant is less critical, and may consist of tissues as diverse as stolons, petioles, stems, leaves, hypocotyls, and ovaries. Otherwise, immature zygotic embryos are the most responsive explant. Very immature zygotic embryos can form somatic embryos upon exposure to a cytokinin (Figure 1A). Species tested for cytokinin-induced embryogenesis are limited to alfalfa and a few clover species, all of which have responded (Maheswaran and Williams 1984, 1986b). As the zygotic embryo becomes more mature, the use of auxin becomes necessary to induce somatic embryogenesis (Figure 1B), and eventually, the embryos of some species mature to a point where somatic embryogenesis can no longer be induced. The entire zygotic embryo may not be embryogenic, such as in pea, in which somatic embryos only form on the axis (Kysely and Jacobsen 1990). In contrast, both axes (Hazra et al. 1989) and cotyledons (Ozias-Akins 1989) of peanut (*Arachis hypogaea*) are embryogenic. The orientation of the cotyledon on the medium can also be significant. In soybean, the greatest number of embryos form when excised cotyledons are placed with the abaxial surface on induction medium (Hartweck et al. 1988).

In some species, embryos may be induced from other tissues, such as shoot apices of pea (Kysely and Jacobsen 1990) or immature leaflets of peanut (Baker and Wetzstein 1992). While pollen-derived embryos have been reported in peanut (Bajaj et al. 1980), there are no verified reports of a haploid plant being obtained from microspore- or pollen-derived embryos of any legume.

Plant genotype can be a critical factor for somatic embryogenesis. For example, soybean genotypes (Komatsuda and Ohyama 1988; Parrott et al. 1989) and alfalfa germplasms and cultivars differ in the frequency of individuals with embryogenic capacity (Mitten et al. 1984; Brown and Atanassov 1985; Meijer and Brown 1985; Chen and Marowitch 1987; Chen et al. 1987; Bianchi et al. 1988). Recurrent selection has been successful for increasing the embryogenic capacity of both tetraploid

(Bingham et al. 1975) and diploid (Ray and Bingham 1989) alfalfa. However, different alfalfa germplasms vary in genetic control of regeneration, and consequently respond dissimilarly to different regeneration protocols, manifested as genotype × protocol interactions (Seitz Kris and Bingham 1988). The genetics of regeneration are probably better defined in alfalfa than in any other plant species. Three separate studies have identified two dominant Mendelian genes conditioning embryogenic capacity. These have been called *Rn1* and *Rn2* (Reisch and Bingham 1980), *Rn3* and *Rn4* (Wan et al. 1988), and *Rna* and *Rnb* (Hernández-Fernández and Christie 1989). These gene pairs have not been tested for allelism. In red clover (*Trifolium pratense*), embryogenic capacity has been determined to be under the control of additive genetic factors (Keyes et al. 1980).

2.2 Histodifferentiation of somatic embryos: Zygotic embryo ontogeny has been described as a sequence of three confluent stages, histodifferentiation, maturation, and desiccation (Kermode 1990), and distinct patterns of gene expression are associated with these stages (Hughes and Galau 1989). Histodifferentiation refers to the stage of extensive cell division starting with the zygote and ending with a differentiated, immature, cotyledon-stage embryo. Maturation is marked by cell expansion and accumulation of storage reserves. Development ends when the embryo desiccates and becomes quiescent (Kermode 1990). Here, somatic embryo ontogeny is described as it parallels to the stages of zygotic embryogenesis. As the process of somatic embryogenesis is better understood and better regulated, zygotic and somatic embryogenesis will become more similar at the molecular, developmental and morphological levels.

Histodifferentiation of somatic embryos begins after induction of the embryogenic state. However, the histodifferentiation stage of some species is suppressed until auxins are removed from the medium (Walker et al. 1979), or, at least, until the auxin concentration is lowered to a threshold that will permit somatic embryo development (Figure 2). In species where embryos are capable of developing in the presence of an auxin, the continued presence of an exogenous auxin is detrimental to normal development (Figure 2). Auxins suppress the development of the apical meristem, probably by the same mechanism involved in the establishment of apical dominance. While the original observation of this phenomenon was made in carrot somatic embryos, the same observation has been made for those of soybean (Parrott et al. 1988) and crownvetch (Dusková et al. 1990).

2.3 Maturation of somatic embryos: Embryo maturation begins after histodifferentiation is complete. In legumes, growth by mitosis stops, and growth through cell expansion begins as cells accumulate storage reserves (Raghavan 1986). There is a traditional tendency in published reports to provide various growth regulators in the medium during this stage. Yet, there is enough information suggesting that exogenous auxin or cytokinin is not necessary for normal embryo maturation, as evidenced by the normal development of legume zygotic (Hsu and Obendorf 1982; Stafford and Davies 1979) or somatic embryos (e.g., Parrott et al. 1988) on medium devoid of all growth regulators. Poorly developed meristems or swollen hypocotyls may result from the application of exogenous auxins or cytokinins, respectively. Consequently, a treatment that binds and removes auxins, such as the addition of activated charcoal to the maturation medium (Ebert and Taylor 1990), can improve embryo normalcy and enhance germination (Buchheim et al. 1989). However, application of activated charcoal at the wrong stage, such as during the induction phase (instead of the development/maturation phase) suppresses embryogenesis (Genga and Allavena 1991).

In contrast to auxins and cytokinins, abscisic acid (ABA) may be necessary during embryogenesis to initiate the synthesis of storage proteins and proteins involved in dessication tolerance (Sussex and Dale 1979; Barratt 1986; Galau et al. 1990). Alfalfa is notable in that exogenous ABA promotes maturation (Denchov et al. 1991), desiccation tolerance (Senaratna et al. 1989), and subsequent conversion (Fujii et al. 1989) of somatic embryos, permitting plant recovery from as many as 80% of somatic embryos.

Optimal ABA applications range from 5 µM (Fujii et al. 1990) to 10 µM (Senaratna et al. 1989). Application of ABA has also aided the recovery of plants from somatic embryos of various lupin species (Nadolska-Orczyk 1992).

The time necessary for somatic embryos to achieve physiological maturity is species-specific, mirroring the maturation period of zygotic embryos *in planta*. Studies of zygotic soybean and bean (*Phaseolus vulgaris*) embryos cultured *in vitro* suggest that seed growth rate and maturation are controlled by the embryo (TeKrony et al. 1979; Egli et al. 1981; Adams and Rinne 1981; Dyer et al. 1987), and parallel that of embryos *in planta* (Long et al. 1981). Accordingly, current protocols for soybean (Buchheim et al. 1989) or peanut (Durham and Parrott 1992) include a 2-3 month maturation period, while white clover only requires 2-3 weeks (Parrott 1991).

2.4 Desiccation, germination, and conversion: Physiological maturity of legume zygotic embryos is followed by a period of desiccation. This desiccation period has been associated with the synthesis of proteins associated with the ability to germinate (Rosenberg and Rinne 1986, 1988). Partial desiccation (Figure 1B) enhanced conversion of somatic embryos of soybean (Hammatt and Davey 1987; Parrott et al. 1988; Buchheim et al. 1989), peanut (Durham and Parrott 1992), and alfalfa (McKersie et al. 1989; Senaratna et al. 1989; Senaratna et al. 1990; Anandarajah and McKersie 1990). Desiccation treatment may not be universally required for all legumes. For example, somatic embryos of birdsfoot trefoil (*Lotus corniculatus*), crown vetch (Arcioni and Mariotti 1982) and white clover (Parrott 1991) germinate readily without desiccation. Nevertheless, the ability to desiccate somatic embryos should eventually facilitate storage and use as synthetic seed.

Finally, the role of photoperiod has received very little attention, but may be a critical factor for daylength-sensitive species. In soybean, the use of a 23 hour photoperiod (Parrott et al. 1988) is necessary to prevent the premature induction of flowering. Once floral induction occurs, germinating somatic embryos cease all growth, even if the resulting seedling is only a few centimeters tall.

2.5 Role of other medium components: The role of other medium components has not received as much attention as growth regulators. Glucose is superior to sucrose for the induction of somatic embryos of scarlett runner bean, *Phaseolus coccineus* (Genga and Allavena 1991). The use of maltose instead of sucrose has been reported to enhance somatic embryo production and conversion in alfalfa (Strickland et al. 1987), and both maltose and glucose are better than sucrose for repetitive embryogenesis in alfalfa (Parrott and Bailey, submitted). The most recent protocols for the maturation of soybean (Finer and McMullen 1991) and alfalfa (Denchov et al. 1991) somatic embryos use maltose instead of sucrose. Conversion of white clover somatic embryos is significantly enhanced when 6% maltose is used instead of the more common 3% sucrose (Weissinger and Parrott, in preparation). While these results suggest maltose enhances embryo development and/or conversion, it is necessary to test maltose on a wider range of species before it becomes possible to evaluate its full potential as a medium component.

Amino acids, notably proline and its analogues such as alanine, glutamine, lysine, and serine, have also been reported to increase the frequency of somatic embryogenesis, with proline resulting in a three-fold increase in embryo number. The use of glutamine, arginine, or alanine increased the size of embryos and improved conversion into plants (Stuart and Strickland 1984a, 1984b). For all these amino acids, except glutamine, enhancing effects were dependent on the presence of NH_4^+ in the medium.

Sufficient ammonium in the basal salt mixture appears to be essential for somatic embryogenesis (Greinwald and Czygan 1991). While induction may occur with as little as 5 mM (Meijer and Brown 1987b), at least 12.5 mM is required for embryo development (Walker and Sato 1981; Meijer and Brown 1987b). If ammonium levels are too high, they may inhibit embryogenesis (Trigiano et al. 1988).

Any basal salt formulation that contains enough ammonium may probably be used, but in general, there has been a lack of critical evaluation of the various media formulations. MS (Murashige & Skoog,

1962), B5 (Gamborg et al. 1968), and SH (Schenk and Hildebrandt 1972) formulations have all been used successfully. B5 has been reported to be superior to MS (Nadolska-Orczyk 1992; Saxena and King 1987) and vice versa (Lehminger-Mertens and Jacobsen 1989). Basal salts formulations optimized for specific legumes have also been developed, such as L2 for red clover (Collins and Phillips 1982), L-6 for the moth bean, *Vigna acontifolia* (Kumar et al. 1988a), and EC6 for immature clover embryos (Maheswaran and Williams 1984). The L-6 formulation later proved to be the best formulation for the culture of tepary bean, *Phaseolus acutifolius* (Kumar et al. 1988b). Finally, the possibility exists that embryogenesis may be best optimized by using different basal salts during different stages of the process. For example, while somatic embryogenesis of white clover is best induced and maintained on EC6 medium, MS salts are better for achieving conversion of embryos into plants (Weissinger and Parrott, in preparation).

2.6 Somatic embryogenesis and somaclonal variation: Hanna et al. (1984), observing a lack of somaclonal variation in Guineagrass regenerated from somatic embryos, postulated that less somaclonal variation is recovered from embryogenic systems than from organogenic systems, plausibly because any severe perturbations caused by somaclonal variation during ontogeny could prevent the recovery of somatic embryos. The actual amount of somaclonal variation recovered probably depends on the regeneration protocol. Somaclonal variation has been reported when somatic embryos are obtained following a callus phase, such as in red clover (Wang and Holl 1988) and alfalfa (Nagarajan and Walton 1987), and this variation increases with time in culture (Bingham et al. 1988). In contrast, somaclonal variation was not found in direct embryogenesis of white clover (Maheswaran and Williams 1987). In soybean, somaclonal variation decreases with increasing amounts of 2,4-D in the induction medium (Shoemaker et al. 1991).

2.7 Repetitive embryogenesis and mass propagation: A common characteristic of embryogenic tissue is that it can remain embryogenic indefinitely (Terzi and Loschiavo 1990), a phenomenon variously termed secondary, recurrent, or repetitive embryogenesis (Figure 1B & 2). Maintenance of an embryogenic state may require exogenous auxin (Figure 3A), or it may occur in the absence of exogenous growth regulators (Figure 3F). The amount of auxin required may vary with culture protocol. For example, on solid medium soybean and white clover (Weissinger and Parrott, in preparation) require 20-40 mg/L of 2,4-D (Figure 3B & C). In liquid medium, soybean (Finer and Nagasawa 1988) and peanut (Durham and Parrott 1992) undergo repetitive embryogenesis at 5 mg/L of 2,4-D (Figure 3D & E). Alfalfa (Figure 3F) undergoes repetitive embryogenesis on solid medium devoid of all growth regulators (Dos Santos et al. 1983; Lupotto 1983, 1986). These particular species also differ in the developmental stage at which they undergo repetitive embryogenesis. White clover and soybean somatic embryos reach a globular stage of development before secondary embryos appear, while peanut somatic embryos reach a heart to torpedo stage before the appearance of new embryos. For white clover, peanut and soybean, secondary embryogenesis is inhibited and embryo differentiation and maturation is promoted when auxin is removed from the medium (Figure #G & H). Alfalfa somatic embryos reach the cotyledonary stage and may even start to germinate before becoming repetitive.

The ability of somatic embryos to become repetitive makes somatic embryogenesis a powerful tool. Aside from its obvious potential for mass propagation, repetitive embryogenesis can be used for genetic transformation, especially in those species that do not regenerate readily from callus (Finer and McMullen 1991). Somatic embryogenic systems may be additionally modified for mass propagation by using micropropagation techniques and exposing somatic embryos to high cytokinin:low auxin ratios to induce multiple shoot formation from the apical meristem, thus increasing the number of plants obtainable from one embryo (Collins and Phillips 1982).

3. Summary and conclusions

Legumes no longer deserve their reputation as being difficult to regenerate from tissue culture. The number of species regenerated from somatic embryos is continually increasing, and the phenomenon of somatic embryogenesis is becoming better understood. Enough is now known about the process to refine previously developed embryogenic protocols and to define embryogenic protocols for additional species. By manipulating the growth regulators in the medium, it is possible to regulate the ontogeny of somatic embryos and induce repetitive embryogenesis. The latter phenomenon is especially useful for genetic transformation, and has permitted the transformation of species which do not regenerate from callus, making them amenable to nonconventional genetic improvement techniques.

4. Protocols for somatic embryogenesis

A detailed description of the various protocols for somatic embryogenesis in legumes would be impractical. Rather, we list examples of representative regeneration schemes.

Glycine max (L.) Merr.

Two protocols (A and B) are described below. They are derived from various sources in the literature (Lazzeri et al. 1985; Parrott et al. 1988; Finer and Nagasawa 1988; Finer and McMullen 1991) and our own most recent experience. Since the two protocols have much in common they are discussed separately only when they differ. All media are solidified with 0.2% (w/v) Gelrite and adjusted to pH 5.8 prior to autoclaving unless otherwise indicated. Cultures are maintained at 2-27 °C, with a light intensity of 10-75 $\mu E\ m^{-2}\ s^{-2}$, and a 23 h photoperiod, unless indicated otherwise.

Growth of source plants

Any growth conditions which support vigorous plants and flowering are suitable. Photoperiod should be extended initially to allow for substantial vegetative growth prior to the induction of flowering, which will in turn lead to a high yield of immature seeds for explants.

Induction of embryogenesis

1. Harvest pods when seeds are 3-6 mm long.
2. Surface-sterilize pods with 70% propanol for 30 sec, followed by 1% sodium hypochlorite (20% commercial bleach) for 15 min. Rinse three times in sterile water.
3. Excise immature seeds from the pods. Cut away the end of the seed containing the embryonic axis. Apply gentle pressure to the narrow end to extrude cotyledons. Select dissected cotyledons 3-5 mm long.

A) 4. Place abaxial surface of each cotyledon on medium containing MS salts, B5 vitamins, 6% sucrose, and 40 mg/L 2,4-D. Adjust medium pH to 7.0 prior to autoclaving.

B) 4. Same as in A, but replace 2,4-D with 10 mg/L NAA.

Maintenance of embryogenesis

A) 1. After 4-6 weeks on induction medium transfer clumps of globular-stage embryos to 10A40N medium (Finer and Nagasawa 1988).

A) 2. Subculture monthly. Select rigorously for nodular, compact, green clumps of embryogenic tissue. Alternatively, clumps of globular stage embryos may be subcultured monthly on solid induction medium with the 2,4-D level reduced to 20 mg/L.

Development of somatic embryos

A) After 4-6 weeks on induction medium, or at any time on maintenance medium, transfer globular-stage somatic embryos to a growth-regulator-free medium consisting of MS salts, B5 vitamins, 6% maltose, and 0.5% activated charcoal.

B) After 10 d on induction medium, transfer whole explants to growth-regulator-free medium with MS salts, B5 vitamins, and 3% sucrose (MS0).

Maturation of somatic embryos

1. After 4 weeks on development medium, transfer cotyledon-stage somatic embryos to growth-regulator-free maturation medium with MS salts, B5 vitamins, and 6% maltose.
2. After 4 weeks on maturation medium transfer mature somatic embryos to a desiccation chamber consisting of a dry Petri plate containing a 1 cm^3 block of maturation medium to maintain high humidity. Seal plate with Nescofilm.

Germination and conversion of somatic embryos

1. After 7 d in a desiccation chamber, transfer embryos to MS0.
2. Transfer seedlings with roots and primary foliage leaves (usually after 1-3 weeks) to a GA-7 Magenta vessel containing MS0.
3. Transfer healthy plants to a 6.35 cm pot containing a sterilized 1:1 mixture of sand and commercial potting mix. Place potted plant inside sterile GA-7 vessel with GA-7 coupler (Magenta).
4. After 1 week in soil, acclimatize plant by progressively loosening the GA-7 coupler.
5. Transfer hardened plants to a greenhouse.

***Medicago sativa* L.**

Here we report only on repetitive somatic embryogenesis, as modified from the protocol of Lupotto (1986). Other protocols for somatic embryogenesis of alfalfa have been described elsewhere (Saunders and Bingham 1972; Meijer and Brown 1987a; Denchov et al. 1991).

Callus induction

1. Harvest the top 5 cm of 10 cm plant.
2. Surface-sterilize in 70% 2-propanol for 30 seconds, followed by immersion in 1.05% sodium hypochlorite for 12 minutes, and three rinses in sterile, deionized water.
3. Place petiole and/or leaf sections on BIINK medium, consisting of Blaydes salts and vitamins (Blaydes 1966), 3% sucrose, 3 g/L Gelrite as solidifying agent, pH adjusted to 5.8, and supplemented with 2 mg/L each of IAA, NAA, and kinetin.
4. Subculture monthly.

Initiation of repetitive somatic embryogenesis

1. Transfer cotyledon-stage somatic embryos which develop on the surface of the callus to MS0 medium.
2. Secondary embryos develop from the surface of primary embryos. Excise and subculture new embryos at monthly intervals.

Conversion of secondary somatic embryos

1. Some embryos germinate and develop foliage leaves prior to becoming embryogenic. Transfer these seedlings directly to 6.35 cm pots containing a sterilized mixture of 1:1 sand and commercial potting mix.
2. Acclimatize and transfer plants to the greenhouse by the soybean protocol (above).

Trifolium repens L.

A) Modified protocols for the cytokinin-based induction of primary (Maheswaran and Williams 1984) and secondary (Maheswaran and Williams 1986a) embryogenesis are described. The genetic fidelity of plants regenerated by both protocols has been documented (Maheswaran and Williams 1987).

B) Regeneration via auxin-induced embryogenesis (Parrott 1991; Weissinger and Parrott, in preparation)

Growth of source plants

Source plants are hand cross-pollinated to produce embryos for dissection.

Initiation of cultures

A) 1. Developing seed pods are harvested 5-7 days after pollination, depending on temperature and growth conditions, to yield torpedo-stage embryos 0.4-0.8 mm in length.

B) 1. Developing seed pods are harvested 7-8 days after pollination, depending on temperature and growth conditions, to yield cotyledon-state somatic embryos. The endosperm has solidified at this point.

2. The pods are surface-sterilized for 30 seconds in a solution of 70% 2-propanol (v/v), followed by 12 minutes in a 1% sodium hypochlorite (20% commercial bleach) solution, then washed thoroughly with sterile water and dissected aseptically.

A) 3. Seed coats are removed, and embryos are placed on EC6 medium containing 0.05 mg/L 6-benzylaminopurine (BAP) (Maheswaran and Williams 1984).

B) 3. Seed coats and embryonic axes are removed, and cotyledons placed adaxial side up, on EC6 basal medium (Maheswaran and Williams 1984) supplemented with 40 mg/L 2,4-D for 10 days.

Embryo development and conversion

A) 1. Somatic embryos arise from the hypocotyl portion of the zygotic embryo 7-9 days after culture initiation.

B) 1. Transfer cotyledons to MS0 medium (growth-regulator-free MS salts, B5 vitamins, and 3% sucrose).

A) 2. Somatic embryos germinate approximately 3 weeks after culture initiation. Transfer germinated embryos to growth-regulator-free EC6 medium.

B) 2. Somatic embryos mature after about 3 weeks. Separate from explant, and transfer individual embryos to MSO until germination occurs.

3. After 1-2 weeks, roots are well developed allowing plantlets to be transferred to soil and hardened off.

Note: Somatic embryos have also been obtained from zygotic embryos of *T. pratense, T. resupinatum, T. subterraneum,* and *Medicago sativa* using cytokinin-stimulated embryogenesis.

Initiation of cultures and conversion of secondary embryos

A) 1. Primary somatic embryos are initiated as described above.

B) 1. Primary somatic embryos are initiated as described above, but the explant cotyledon remains on EC6 medium with 40 mg/L 2,4-D for 30 days.

A) 2. Primary embryos at the torpedo to cotyledonary stage are removed from the zygotic embryo and placed on EC6 medium with 2 mg/L BAP.

B) 2. Primary embryos at the globular to heart stage are removed from the zygotic cotyledon and placed on EC6 medium with 20-40 mg/L 2,4-D.

A) 3. After 2 weeks, secondary embryos at various stages of development are apparent on all parts of the primary embryo that directly contact the medium. Transfer new embryos to EC6 medium with BAP to repeat the cycle up to one additional time, or transfer to germination medium (below).

B) 3. New somatic embryos form after 2-4 weeks. Transfer the new embryos to EC6 medium with 2,4-D to repeat the cycle, or transfer to development/maturation medium (below).

A) 4. Upon germination, transfer secondary embryos to growth-regulator-free EC6 medium for root development, then transfer to soil.

B) 4. Transfer embryos to growth-regulator-free medium consisting of MS salts, B5 vitamins, and 6% maltose for germination.

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6. Figures

1. Some possible embryogenic pathways in legumes. The youngest zygotic embryos respond to cytokinin, while older zygotic embryos respond to auxin. Tissue from seedlings responds to combinations of auxins and cytokinins by forming callus. Somatic embryos may be matured, desiccated, and germinated. Alternatively, enough auxin may be used to induce repetitive embryogenesis, which continues until auxin is withdrawn from the medium, permitting the somatic embryos to resume their development. (Drawing by J. Adang)

2. Effect of exogenous auxins on somatic embryo development. (Drawing by J. Adang)

3. Somatic embryos of legumes.

   A) Embryos arising from cultures of proembryogenic masses of black locust (*Robinia pseudoacacia* L.). The cultures require 3 mg/l 2,4-D to remain embryogenic. Bar=2.5 mm. Photo courtesy of S. A. Merkle.
   B) Primary somatic embryos of white clover following exposure to 40 mg/l 2,4-D. Bar=5.0 mm.
   C) Mature secondary somatic embryo of white clover. Bar=1.0 mm.
   D) Clumps of globular somatic embryos from a suspension culture of soybean. The cultures remain embryogenic when exposed to 5 mg/l 2,4-D. Bar=5.0 mm.
   E) Embryogenic clumps from a suspension culture of peanut. The cultures remain embryogenic when exposed to 5 mg/l 2,4-D. Bar=5.0 mm.
   F) Recurrent somatic embryogenesis of alfalfa from a late cotyledonary stage embryo plated on growth-regulator-free medium. Bar=5.0 mm.
   G) Maturation of soybean somatic embryos after removal of auxin and exposure to 6% maltose and .5% activated charcoal Bar=5.0 mm.
   H) Maturation of peanut somatic embryos after removal of auxin and 8 weeks culture on growth-regulator-free medium. Bar=5.0 mm.

7. Tables

Table 1. Explant source for somatic embryogenesis observed for various species of legumes from which whole plants have been obtained.

Species	Explant[1]	Reference
Albizia lebbeck (L.) Benth	H	(Gharyal and Maheshwari 1983; Tomar and Gupta 1988)
A. richardiana King	H	(Tomar and Gupta 1988)
Arachis hypogaea L.	A, C, CN, EA, S, ZE	(Bajaj et al. 1980; Bajaj 1983; Baker and Wetzstein 1992; Durham and Parrott 1992; Hazra et al. 1989; Ozias-Akins 1989; Sellars et al. 1990)
A. paraguariensis Chod. et Hassl.	A, ZE	(Sellars et al. 1990; Still et al. 1987)
A. villosa Benth.	A	(Bajaj et al. 1981; Bajaj 1983)
Cercis canadensis L.	ZE	(Geneve and Kester 1990)
Chamaecytisus purpureus (Scop.) Link	H, L, R	(Greinwald and Czygan 1991)
C. austriacus (L.) Link	SC, H, L, R	(Greinwald and Czygan 1991)
Cladrastis lutea (Michz.) K. Koch	C	(Weaver and Trigiano 1991)
Clitoria ternatea L.	H, R	(Dhanalakshmi and Lakshmanan 1992)
Coronilla varia L.	Ca, L, R	(Arcioni and Mariotti 1982; Arcioni et al. 1988; Dušková et al. 1990)
Crotalaria juncea L.	CP	(Ramanuja Rao et al. 1982)
Glycine canescens F.S Herm.	C	(Grant 1984)
G. max (L.) Merr.	C, EA	(Barwale et al. 1986; Buchheim et al. 1989; Christianson et al. 1983; Christou and Yang 1989; Feng et al. 1989; Ferreira et al. 1991; Finer 1988; Finer and Nagasawa 1988; Ghazi et al. 1986; Hammatt and Davey 1987; Hartweck et al. 1988; Hepher et al. 1988; Kien et al. 1989; Komatsuda and Ko 1990; Komatsuda and Ohyama 1988; Lazzeri et al. 1985, 1987a, 1987b, 1988; Li et al. 1985; Parrott et al. 1988, 1989; Ranch et al. 1985; Shoemaker et al. 1991; Shoemaker and Hammond 1988; Tétu et al. 1987)
G. soja Sieb. et Zucc.	H	(Gamborg et al. 1983)

	Species	Explant[1]	Reference
1	*Lathyrus sativus* L.	ST	(Gharyal and Maheshwari 1983)
2	*Lens culinaris* Medik.	EA	(Saxena and King 1987)
3	*Lotus corniculatus* L.	L	(Arcioni and Mariotti 1982; Arcioni et al. 1988)
4	*Lupinus albus* L.	C	(Nadolska-Orczyk 1992)
5	*L. angustifolius* L.	C	(Nadolska-Orczyk 1992)
6	*L. mutabilis* Sweet	C	(Nadolska-Orczyk 1992)
7	*Medicago coerulea* Less.	LP	(Arcioni et al. 1982)
8 9	*M. difalcata* Sinsk. (=*M. sativa* spp. *difalcata*)	LP	(Gilmour et al. 1987)
10	*M. falcata* Arcengeli (=*M. sativa* spp. *falcata*)	LP	(Denchov et al. 1991; Gilmour et al. 1987)
11	*M. glutinosa* M.B. (=*M. sativa* spp. *glutinosa*)	LP	(Arcioni et al. 1982; Gilmour et al. 1987)
12 13	*M. hemicycla* Grossh. (=*M. sativa* spp. *hemicycla*)	LP	(Gilmour et al. 1987)
14	*M. media* Pers.	R, H	(Nagarajan and Walton 1987)
15	*M. sativa* L.	C, H, L, LP, O, P, S, SC, ST	(Anandarajah and McKersie 1990; Arcioni et al. 1982; Bianchi et al. 1988; Binarová et al. 1990; Brown and Atanassov 1985; Chen and Marowitch 1987; Chen et al. 1987; Denchov et al. 1991; Dos Santos et al. 1980, 1983; Frame et al. 1991; Fujii et al. 1989, 1990; Hartman et al. 1984; Hernández-Fernández and Christie 1989; Johnson et al. 1981; Kao and Michayluk 1980, 1981; Latunde-Dada and Lucas 1988; Lu et al. 1982, 1983; Lupotto 1983, 1986; McKersie et al. 1989; Meijer 1989; Meijer and Brown 1985, 1987a, 1987b, 1988; Meijer and Simmonds 1988; Mezentsev et al. 1982; Mitten et al. 1984; Nagy et al. 1991; Novák and Konecná 1982; Ray and Bingham 1989; Reisch and Bingham 1980; Saunders and Bingham 1972; Seitz Kris and Bingham 1988; Senaratna et al. 1989, 1990; Slade et al. 1989; Song et al. 1990; Stavarek et al. 1980; Strickland et al. 1987; Stuart and Strickland 1984a, b; Walker and Sato 1981; Walton and Brown 1988; Wan et al. 1988; Xu et al. 1982; Yu et al. 1990)

	Species	Explant[1]	Reference
1	*M. truncatula* Gaertn. L		(Nolan et al. 1989)
2	*M. varia* Mart. (=*M. sativa* spp. *varia*)	LP	(Gilmour et al. 1987)
3	*Phaseolus acutifolius* Gray	L	(Kumar et al. 1988b)
4	*P. coccineus* L.	C	(Genga and Allavena 1991)
5	*Pisum sativum* L.	LP, ST, ZE	(Kysely et al. 1987; Kysely and Jacobsen 1990; Lehninger-Mertens and Jacobsen 1989; Tétu et al. 1990)
6	*Robinia pseudoacacia* L.	ZE	(Merkle and Wiecko 1989)
7	*Trifolium arvense* L.	H	(Bhojwani et al. 1984)
8	*T. alpestre* L.	C, H, R	(Yamada and Higuchi 1990)
9	*T. amabile* Humb., Bonpl. et Kunth	C, H, R	(Yamada and Higuchi 1990)
10	*T. apertum* Bobrov	C, H, R	(Yamada and Higuchi 1990)
11	*T. caucasicum* Tausch	C, H, R	(Yamada and Higuchi 1990)
12	*T. cherleri* L.	C, H, R	(Yamada and Higuchi 1990)
13	*T. heldreichianum* Hausskn.	C, H, R	(Yamada and Higuchi 1990)
14	*T. incarnatum* L.	H	(Pederson 1986)
15	*T. medium* L.	P	(Choo 1988)
16	*T. montanum* L.	C, H, R	(Yamada and Higuchi 1990)
17	*T. pratense* L.	Ca, H, P, SC, ZE	(Bhojwani et al. 1984; Collins and Phillips 1982; Keyes et al. 1980; Maheswaran and Williams 1986b; McGee et al. 1989; Phillips and Collins 1980; Wang and Holl 1988)
18	*T. repens* L.	H, L, ZE	(Bhojwani et al. 1984; Bond and Webb 1989; Maheswaran and Williams 1984, 1986a, 1987; Parrott 1991)
19	*T. resupinatum* L.	ZE	(Maheswaran and Williams 1986b)

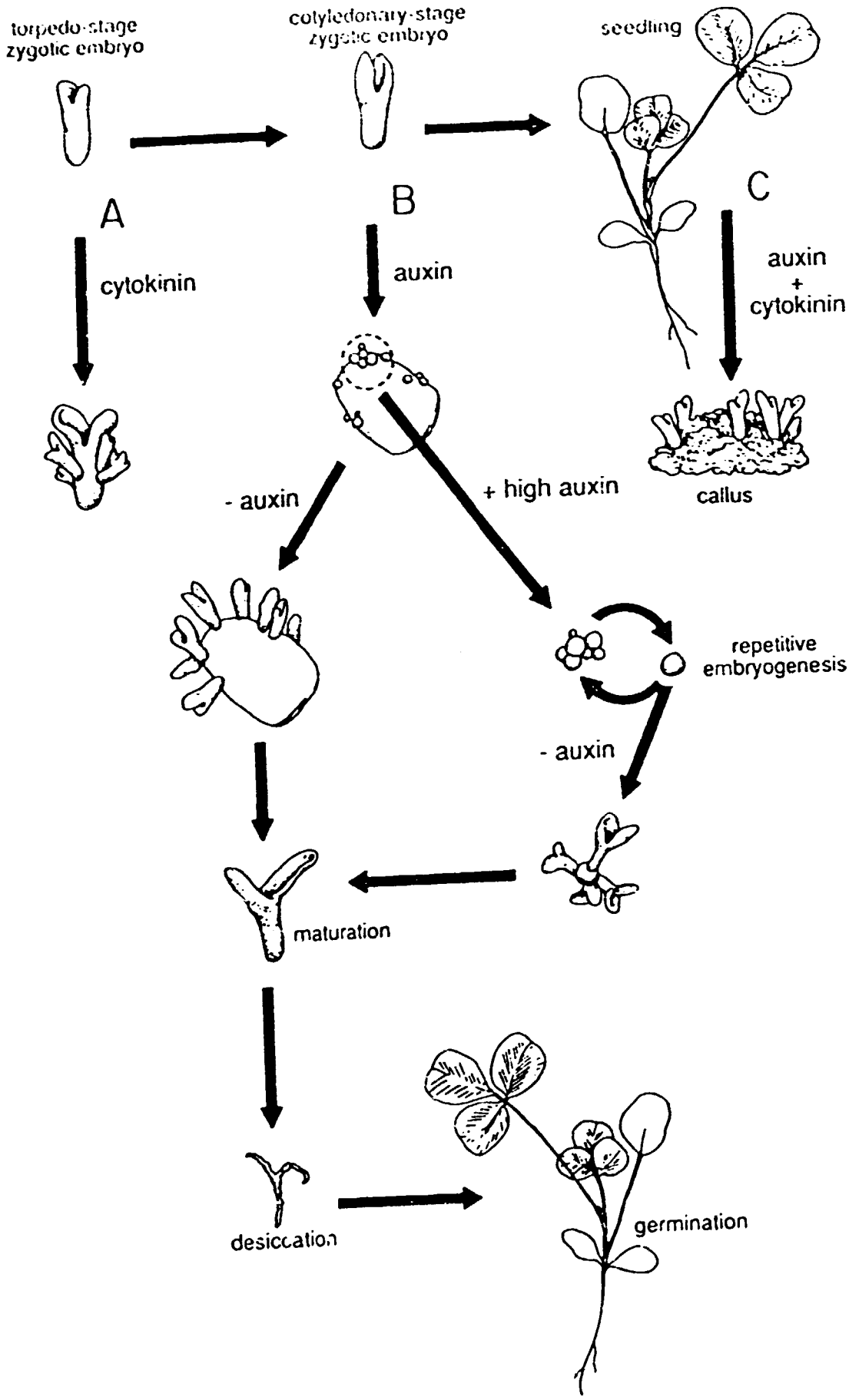
	Species	Explant[1]	Reference
1	*T. rubens* L.	H	(McGee et al. 1989; Parrott and Collins 1983)
2	*T. subterraneum* L.	ZE	(Maheswaran and Williams 1986b)
3	*T. vesiculosum* Savi	H	(Pederson 1986)
4	*Trigonella corniculata*		(Lu et al. 1982)
5	*Vicia faba* L.	C	(Griga et al. 1987)
6	*V. narbonensis* L.	ST	(Pickardt et al. 1989)
7	*Vigna aconitifolia* (Jacq.) Meréchal	L, LP	(Eapen and George 1990; Kumar et al. 1988a; Shekhawat and Galston 1983)
8	*V. glabrescens* × *V. radiata* (L.) Wilczek	ZE	(Chen et al. 1990)
9	*V. mungo* (L.) Hepper	C	(Eapen and George 1990)
10			

11 [1]A=anther, C=cotyledon, CN=cotyledonary node, CP=cotyledon protoplast, Ca=callus, CaP=callus protoplast, E=epicotyl, EA=embryonic axis, H=hypocotyl,
12 HP=hypocotly protoplast, I=internode, L=leaf, LP=leaf protoplast, O=ovary, P=petiole, R=root, Ra=radicle, S=stem, SC=suspension culture, ST=shoot tip,
13 St=stolon, ZE=zygotic embryo

Table 2. Explant source for somatic embryogenesis observed for various species of legumes from which whole plants were not obtained.

Species	Explant[1]	Reference
Acacia koa Gray	H, ST	(Skolmen 1986)
Arachis glabrata Benth.	A	(Martin 1970)
A. hypogaea L.	A	(Martin 1970)
Cercis canadensis L.	ZE	(Trigiano et al. 1988)
Cyamopsis tetragonoloba L.	SC	(McHughen and Swartz 1984)
Glycine gracillis Skvortz	C	(Komatsuda 1990; Komatsuda et al. 1991)
G. max (L.) Merr.	C, H, SC	(Beversdorf and Bingham 1977; Kerns et al. 1986; Komatsuda 1990; Phillips and Collins 1981; Yang et al. 1991)
G. soja Sieb. et Zucc.	H, SC	(Beversdorf and Bingham 1977; Phillips and Collins 1981)
G. tabacina	H	(Beversdorf and Bingham 1977)
Medicago sativa L.	P, SC, ZE	(Binarová and Dolezel 1988; Maheswaran and Williams 1984)
Phaseolus vulgaris L.	ST	(Martins and Sondahl 1984)
Trifolium ambiguum Bieb.	H	(Pederson 1986)
T. arvense L.	H	(Bhojwani et al. 1984)
T. pratense L.	ZE	(Maheswaran and Williams 1984)
T. repens L.	H, ZE	(Maheswaran and Williams 1985; Pederson 1986)
T. rubens L.	P	(Cui et al. 1988)
Vicia faba L.	C	(Griga et al. 1987)
Vigna radiata (L.) Wilczk	C	(Eapen and George 1990)

[1]A=anther, C=cotyledon, H=hypocotyl, P=petiole, SC=suspension culture, ST=shoot tip, ZE=zygotic embryo.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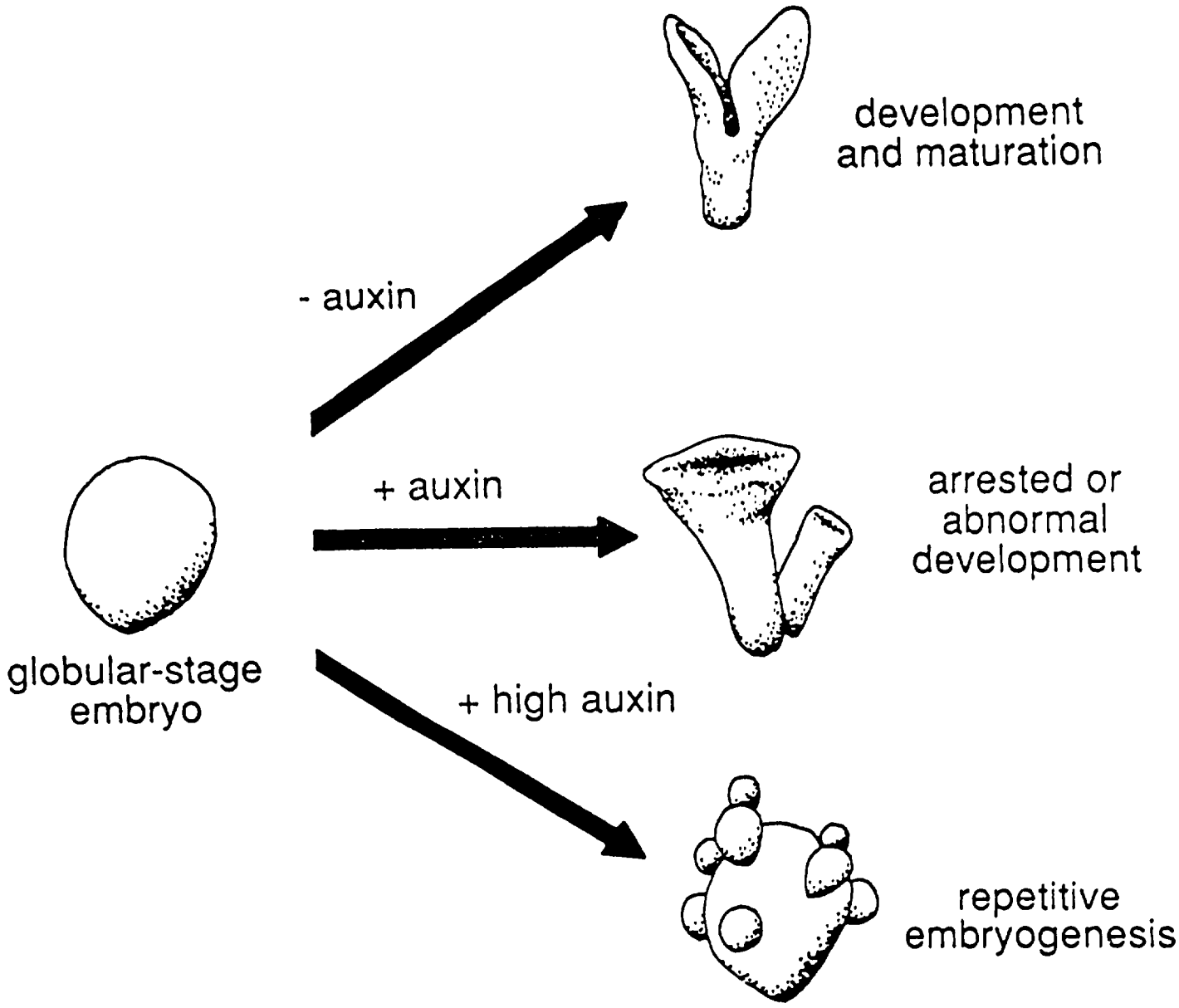
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Methods Manual prepared for the CAMBIA
UNIDO/ICGEB/Course

Plant Biotechnology:
Tissue Culture And Beyond

Cairo, Egypt October 9 - 21, 1993

Part II

The Use of Reporter Genes for the Generation and Analysis of Transgenic Plants

Release 25 - 9 - 93

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Preface

This manual has been assembled by CAMBIA as an aide for the International Course on Plant Biotechnology: Tissue Culture and Beyond, Cairo, Egypt held October 9 - 21, 1993. It is modeled on a version produced as a service to Rockefeller Foundation's Rice Biotechnology Network. In the current manual, assays that are used in the isolation, demonstration and assay of transgenic plants are described. The principle focus is on the use of reporter genes, particularly GUS (ß-glucuronidase) and will be expanded in future updates to include the new non-destructive versions of GUS, as well as a new non-destructive marker. Assays for these enzymes, both spatial and quantitative are described, and extensive background biology and biochemistry is given. Additionally, the principle underlying the use of different selectable markers in the production of transgenic plants will be discussed in future updates.

This Manual is under constant revision. Registered users will be eligible for quarterly updates and new vectors and methods

Introduction

Gene Fusion Systems

Gene fusions are DNA constructions in which DNA sequences from two (or more) genes are combined such that the coding sequences of one gene (the responder) are transcribed and/or translated under the direction of another gene(s) (the controller). In one general class of experiments, the purpose of incorporating the responder gene is to facilitate and enhance detection of gene activity; in such cases the responder gene is acting as a reporter of gene activity. In other experiments the responder gene is included with the aim of manipulating a desired change in cell phenotype and is thus acting as an effector.

Reporter genes usually encode enzymes and the appearance of the reaction products is used to make deductions about the activity of the gene fusion and hence about the controller gene sequences. The use of responder genes as effectors in a manipulative sense can be exemplified by chimeric genes encoding antibiotic resistance. At present GUS is used only as a reporter gene, but work is currently in progress to synthesis substrates that would enable it to be also used as an effector gene. It is to emphasize the diverse ways in which GUS may be used that we refer to it as a gene fusion system.

The reasons to use gene fusions are many. The overall purpose, however is to both facilitate and expand the ways in which gene activity may be studied. For example, it is often extremely difficult and/or laborious to devise and implement methods for the detection of a particular gene product. By using gene fusions, one set of methods that have been fully developed in other systems are generally applicable to the analysis of expression of almost any gene of interest. Moreover, a powerful reporter gene, with simple and quantitative assays, can make the logistics of analyzing large numbers of transgenic plants much more reasonable, a feature which is going to become of increasing importance as the extent of natural variation in gene expression begins to emerge. Finally, a powerful and sensitive gene fusion marker makes possible the ready and reliable detection of transgenic tissue following the introduction of foreign DNA into plant material.

A powerful gene fusion system does not just make standard forms of gene analysis available, but actually enables forms of analysis that are difficult or impossible by other means. One example derives from the extreme sensitivity of an enzyme (reporter gene) assay, which allows gene activity to be analyzed even in cases where the gene product itself is present in such low amounts that it would be almost impossible to detect by other means. Another example is the analysis of genes that are members of multi-gene families whose products are very similar but may be regulated differentially during development. By using gene fusions to individual members of such families and introducing these fusions into the genome, one can distinguish and hence study the expression of individual genes separately from the background of the other members of the gene family (e.g. Forde *et al.* 1989). An even more exciting application of gene fusion technology, which should become available in the near future, will be the possibility of monitoring gene activity in vivo in living plants.

Properties of an ideal gene fusion system

Gene fusion technology is useful only to the extent to which it facilitates and/or extends the possibilities for the analysis of gene function. This is largely determined by the properties of the protein encoded by the chosen responder gene. The minimal requirements for a good reporter gene are that there should be no intrinsic background activity in the organism being studied and that the enzyme should be able to be assayed routinely and reliably in the laboratory. In addition, the enzyme should not interfere with normal physiological functioning of the organism, nor affect the biochemistry adversely. To ensure that the assays are quantitative i.e. that the rate of end product accumulation is directly proportional to the level of gene expression, the enzyme activity should not be affected by any post-translational modifications nor be subject to any other form of regulation. There should also not be any endogenous substrates that could compete with the substrate being supplied, or any endogenous enzymes that could further metabolize the reaction product(s), as either would affect the quantitative nature of the assay.

There are many additional features that can greatly enhance the power of a gene fusion system. Ideally the enzyme should have numerous, simple assays, some of which are sensitive enough to measure moderate to low levels of gene expression in single cells, and others which allow spatial discrimination of enzyme activity within the complex cellular patterns of tissues and organs. The enzyme should be able to tolerate amino terminal fusions, so that it can be used in translational as well as transcriptional fusions - for example to facilitate studies on translational initiation, intracellular targeting, translocation of proteins across membranes or selective turnover of proteins. There should be the possibility of using the system as a true responder, providing both reporting and effecting functions - thereby allowing genetic selections to be applied. Finally, there should be methods available to use the system in live organisms, rather than having always to study gene expression in homogenized, lysed, permeabilized, frozen or otherwise abused tissues.

GUS

The *E. coli* gene encoding ß-glucuronidase (GUS) is now widely used as a reporter in transgenic plants, bacteria and fungi, and has many properties that makes it nearly ideal for gene fusion experiments. Intrinsic ß-glucuronidase is absent from most, if not all higher plants, fungi of industrial and agricultural importance, and most bacteria, and hence the GUS activity due to expression of an introduced gene or gene fusion can be measured with great sensitivity. The enzyme is very stable and can be assayed using numerous spectrophotometric, fluorometric and histochemical assays. A virtually unlimited number of new substrates for GUS can be synthesized by a variety of novel routes to further increase the scope of gene fusion experiments.

Many of the major logistical hurdles in agricultural biotechnology - including the reliable detection of rare events such as homologous recombination, transformation of recalcitrant crop species, or optimizing lengthy procedures such as the development of conditions for *Agrobacterium* infection - can be overcome by the

creative use of this fusion system. More importantly, because of the cheapness, versatility, accuracy and speed of assays, the use of the GUS system will do much to eliminate the greatest barrier in agricultural biotechnology namely our great ignorance of the molecular biology of plants, fungi, bacteria in the field, and their interactions with each other and the environment.

In this manual and in the cited references, we review many features of the GUS system and its use for plant, fungal and bacterial studies, and describe detailed protocols for the assay of ß-glucuronidase in transgenic organisms.

Introduction to the GUS system

Chemistry of Substrates for GUS;

GUS catalyzes the hydrolysis of a very wide variety of ß-glucuronides, and with much lower efficiency some ß-galacturonides; the reaction and a small selection of the available substrates for routine assay of the enzyme are diagrammed in Figure xx. Almost any aglycon conjugated in a hemiacetal linkage to the C1 hydroxyl of a free D-glucuronic acid in the ß configuration serves as a GUS substrate. Glucuronides are generally very water soluble, due to the ionizable carboxylic acid group at the 6-carbon position in the glycon.

Most aromatic and aliphatic glucuronides are remarkably stable relative to other types of glycoside conjugates. It is speculated that this is due to the inductive effect of the carbonyl group at C-6 on the hemiacetal linkage at C-1. Many ß-glucuronides can be prepared free of other contaminating glycosides by vigorous acid hydrolysis, which cleaves glucosides, galactosides and other glycosides, but leaves most glucuronides intact. For example, complex carbohydrate polymers such as gum arabic can be reduced to a collection of monosaccharide components, and the single ß-glucuronyl disaccharide aldobiuronic acid, simply by boiling gum arabic in sulfuric acid overnight. This characteristic stability is extremely convenient not only in the preparation of glucuronides but also in the analysis of GUS. Colorigenic and fluorogenic substrates such as p-nitrophenyl ß-D-glucuronide and 4-methylumbelliferyl ß-D-glucuronide are much more stable in aqueous solution than the corresponding ß-D-galactosides or ß-D-glucosides, making background due to spontaneous hydrolysis much less of a problem.

Occurence of Glucuronides in Nature

Glucuronides in Polysaccharides

ß-Glucuronides in polysaccharide form are common in nature, most abundantly in vertebrates, where they are major constituents of connective and lubricative tissues (eg. chondroitan sulfate of cartilage, and hyaluronic acid - the principle constitutent of synovial fluid and mucus) in polymeric form with other sugars such as N-acetylglucosamine. ß-Glucuronides are relatively uncommon in plants. However, some plant gums and mucilages produced by wounded trees, notably gum arabic from *Acacia senegal*, do contain significant fractions of ß-glucuronides in polymeric form, although rarely if ever as terminal residues that would serve as

GUS substrates. Glucuronides and galacturonides found in plant cell wall components (such as pectin) are generally in the alpha configuration, and are frequently substituted as the 4-O-methyl ether; hence these are not substrates for ß-glucuronidase.

Glucuronidation as a Detoxication Pathway

Glucuronides in Simple Chemical Conjugates

As simple glycosides, ß-glucuronides are extremely important as of of the two principal forms in which xenobiotics (compounds that are foreign to the body) and endogenous phenols and aliphatic alcohols are made biologically inert (detoxified) and excreted in the urine and bile of vertebrates (reviewed by Dutton, 1966,1981). The second most important form of detoxification is by addition of a sulfate residue, and this will be discussed further under the section on the aryl sulfatase gene fusion system.

The principal problem underlying detoxification in vertebrates, is that many compounds within the body, including endogenous biologically active molecules such as steroid hormones, bio-degradation products such as bilirubinsteroid hormones, and foreign compounds (xenobiotics) that may have been introduced into the body in food or medicine, are lipophilic or fat soluble. Hence, they do not dissolve readily in urine or bile, the two major routes to removal of waste products from the body. This problem is oversome by conjugation of the lipophilic compounds to highly polar residues, such as glucuronic acid or a sulfate residue, making the resulting conjugate highly water soluble, and thus able to be excreted from the body.

Glucuronidation occurs in many tissues in vertebrates, particularly in the liver. The reaction is carried out by a set of membrane-bound enzymes that catalyze the transfer of a glucuronate residue from uridine diphosphate 1-α-D-glucuronate to the aglycon (the **aglycon** is the residue being detoxified, to which the sugar molecule or **glycon** is bound). Several isozymes of UDP-glucuronyl transferase have been characterized, and these are reviewed in detail in Dutton (1980). These enzymes frequently form part of a collection of detoxifying enzymes, including hydroxylases and mixed-function oxidases, that work together to metabolize lipophilic, relatively insoluble compounds into the highly water-soluble glucuronide conjugates (as well as into sulfates and other derivatives). These conjugates are then excreted into the bile (for the larger glucuronide conjugates) or the urine.

Several thousand ß-glucuronides have been identified in urine and bile as detoxication products. This includes many that form following oral administration of the free aglycon or a related compound, for example as a drug during medical treatment, and an extensive list of known glucuronides can be found in Dutton (1966). In addition, many endogenous steroid hormones and bioactive substances, or bio-degradation products such as bilirubin, are conjugated and excreted as ß-glucuronide conjugates.

This process of conjugation with glucuronides is of coursed reversed by activity of the enzyme ß-glucuronidase (GUS). Interestingly, ß-glucuronidase activity is reliably reported almost exclusively from those organisms that have, or are associated with organisms that have glucuronidation as a detoxication pathway. Thus vertebrates, which all use glucuronidation as the principle conjugation mechanism, together with their endogenous microbe populations (particularly *E. coli*) have GUS activity. By contrast, insects and plants conjugate xenobiotics with glucose, rather than glucuronic acid, as their detoxication and derivatization mechanism, and ß-glucuronidase is rarely if ever reported in these organisms or their associated microbial populations.

The Enzyme ß-Glucuronidase

Animals

ß-glucuronidase activity is extremely common in almost all tissues of all vertebrates and many molluscs (Levvy and Conchie, 1966). The enzyme has been purified from many mammalian sources (eg. Tomino *et al*, 1975) and shows a homotetrameric structure, with a subunit molecular weight of approximately 70 kDa. The enzyme from these sources is synthesized with a signal sequence at the amino terminus, and is then transported to and glycosylated within the endoplasmic reticulum and ultimately localized intracellularly within vacuoles. Unlike the bacterial enzyme, mammalian and molluscan GUS can cleave thioglucuronides. In general, however, the *E. coli* GUS is much more active than the mammalian enzyme against most biosynthetically derived ß-glucuronides (Tomasic and Keglevic, 1973; Levvy and Conchie, 1966). The genetics of GUS in mammals have been extensively characterized (reviewed in Paigen, 1979).

Plants

GUS activity is largely if not completely absent from higher plants (Jefferson *et al*, 1987), mosses (D. Schaefer, pers. comm.) and ferns (A. Silberstein, pers. comm.) There are a few reports of endogenous activity in plants but they rarely include quantitative tests with more than one substrate, to ensure that the activity is a true ß-glucuronidase, not an activity specific for the aglycon of the test substrate (eg. Schulz and Weissenbock, 1987), nor do they often make use of specific inhibitors of GUS such as saccharolactone (see below). Such reports should also be interpreted cautiously because only rarely do plants exist without numerous exo- and endophytic organisms, many not yet classified, which could be contributing GUS activity. Even "sterile" tissues in culture can have numerous endophytic organisms. Often conflicting reports concerning GUS activity have been traced to these differences, hence it is always worth repeating such observations on local test material. Still, it must be remembered that, though rare, intrinsic activity may occur and must be objectively dealt with and characterized. Specific glucuronidases that recognize endogenous substrates such as glycyrrhizin conjugates have been described, but are not capable of cleaving GUS assay substrates.

Nematodes

The free-living soil nematode, *Caenorhabditis elegans*, has an endogenous ß-glucuronidase activity (Sebastiani *et al*, 1987; Jefferson *et al*, 1987), which occurs at low levels in the intestine of the worm. Enzyme activities in other nematodes have apparently not been investigated.

Insects

Very few insects have been investigated for intrinsic GUS activity. Studies on *Drosophila melanogaster* embryos, pupae and larvae showed no detectable activity under conditions that gave very high levels of ß-galactosidase (Jefferson, 1985). More recent studies show that even in adult Drosophila, GUS is largely if not completely absent. Other insects from diverse tax are also GUS negative (P Atkinson, in preparation).

Locust crop fluid liquor is a source of GUS but it is not clear whether this is an intrinsic activity, or due to microorganisms in the crop fluid.

Fungi and Moulds

GUS activity has not yet been found in any fungi, including *Saccharomyces*, (Jefferson, 1985; Schmitz *et al*, 1989;) *Schizosaccharomyces*, *Aspergillus*, *Neurospora*, *Cladosporium*, *Leptosphaeria* (Roberts *et al.*, 1989; R. Oliver, pers. comm.) and other Ascomycetes such as barley powdery mildew (Jefferson and M. Wolfe, unpublished data), or Oomycetes such as *Bremia lactuca* (R. Michelmore, pers. comm). There is also no detectable activity in the slime mould, *Dictyostelium discoidum* (Datta *et al*, 1986; Jefferson, 1985)

Bacteria

GUS activity is not present in most bacterial genera examined. In fact, GUS assays are routinely used as diagnostic assays for the specific detection of *E. coli* and *Shigella* species in clinical and environmental samples (e.g. Rice *et al.* 1990; Cleuziat and Robert-Baudouy 1990). There were earlier reports of some GUS$^+$ *Salmonella* species (Killian and Buellow 1979) but these appear not to have been confirmed by later studies (e.g. Perez *et al.* 1986; Cleuziat and Robert-Bauduoy 1990). Thus, as the eighth edition of Bergey's Manual states that "it is taxonomically difficult to justify separate genera or even separate species status for (*E. coli* and *Shigella*)" (Brenner 1984), GUS activity can reasonably be said to be restricted to a single taxonomic group - that of *E. coli* and *Shigella* species - among the Enterobacteriaceae.

The natural habitat of *E. coli* is the gut, and the GUS activity of *E. coli* plays a specific and very important role in its natural history. As discussed above, the gut is a rich source of glucuronic acid compounds, providing a carbon source that can be efficiently exploited by *E. coli*. Glucuronide substrates are taken up by *E. coli* via a specific transporter, the glucuronide permease (see below), cleaved by ß-glucuronidase and the glucuronic acid residue thus released is used as a carbon source.

In general, the aglycone component of the glucuronide substrate is not used by *E. coli* and passes back across the bacterial membrane into the gut to be reabsorbed into the bloodstream. This circulation of hydrophobic compounds resulting from the opposing processes of glucuronidation in the liver and deglucuronidation in the gut is termed enterohepatic circulation (Figure xx). This phenomenon is of great physiological importance because it means that, due in large part to the action of microbial ß-glucuronidase, many compounds including endogenous steroid hormones and exogenously administered drugs, are not eliminated from the body all at once. Rather, the levels of these compounds in the bloodstream oscillate due to this circulatory process. This process is of great significance in determining pharmaceutical dosages, and indeed some drugs are specifically administered as the glucuronide conjugate, relying on the action of ß-glucuronidase to release the active aglycone (Draser and Hill 1974). Enterohepatic circulation is also important in the day-to-day physiological state of the body, probably being a prime cause of the physiological impact of variations in diet or in gut flora (Goldin 1986).

GUS activity is found in certain other bacterial species. In particular, it is found in other, non-enterobacterial, anaerobic residents of the gut, primarily in *Bacteroides* and *Clostridium* species (Hawkesworth *et al.* 1971). Although these species exhibit lower ß-glucuronidase activity per cell than *E. coli*, they are apprcximately 100-fold more abundant in the gut, and hence it was suggested that they might make a more significant contribution overall to enterohepatic circulation. However, it is difficult to judge the relative contributions of the different groups of bacteria based on a single set of measurements of their GUS activity with one glucuronide substrate. It is not known, for example, whether these organisms possess a glucuronide permease and whether their GUS activity, or any permease activity, possesses the same substrate versatility as the *E. coli* enzyme.

There are reports of GUS activity in strains of *Streptococcus*, *Staphylococcus* and *Corynebacteria* (Dutton 1966), and we have found certain bacteria associated with plants that are GUS$^+$ (see below). However, GUS activity is not found in most of the bacterial species that are commonly studied because of their importance in agriculture, such as *Rhizobium*, *Bradyrhizobium*, *Agrobacterium* and *Pseudomonas* species. Thus GUS is now being used as a reporter gene in these organisms, allowing studies of the spatial localization of gene activity of these bacteria in association with their plant hosts.

Properties of ß-Glucuronidase from Escherichia coli

The ß-glucuronidase of *E. coli* was first described in any detail by F. Stoeber (1961) who demonstrated key features of its regulation and biochemistry. The enzyme has been purified to near homogeneity and shows many properties that make it excellent for gene fusion experiments (Jefferson, 1985; Jefferson *et al.* 1986). Purification can be carried out very simply by conventional ion exchange (DEAE) and gel filtration methods. Several additional methods have been develcped (eg. Blanco and Nemoz, 1987).

E. coli ß-glucuronidase (GUS) has a monomer molecular weight of about 68,200 daltons, although under certain conditions of SDS-PAGE it migrates a bit slower than would be predicted (around 72-74 kDa). The behaviour of the native enzyme

on gel filtration columns indicates that the active form is probably a tetramer. It is not processed at the amino terminus in *E. coli*, and is found exclusively in the cytoplasm. Although GUS is not glycosylated, there is at least one cryptic glycosylation site. GUS fusions that incorporated amino-terminal addition of a signal peptide have shown that these sites can be utilized in eukaryotes in the endoplasmic reticulum, leading to great reduction of enzyme activity (Iturriaga *et al*, 1989).

GUS is an exo-hydrolase; it will not cleave glucuronides in internal positions within polymers. GUS is specific for ß-D-glucuronides, with some tolerance for ß-galacturonides. It is inactive against ß-glucosides, ß-galactosides, ß-mannosides, or glycosides in the alpha configuration.

ß-Glucuronidase is very stable, and will tolerate many detergents, widely varying ionic conditions, and general abuse. It is most active in the presence of thiol reducing agents such as ß-mercaptoethanol or dithiothreitol (DTT). GUS has no cofactors, nor any ionic requirements. GUS is inhibited by some divalent metal ions; 70% inhibition by Mn^{2+} and Co^{2+} at 10 mM, and completely by Cu^{2+} and Zn^{2+} at comparable concentrations (Stoeber, 1961). ß-Glucuronidase can be assayed at any physiological pH, with an optimum between 5.0 and 7.8. The enzyme is about 50% as active at pH 4.3 and at pH 8.4. GUS from *E. coli* K12 is reasonably resistant to thermal inactivation with a half-life at 55 C of about two hours and at 60 C, about 15 minutes (Jefferson 1985). The specific inhibitor, glucaric acid 1.4 lactone (saccharic acid lactone, saccharolactone) is a very useful reversible competitive inhibitor of GUS (see below).

The E. coli gus Operon

ß-glucuronidase is encoded by the *gus*A (formerly *uid*A) locus of *E. coli* (Novel and Novel, 1973) at 35.6 minutes on the *E. coli* K12 chromosome map (Bachmann, 1987). This region of the chromosome lies between *add* and *man*A and the newly discovered *man*B locus (Jefferson and Liang, in preparation). Interestingly, this entire region is also completely absent from the genome of the closely related bacterium, *Salmonella typhimurium*. Since the initial mapping of the gene, it has been determined that *gus*A is one member of an operon, consisting of three protein-encoding genes. The second gene encodes a specific permease for glucuronides (see Stoeber, 1961; Jefferson, Liang and Roscoe, 1989, in preparation). The third gene encodes a periplasmic or peripheral membrane protein of approximately 50 kDa that may be involved in permease action (Liang, Roscoe and Jefferson, in preparation). The principle repressor for the *gus* operon, *gus*R, maps immediately upstream of the operon.

Since the discovery, sequencing and characterization of the additional genes of the operon, the *uid* locus has been renamed the *gus* locus, consisting of *gus*R (repressor of the *gus* operon), *gus*A, (GUS - ß-glucuronidase) *gus*B (PER - ß-glucuronide permease) and *gus*C (MOP - tentatively a modulator of permease activity). As the use of the mnemonic GUS to refer to the enzyme is already in very wide usage due to the implementation of the gene fusion system, because the extensively characterised genetic loci encoding ß-glucuronidase in humans, mice and other mammals are called *gus* (reviewed by Paigen, 1979), and because *uid*

has little mnemonic value, we feel that the renaming of the locus in *E. coli* is warranted.

Regulation of GUS activity in E. coli

ß-glucuronidase activity is not constitutively expressed in *E. coli*: rather, there appear to be three different factors regulating transcription of the operon. The primary mechanism of control is induction by glucuronide substrates. GUS activity is almost undetectable in cells which have been grown in the absence of glucuronides; however, incubation of *E. coli* in the presence of a glucuronide substrate leads to induction of high levels of GUS activity (Stoeber 1961).

This regulation is due to the action of the product of the *gusR* (formerly *uidR*) gene which encodes a repressor that is specific for the *gus* operon (Novel and Novel 1976a). Inactivation or deletion of *gusR* leads to constitutive ß-glucuronidase activity (Novel and Novel 1976a; KJW and RAJ unpublished results). *gusR* maps to the same region of the chromosome as *gusA*, lying upstream of *gusA* and being separately transcribed. The direction of transcription is the same as *gusA* (Blanco *et al.* 1985; KJW and RAJ unpublished) and we have recently completed the DNA sequence of this region and have found that *gusR* is most likely encoded by an open reading frame of 195 amino acids.

GusR repression of ß-glucuronidase activity has been shown by Northern analysis to be mediated by transcriptional regulation. RNA from uninduced cultures of *E. coli* showed no hybridization to a *gusA* probe, in contrast to the strong hybridization observed to RNA extracted from cultures that had been induced with methyl ß-D-glucuronide (Jefferson 1985). Presumably, therefore, GusR acts by binding to *gusA* operator sequences so preventing transcription, this repression being relieved when a glucuronide substrate binds to the repressor and inactivates it. While the exact operator site remains to be defined, there are several candidate regions of dyad symmetry upstream of the *gusA* ATG (Jefferson *et al.* 1986).

A second key level of control is that of catabolite repression. *E. coli* grown in the presence of 1% glucose does not express ß-glucuronidase activity even in the presence of a glucuronide inducer (Stoeber 1961). A putative CAP binding site has been identified in the *gusA* upstream sequences (Jefferson *et al.* 1986).

A third level of regulation of *gus* transcription appears to be exerted by the product of the *uxuR* gene. This gene, which maps elsewhere on the *E. coli* chromosome at minute 98, is primarily concerned with regulation of the *uxuAB* operon which encodes enzymes involved in the further metabolism of glucuronic acid (Novel and Novel 1976b). Repression of transcription of the *uxuA* and *B* genes is relieved by incubation with glucuronic acid, which presumably binds to and inactivates UxuR. In *E. coli* K-12, mutations in *uxuR* cause derepression of ß-glucuronidase to only 1-4% of the full glucuronide-induced level (Novel and Novel 1976b). As induction of transcription of a gene encoding an enzyme activity by the product of that enzyme activity is hard to understand, and as the level of regulation is < 5% of that exerted by GusR and by CAP, it is not certain whether regulation by UxuR is of primary importance or is a secondary effect, perhaps resulting from some degree of homology between the two gene products. This latter possibility is supported by the

observation that expression of *uxuR* on a multicopy plasmid can largely suppress the effect of a *gusR* mutation (Ritzenhaler *et al.* 1983).

A glucuronide specific permease is encoded by gusB

The existence of a glucuronide-specific permease was first demonstrated in the late 1950s by F. Stoeber working in the laboratory of Jacques Monod at the Pasteur Institute, Paris. Stoeber measured accumulation of ^{35}S phenyl ß-D-thioglucuronide, a glucuronide that is not hydrolyzed by GUS, in a faecal *E. coli* isolate and showed that this compound could be accumulated from external concentrations as low as 5 µM, giving over 200 fold concentration in the cell. This accumulation was inhibited in the presence of sodium azide, indicating that it is an active transport process. Using uptake competition studies he demonstrated that different glucuronides have different affinities for the permease, and some, such as phenolphthalein ß-D-glucuronide, are not taken up at all. Like GUS activity, the permease activity is induced by pre-incubation with a glucuronide substrate (Stoeber 1961).

Interestingly, Stoeber found that *E. coli* K-12, in contrast to the faecal strain, had very poor uptake ability (Stoeber 1961). We have repeated this observation with the cloned *gus* operon from a K-12 strain and from a faecal strain. When expressed in a host strain that is deleted for the *gus* operon, the cloned K-12 genes conferred almost no ability to accumulate phenyl thioglucuronide in the cells, in contrast to the strong accumulation observed on expression of the operon from the faecal isolate (W-J Liang, P J Henderson, KJW and RAJ unpublished results). This observation is of considerable importance in induction assays (see below).

Molecular-genetic evidence concerning the glucuronide permease was first obtained when 340 bp of an open reading frame, encoding a highly hydrophobic protein, was identified immediately downstream of *gusA* - in fact there is a four base-pair overlap (ATGA) between the two genes (Jefferson *et al.* 1986). The sequence of *gusB* from *E. coli* K-12 has now been completed and indicates a protein with twelve membrane spanning domains, that has 25% identity at the amino acid level to the *E. coli* melibiose transporter encoded by *melB* (RAJ and W-J Liang unpublished data; Liang 1989). From the homology to the melibiose transporter (a sodium symporter) and from the dependence on membrane potential, it is likely that the glucuronide permease also functions as a cation symporter coupled to the electrochemical gradient.

The range of gus operon inducers

We now know that efficient induction of expression of ß-glucuronidase and of the whole operon depends on two key steps:

1) The substrate must be taken up into the cell via the glucuronide permease.
2) The substrate must be able to alleviate repression by the *gus* repressor.

Stoeber tested the ability of a number of different glucuronides to induce GUS activity, and found that it varied greatly, methyl ß-D-glucuronide at 1 mM

concentration inducing a level of GUS activity approximately 15 times that of phenyl ß-D-thioglucuronide (a gratuitous inducer). He also found that some GUS substrates, such as phenolphtlathein ß-D-glucuronide, and aldobiuronic acid (galactosyl ß-D-glucuronide) do not act as inducers of ß-glucuronidase activity (Stoeber 1961). In the case of phenolphthalein ß-D-glucuronide, this is clearly due to the inability of this substrate to be transported by the permease.

We have tested the ability of GUS substrates now commonly used in quantitative and spatial analysis of GUS activity to act as inducers of the *gus* operon in *E. coli*, and have found that 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-Gluc), p-nitrophenyl ß-D-glucuronide (PNPG), 4-methylumbelliferyl ß-D-glucuronide (MUG) and resorufin glucuronide all act as powerful inducers. In general, values of GUS activity measured after 90' induction, starting with 1 mM external concentrations of these glucuronides, are of the order of 1-50 nmols PNPG hydrolyzed per minute per OD_{600} unit of bacterial culture. We have also tested a number of glucuronides that would occur naturally in the body, including oestrogen glucuronide and testosterone glucuronide, and found that they too appear to have low inducing power (Liang 1989).

This range of inducers illustrates a remarkable fact about the glucuronide permease, namely that it is able to recognize and actively transport an extraordinary range of glucuronides with different aglycone residues. Likewise, the *gus* repressor must be able to recognize the same range of glucuronides. Thus, for example, while X-Gal does not act to induce the *lac* operon in *E. coli* because it is not transported by the *lac* permease, X-Gluc is a powerful inducer of the *gus* operon, indicating that it is actively accumulated within the cell by the glucuronide permease.

All the measurements discussed above, carried out both by Stoeber and by ourselves, have been on faecal isolates of *E. coli*. In contrast, *E. coli* K-12 strains show only very low levels of GUS induction in similar conditions. This can readily be visualized by streaking a faecal sample on an LB plate containing 50 µg/ml X-Gluc: overnight, dark blue colonies will appear. In contrast, an *E. coli* K-12 strain streaked on the same plate will show no or very little blue coloration in the colonies even after several days incubation. This is because the K-12 permease fails to concentrate the X-Gluc from the low external concentration in the plate (about 120 µM) and thus fails to induce the K-12 ß-glucuronidase activity. To obtain high levels of K-12 ß-glucuronidase induction it is necessary to use external concentrations of inducer of at least 10 mM.

Other bacteria show inducible GUS activity: possible contribution to "background" activity in GUS assays

As discussed above, there are bacterial species other than *E. coli* that possess ß-glucuronidase activity, particularly among the gram-positive genera *Staphylococcus* and *Streptococcus*. While the natural habitat of *Staphylococcus* is the skin and mucosal membranes of mammals, and that of *Streptococcus* is the intestinal tract and other organs also of mammals, it is important to remember that such microorganisms are not restricted in their distribution to these habitats but can be found elsewhere in stable and transient niches.

Solid rich media containing X-Gluc (50 µg/ml) provides a convenient means of screening for GUS positive microorganisms. We have screened samples of soil, faecal matter and some plant tissue in this way, and all, depending on provenance, have been shown to contain them. Here we give four examples of non-coliform GUS^+ bacteria which we have found in association with plants and which are illustrative of the false positive results that can occur in carrying out GUS assays with putatively transgenic plant material.

The first bacterium was found among the organisms which grew when pollen was shaken from the flowers of greenhouse-grown tobacco directly onto X-Gluc plates. It had the characteristic colonial morphology associated with highly mobile or gliding bacteria. At 30 C finger-like projections advanced from the point of inoculation at the rate of about 2 cm per day. The mobile circular mound of cells at each end of the projection exhibited GUS (or GUS-like) activity. Of the recognized gliding bacteria, this organism most closely resembles the Myxobacteria. However, although spores were observed within the mounds, we did not observe the larger, sculpted fruiting bodies often found in this group. Of particular interest is the observation that when pollen grains of tobacco placed on X-Gluc plates are engulfed by these bacterial swarms, an intense deposition of blue pigment characteristic of a GUS X-Gluc reaction appears within the grains (Figure 3). Whether or not this observation represents genuine GUS activity, the phenomenon clearly illustrates the capacity of microorganisms to confound histochemical procedures, and emphasizes the need for caution. It is certainly possible that some of the observations of GUS or GUS-like activity in control plants could be explained by the presence of this type of organism.

The second and third organisms were both isolated directly from plant tissues that had been subjected to particle bombardment. More specifically they were recovered from blue zones that developed around the points of impact of shrapnel following histochemical staining with X-Gluc. Fatty acid profiles did not exactly match any characterized microorganisms, but one showed similarity to a *Micrococcus* or *Clavibacter* species and the second to a *Staphylococcus* or *Brevibacterium* species. These assignments are consistent with the results of fermentation and enzyme activity profiles. Both organisms could be cultured on the surface of potato tubers and gave blue zones when the slices were subjected to histochemical staining with X-Gluc.

The fourth organism was isolated from yam (*Dioscorea cayanenesis*), again from tissue that had been subjected to particle bombardment. Analysis of the fatty acid content of this organism identified it as a *Curtobacterium* species, a genus of bacteria that is commonly isolated from plants. The GUS activity of this bacterium was studied more carefully and it was found not to be constitutive but to be inducible by incubation with each of the GUS substrates X-Gluc, MUG and PNPG - albeit to levels below those observed in *E. coli* (M. Tor and KJW unpublished results). This induction of GUS activity was completely repressible by inclusion of 100 µg/ml chloramphenicol in the medium.

The studies on the latter microorganism indicate that GUS activity is inducible not just in *E. coli* but also in other microorganisms. Thus, much of the "background" activity that is observed when samples of tissue are subjected to long incubation in

GUS substrates - often overnight - may result from the induction and subsequent activity of GUS in plant-associated microorganisms. If the enzyme is inducible rather than constitutive then it is easy to overcome the problem by the inclusion of chloramphenicol in the incubation medium, as this specifically inhibits bacterial, but not eukaryotic, protein synthesis.

Gene Fusions Using GUS in Transgenic Systems

The *gus*A gene is 1806 bp long, and encodes the 68 kDa GUS enzyme. The DNA sequence of *gus*A from *E. coli* K12 and several derivatives of the *gusA* gene are shown in the Appendix. The first gene fusions that used GUS as a reporter enzyme used the control sequences from the *E. coli lac* operon to direct GUS expression in *E. coli* (Jefferson *et al.*, 1986). These experiments were followed by the system's first implementation in eukaryotic systems. Chimeric expression vectors were constructed consisting of control sequences from various nematode genes directing the expression of GUS, and were microinjected into strains of the nematode *Caenorhabditis elegans* that lacked intrinsic ß-glucuronidase activity. Although expressed at very low levels, GUS activity was accurately measured fluorometrically in the resulting transgenic nematodes and visualized using histochemical techniques (Jefferson *et al.*, 1987). One of the goals of these experiments was to develop methods for in vivo analysis of gene fusion activity during development. Because the concurrently-developed transformation methods for nematodes proved inadequate to fully test the system, the use of GUS in plants was explored.

Present and future uses of GUS

Many of the major technical and logistical hurdles in agricultural biotechnology - including the reliable detection of rare events such as homologous recombination, transformation of recalcitrant crop species, or optimizing lengthy procedures such as the development conditions for *Agrobacterium* infection - can be overcome by the creative use of this fusion system. The power of the GUS gene fusion system has already been demonstrated in many of the publications in plant molecular biology over the past five years that have employed GUS simply as a reporter gene.

Quantitative GUS assays have been used to study the expression of genes such as those encoding the small subunit of ribulose bisphosphate carboxylase (Jefferson *et al.* 1987b), the major protein present in potato tubers, patatin (Rocha-Sosa *et al.* 1989, Wenzler *et al.* 1989, Jefferson *et al.* 1989), and glutamine synthetase (Forde *et al.* 1989). They have been used to demonstrate the subcellular, and even sub-organelle, location of GUS that had been directed to chloroplasts or mitochondria by the addition of the appropriate target sequences (Kavanagh *et al.* 1988; Schmitz and Lonsdale 1989) or to the endoplasmic reticulum by addition of a signal sequence (Ittarugia *et al.* 1989). The logistical power of the GUS system in assaying gene expression in very large numbers of transgenic plants was demonstrated in a field trial carried out in Cambridge, U.K., in 1987, in which the expression of patatin-GUS fusions in 24,000 different samples of transformed, field-grown potato plants was determined (Jefferson 1989) to obtain statistically reliable estimates of variation in gene expression.

Histochemical assays have been used to localize patterns of expression of genes such as those encoding petunia 5-enolpyruvylshikimate-3-phosphate synthase (Benfey and Chua 1989), a bean cell-wall glycine-rich protein (Keller *et al.* 1989) and bean phenylalanine ammonia lyase (Bevan *et al.* 1989) among others. One unexpected outcome of using such assays was the discovery that the nominally constitutive CaMV 35S promoter does in fact show considerable tissue variation in activity (Jefferson *et al.* 1987b). A particularly elegant demonstration of the power of gene fusions to distinguish between different members of a multigene family was the use of GUS fusions to show that two different glutamine synthetase genes from *Phaseolus vulgaris* are expressed in distinct tissues of the root nodule of transgenic *Lotus corniculatus* (Forde *et al.* 1989).

GUS has also been used to great effect in other capacities. It has been used as a marker for the transformation of important and recalcitrant crop plants such as soybean and rice (Hinchee *et al.* 1988; McCabe *et al.* 1988; Toriyama *et al.* 1988), and in the optimization of transformation of maize using high-velocity microprojectiles (Klein *et al.* 1988). It has also been used to develop a powerful visual assay for the movement of transposable elements, GUS activity being restored when a transposable element inserted between a plant promoter and the initiation codon of the GUS structural gene excises (Masson and Federoff 1989; Finnegan *et al.* 1989). This technique is becoming a versatile tool for studies of plant development by providing a simple and reliable marker for plant cell lineages (Finnegan *et al.* 1989) and for chimeras (W. Swain, Agracetus, pers. comm.; Christou *et al.* 1989).

The lack of intrinsic GUS activity in both plants and the majority of microorganisms that interact with them will enable GUS to be used both as a marker to trace plant-associated microorganisms and also to monitor expression of particular genes in these microorganisms. For example, the use of GUS in the fungus *Cladosporium fulvum*, a pathogen of tomato, was recently described (Roberts *et al.* 1989) and GUS fusions have been used to localize the expression of *Rhizobium* genes in alfalfa root nodules (Sharma and Signer in preparation).

A virtually unlimited number of new substrates for GUS can be prepared, both by chemical synthesis and more interestingly by biosynthetic methods (e.g. Mead *et al.* 1957), to further increase the scope of gene fusion experiments. In the short term, new substrates will mainly be those that enhance the versatility of GUS when used as a reporter gene. For instance, chromogenic substrates that give a different color on cleavage than X-Gluc, or fluorogenic substrates with different absorption and emission maxima to allow maximal separation of the measured signal from the background fluorescence.

In the long run, other substrates will be synthesized that allow both positive and negative selection of GUS-expressing cells, organs and whole plants, as well as substrates that release other bioactive molecules on cleavage, such as plant growth regulators. Further dramatic enhancements of the GUS gene fusion system will be the development of methods that allow the detection of GUS activity in living tissue. The most immediate route by which this will be achieved will be the development of a form of the enzyme that can be secreted and which retains full activity following passage through the endoplasmic reticulum and cleavage of the signal peptide. A longer term objective that will vastly expand the utility of the system is the use of the glucuronide permease (*gusB*) in heterologous systems to actively accumulate GUS substrates into living cells in plants. It will be with the development and application of these more sophisticated uses of the GUS gene fusion system that GUS will become a truly powerful tool in agricultural molecular biology.

Methods

Quantitative assays of reporter enzymes

Detailed protocols for quantitative GUS assays are given later. In this section we discuss important general features of quantitative enzyme assays.

Choice of substrates

Two factors govern the choice of substrates for quantitative enzyme assays. The first is that the rate of product accumulation should be directly proportional to enzyme concentration under the conditions of the assay (see above). The second is that there should be a reliable and sensitive method of detecting the product. The two GUS substrates mentioned above, PNPG and MUG, are both excellent substrates for quantitative assays, giving linear rates of product accumulation over long time periods. Thus the choice of substrate for a GUS assay depends largely on the conditions of the assay and the availability of equipment.

Spectrophotometric versus Fluorometric Assays

The key advantage of fluorescence assays is that they can give at least two to three orders of magnitude (100 - 1,000 X) greater sensitivity than spectrophotometric methods. This has obvious practical implications. First, such assays are remarkably fast. Because 100-1000 times less product can be detected than is required for a color production assay, the results can be obtained 100 - 1000 times faster. In addition, the quantity of material needed for accurate and reliable assays, measured in terms of amount of tissue or levels of gene expression, is substantially reduced.

This difference in sensitivity results primarily from the nature of the measurement used to calculate the amount of enzyme product present. Both types of assays depend on absorption of light of a particular wavelength by the compound being assayed. However, in spectrophotometry, it is the fraction of incident light that is absorbed that is measured -- in other words, a small difference between two large values must be detected. By contrast, a fluorescent compound absorbs light of a particular wavelength and re-emits it as light of a longer wavelength (i.e. lower energy). It is the amount of light of the emission wavelength that is measured in fluorescence spectroscopy -- in other words an absolute value over an arbitrarily small background. Thus the signal to noise ratio, which is the limiting factor in determining the levels of product detectable by any means, is much higher for fluorescence spectroscopy.

Despite the clear advantages of fluorescence methods, they do require more skill than absorption methods, and more attention to controls. Whereas absorption methods produce a number that is absolute for a given cuvette cell size, and that can be used with a knowledge of the extinction coefficient of the compound to establish a concentration of a substance according to the Beer-Lambert law, this is not true with fluorescence methods. While fluorescence output is generally proportional to the concentration of fluorochrome, the actual amount of fluorescence produced for a given amount of fluorochrome will depend on the exact

excitation intensity and wavelength. These parameters will vary between different machines and even on different days, and hence it cannot be overemphasized that internal calibration of the machine with standard solutions is essential at each use. This will enable enzyme activity to be reliably expressed in terms of absolute quantities of fluorochrome produced. The concentration ranges over which fluorescence output is proportional to the concentration of the fluorochrome usually extend for six orders of magnitude or more (10^6 - or more), although this does depend on internal parameters. For a good reference that covers the basics of fluorescence measurements see Guilbault (1973).

One common and important phenomenon associated with fluorescence assays is *quenching*. As described above, the absolute amount of fluorescence emission is dependent on the intensity and wavelength of the excitation light. Thus, any factors in the assay mixture that affect these parameters will correspondingly affect the apparent fluorescence output. For instance, methylumbelliferone (MU - the product of MUG cleavage by GUS) absorbs light very effectively at 365 nm, and emits with a high quantum efficiency at 455 nm. In concentrated extracts made from plant leaves there is a significant level of chlorophyll, which has an absorption maximum around 400 nm, but which also shows significant absorption at both 365 nm and at 455 nm. Thus, if a MUG assay is carried out in such extracts, the chlorophyll will absorb some fraction of the excitation light, so reducing the amount of light available to excite the MU produced by GUS activity, and thereby reducing the MU fluorescence output. In addition, the chlorophyll can absorb some of the emitted fluorescent light from the MU (at 455 nm), so reducing further the apparent fluorescence of the MU. This phenomenon is called quenching and can be a serious problem if one is unaware of it. However, it is a simple matter to control and eliminate quenching, either by reducing the concentration of extract, by eliminating the absorbing and quenching molecules from the extract prior to assaying (e.g. by a spin-column) or by performing internal calibrations with known quantities of MU in the same extract conditions as the assay.

The spectrophotometric assay is a good alternative in many circumstances. It is very cheap, easy to automate and easy to quantitate without sophisticated instrumentation. It is also easy to monitor progress of the reaction by observing the development of color. Its chief disadvantages are the intrinsic lack of sensitivity of methods based on absorption of light and the problems caused by light absorption by pigments in extracts. However, because of the remarkable stability of GUS, the sensitivity can be enhanced quite significantly by using very long assays (even overnight or longer).

Presentation and analysis of data

Importance of kinetics

Whichever assay is chosen for the quantitative measurement of GUS activity, kinetics should always be used to determine rates of GUS activity --derived from measurements of total product accumulated carried out at least three time points. The main reason for this is the greatly enhanced significance of a slope generated from several points rather than a single end-point. Another major advantage to using kinetics is that product accumulation specifically due to GUS activity can be measured. For example, in a fluorescence assay, the rate of

increase of fluorescence due to GUS activity can be measured independent of the intrinsic fluorescence of the extract, or fluorescence increases due to spontaneous hydrolysis of substrate or non-linear accumulation of fluorochromes.

Normalization of data

GUS activity is usually expressed as nmoles product produced per minute. This activity can be normalized to a variety of factors, including mg protein, µg DNA, or unit fresh weight, depending on the particular requirements of the experiment. The choice of denominator is critical as it can completely bias the interpretation of data. For example, consider a comparison of activity of a particular GUS fusion in leaf mesophyll versus leaf epidermal cells. If normalized to protein, similar levels of GUS activity per cell would give a much lower specific activity in the mesophyll cells due to the high levels of protein in photosynthetically active tissues, and one might conclude that expression of the gene fusion was lower in these cells. By contrast, normalization to DNA content would give equal levels of GUS activity per cell. Thus, where feasible, we recommend normalization to DNA (Labarca and Paigen 1980) content as, unlike protein concentration, this will generally be proportional to cell number.

The direct relation between DNA content and cell number does break down in plant organs where some of the cells may become polyploid, e.g. in legume root nodules (see Forde *et al.* 1989). Nevertheless, normalization to DNA content still gives a measure of GUS activity per gene, which in most cases is more meaningful than activity per unit protein. However, as protein assays are more straightforward than DNA assays, normalization of GUS activity to protein content may be adequate if the material being compared can reasonably be assumed to have similar, uniform protein concentrations - for example leaves from two different transgenic plants.

Cautions in interpretation of data

In all types of quantitative experiments there are certain dangers inherent in the use of a powerful and sensitive reporter gene. These stem from the seduction of being able to generate large amounts of numerical data purporting to reflect differing levels of gene activity. Thus a sensitive gene fusion assay will allow quantitative differences to be detected between almost any pair of constructs that differ in their controller sequences. For example, many laboratories routinely use fusions of the Cauliflower Mosaic Virus (CaMV) 35S promoter to GUS as controls in experiments designed to look at cell or organ specific expression of GUS fusions to other genes. However, the exact construction of these fusions, and even the source of the 35S promoter, varies between different laboratories, and hence an experiment designed to assay the level of GUS activity expressed from these different "control" plasmids would also almost certainly detect significant differences.

This observation emphasizes the paramount importance of careful construction of gene fusions. Gene fusions can be very powerful tools in the analysis of factors that affect gene expression -- their primary use to date. With the use of precise gene fusions, sequences affecting not only the initiation of transcription, but other components of the regulatory pathway can be studied. For

example, factors affecting mRNA processing and stability, (such as polyadenylation signals or introns), or translational efficiency (such as the context of the initiator codon or mRNA secondary structure), will inevitably affect reporter enzyme levels. However, it is crucial that gene fusions be strictly comparable in other aspects before firm conclusions about the role of specific sequences can be drawn.

A second and related caveat is that recent work using GUS has shown that clonal propagants of a single transformed plant, grown ostensibly in identical conditions, can show a greater than 100-fold variation in levels of GUS activity. This variability would have been much more laborious and difficult to detect with an assay that lacked either the sensitivity or the ease of obtaining reliable, quantitative results, of GUS assays. It is clear however that such variation is both important and common, and not an artifact of GUS measurements (Dean *et al.* 1988). This observation emphasizes the need for assaying, wherever possible, large numbers of transformants (both independent and clonally propagated) and for using rigorous statistical protocol in analysis and presentation of data. The temptation to select values that support the experimenter's theories, and to discard other "rogue" values, must be scrupulously avoided.

Analysis of GUS Expression

Quantitative GUS assays

Procedures

Preparation of extracts

Almost any method of tissue homogenization can be used although the use of repeated freeze-thaw cycles to disrupt cells may be detrimental to enzyme activity. Grinding the tissue with sand in a pestle and mortar works very well. If the tissue contains high concentrations of polyphenolics or other light-absorbing or fluorescent compounds that might interfere with either the spectrophotometric or the fluorescent assay, many of these can be removed by adsorption onto Polyclar AT (insoluble polyvinyl pyrollidone) by including a pinch of Polyclar in the extraction buffer.

Steps in the procedure

1) Homogenize tissue in GUS extraction buffer with the optional addition of Polyclar AT.

2) Centrifuge briefly to remove cell debris and Polyclar if used.

3) If endogenous fluorescence is likely to be a problem, centrifuge the extract through a spin-column of Sepharose CL6B, BioGel P6 or comparable resin (see below) to eliminate remaining low molecular weight fluorescent compounds.

4) P.oceed with assay or store extract at -70°C after quick freezing in liquid nitrogen.

Notes

1) The extraction buffer given here is one that has been found to work well for plant, fungal and bacterial studies. However, the composition of the buffer can be varied to suit the properties of different extracts, as long as the parameters that affect GUS activity are considered. The pH should be maintained at or near neutrality where GUS is fully active and stable. DTT or ß-mercaptoethanol (10-100mM) help maintain the sulfhydryl groups of GUS in a reduced state, as some plant extracts can be highly oxidizing, leading to a significant reduction in GUS activity. EDTA is included as a prophylactic measure as GUS is inhibited by high concentrations of certain divalent cations -- 70% inhibition by Mn^{2+} and Co^{2+} at 10 mM, and completely by Cu^{2+} and Zn^{2+} at comparable concentrations (Stoeber, 1961). EDTA also ensures that DNAse, which requires Mg^{2+} ions for its activity, is inactive, if the DNA in the extract is also to be analyzed. Detergents are included to increase the efficiency of extraction and also to prevent aggregation of the enzyme. Triton efficiently lyses organelles such as chloroplasts, where as nuclei are efficiently lysed by Sarcosyl but not by Triton. SDS is also tolerated at low concentrations (~0.05%). Finally, GUS is remarkably resistant to protease action, but if proteases are a potential problem, PMSF (phenylmethyl sulfonyl fluoride -- a potent inhibitor of serine proteases) at a final concentration _2mM can be included. Other protease inhibitors such as leupeptin (1mM) and aprotinin (100 µg/ml) have also been used successfully.

4) If the extracts are to be stored, the reliability of the method of storage should be determined for different tissues.

Solutions

GUS extraction buffer	Stock solutions	Volumes
50 mM $NaHPO_4$, pH 7.0	1M $NaHPO_4$, pH 7.0	50 ml
5 mM DTT (Dithiothreitol)	1M DTT in H_2O	5 ml
1 mM Na_2EDTA	0.5 M Na_2EDTA	2 ml
0.1% Sodium Lauryl Sarcosyl	10% Sarcosyl	10 ml
0.1% Triton X-100	10% Triton	10 ml
	H_2O	923 ml

Preparation of Spin Columns

1) Prepare one 0.75 ml Eppendorf for each spin column by gently piercing the base with a 20 - 26 guage syringe needle - do not poke right through.

2) Fill each tube to about 3mm depth with acid-washed 0.7 - 1.2 mm diameter glass beads. Fill to the top with a thick slurry of a resin such as Sepharose CL-6B or BioGel P6 that has previously been equilibrated in the buffer in which you wish the sample to elute, in this case GUS Extraction Buffer.

3) Seat each column inside a 1.5 ml Eppendorf tube from which the bottom has been cut off, and place these inside tubes that fit a bench-top centrifuge.

4) Spin at low speed, about 2,000 r.p.m., for a set period of time, usually 2 minutes, to pack the column. Cap tubes and store columns in the refrigerator until use.

5) To use, add 25 - 80 µl of extract to the top of the column, place inside a new, intact (important!) 1.5 ml Eppendorf tube and support these inside the larger tubes for the bench-top centrifuge. Spin exactly as before. The clarified extract will be collected in the Eppendorf tube.

Notes

1) Spin columns can also be used very effectively in many DNA manipulations, for example to remove residual organic compounds such as phenol or to remove other small molecules such as unincorporated nucleotides or short oligonucleotides. For these pre-equilibrate the columns in $T_{10}E_1$.

4-Methylumbelliferyl-ß-D-glucuronide (MUG) Fluorescence Assay

The only commercially available fluorogenic substrate that has been used extensively for GUS assays is 4-methylumbelliferyl glucuronide (MUG). GUS cleavage of MUG releases 4-methylumbelliferone (MU, 7-hydroxy-4-methylcoumarin) which is fluorescent when its hydroxyl group is ionized. The pKa of this hydroxyl is between 8 and 9 and maximal fluorescence will only be obtained if the product is in a solution with a pH greater than the pKa; fluorescence is relatively low at the neutral pH of the living cell or of a GUS assay (about 15% of the maximum fluorescence in Na_2CO_3 at pH 10.5).

The fluorogenic assay can be performed in Eppendorf tubes or in microtiter plates. The following protocol is for Eppendorfs, but can easily be scaled down for microtiter plates.

Steps in the procedure

1) Prepare one 0.5 ml aliquot of Assay Buffer for each sample to be assayed and incubate at 37°C to pre-warm. Prepare *x* times this number of tubes containing 0.9 ml stop buffer, where *x* is the number of time points to be taken.

2) To start the reactions add 1-50 µl extract to 0.5 ml Assay Buffer and mix thoroughly with a pipet tip or vortexer.

3) After 1-2 minutes, remove 100 µl into an Eppendorf tube containing 0.9 ml Stop Buffer. This will be the zero time reading.

4) At regular time intervals after this remove successive 100 µl aliquots into labeled Eppendorf tubes containing 0.9 ml Stop Buffer. Typically 5 minute intervals for high-levels of GUS, or 30-60 minute intervals for lower levels. Take at least three time points.

5) Calibrate the spectrofluorimeter with freshly-prepared 1 µM and 100 nM MU standards in Stop Buffer. Set the excitation wavelength to 365 nm and the emission detector to 455 nm. On a machine with a digital readout up to 9999 (such as the Perkin-Elmer LS series) it is convenient to set 1 µM MU equal to 1000 relative fluorescence. Then the relative fluorescence reading from the samples can be read directly as nM MU.

6) Read the relative fluorescence of the time course for each extract in ascending order. Convert the values obtained to nanomolar MU, and then to nanomoles MU, after correcting for the assayed volume. Express values as nmoles MU per minute and normalize to DNA, fresh weight, or protein.

Notes

3) The addition of Na_2CO_3 serves the dual purpose of stopping the reaction and maximizing the fluorescence of the product, 4-MU, as described above.

3) GUS activity remains linear (even in crude extracts) for a very long time, sometimes days. Hence the "time=0" point does not need to be at the moment of addition of extract to substrate. In fact, it is often better to allow the reaction to proceed at 37°C for several minutes to equilibrate and to achieve V_{max} before taking the initial time point.

4) The stopped reactions and the calibration standards are stable for at least a few days, if kept cold and dark.

5) Readings taken with a spectrofluorimeter are relative fluorescence, and must be calibrated at each use with known standards.

6) Linearity of the assay is excellent from as lc.v as the machine can measure (usually 1 nm MU or less) up to 2 - 10 µM MU. Measurements of values greater than this with a conventional 1 cm path length will be artifactually low, due to inner-cell effects (quenching of fluorescence in the inside of the cuvette by absorption of some of the excitation light by the high concentrations of product at the outer face of the cuvette) and other factors. Values up to ten times higher are actually linear with respect to enzyme activity, but need to be diluted in 0.2 M Na_2CO_3 before reading on most fluorimeters. Initial levels of fluorescence in the extract (at time=0) are often largely contributed by the traces of pre-hydrolyzed substrate (i.e. MU) in commercial substrate preparations, or coumarins and other endogenous compounds in the extract. GUS produced by one transformed plant cell can easily be measured using unpurified commercial MUG and a simple fluorimeter, using large volume cuvettes, but with long assays.

6) For those without access to a fluorimeter, a very convenient and sensitive qualitative assay can be done by setting the tubes on a long-wave (365 nm) U.V. light box and observing the blue fluorescence. This assay can be scaled down easily to assay very small volumes (reaction volume 50 µl or less, stopped by addition of 25 µl 1M Na_2CO_3) in microtiter plates or Eppendorf tubes. Use a series of known standards of MU in 0.2 M

Na_2CO_3, and interpolate the MU concentration of your assays by eye. Use concentrations of MU ranging from about 50 nM up to at least 20 µM.

Quantitative Microtiter Plate Fluorescence Assays

The Fluoroskan II, manufactured by Labsystems Oy in Finland and distributed by Flow Laboratories, is an excellent machine with the ability to quantitate fluorescence assays in the wells of microtiter plates quickly and reliably. This capability can be used to allow automation of fluorescence assays for GUS, and thereby facilitate the logistics of multiple assays. The use of microtiter plates for GUS assays, from bacteria to fungi and plant extracts should seriously be considered as a cost-effective, time saving method.

Solutions

-Assay Buffer (MUG): 1 mM MUG in Extraction Buffer.

-Stop Buffer: 0.2 M Na_2CO_3

-1 mM Methylumbelliferone Stock Solution: 1 mM in DDH_20. Wrap the bottle in aluminum foil and store at 4 C. This stock is good for at least one month, but be careful of its tendency to crystalize out with time.

-MU Standards: 1 µM & 100 nM in 0.2 M Na_2CO_3
Add 100 µl 1 mM MU Stock Solution to 900 µl Stop Buffer, mix well.
1 µM MU: Add 100 µl of this dilution to 9.9 ml Stop Buffer.
100 nM MU: Add 1.0 ml of 1 µM MU to 9.0 ml Stop Buffer.

Verification of GUS Activity Using Specific Inhibitors

If background activity is suspected, or if measured levels of activity are extremely low, several tests can be carried out to verify that increases in fluorescence are due to a true ß-glucuronidase activity. First, the increase in fluorescence should obviously be MUG dependent. Second, other GUS substrates should be hydrolyzed at comparable rates. An additional and sometimes very useful technique is to use the specific ß-glucuronidase inhibitor saccharolactone (Levvy, 1952) (Sigma S-0375, saccharic acid 1-4 lactone, glucaric acid 1-4 lactone; glucarolactone) to prove the GUS-dependence of the fluorescence increase. This inhibitor will eliminate glucuronidase activity at concentrations much less than one millimolar, but the compound is unstable at neutral pH, so beware of lengthy assays. Because of this instability, use saccharolactone at up to 5 mM for assays up to half an hour. Alternatively, perform the reaction and the inhibited reaction at pH 6.0 or below. GUS activity is not affected by these conditions and saccharolactone is much more stable at acid pH.

Spectrophotometric Assays using p-nitrophenyl ß-D-glucuronide

The currently preferred substrate for spectrophotometric measurement of GUS is p-nitrophenyl ß-D-glucuronide which, when cleaved by GUS, releases the

chromophore p-nitrophenol. At a pH greater than its pKa (around 7.15) the ionized chromophore absorbs light at 400-420 nm, giving a yellow color. Good color development can occur at the pH of the GUS reaction (7.0) but is enhanced by alkalinization of the reaction mixture.

1) Use 1 mM p-nitrophenyl ß-D-glucuronide in Extraction Buffer as Assay buffer.

2) Proceed as for MUG assay, but make the final volume of assay buffer + stop buffer 0.9 ml (not 1 ml).

3) Measure absorbance at 405 nm against a substrate blank (or, if turbidity of the extract is a problem, against a stopped blank reaction to which an identical amount of extract has been added). Under these conditions the molar extinction coefficient of p-nitrophenol is assumed to be 18,000. Thus in the 0.9 ml final volume, an absorbance of 0.020 represents one nanomole of product produced.

Notes

1)This assay can very easily be automated using commercially available ELISA plate readers and microtiter equipment.

1)For very long assays (a few hours or longer), add 0.02% NaN_3 to prevent microbial growth or induction of microbial GUS, and 100 - 200 µg/ml BSA (bovine serum albumin) to stabilize the enzyme. It has not been demonstrated that the BSA is important, but it may be prudent to include it to compete with endogenous proteases and oxidizing agents.

3) Unfortunately, much plant tissue is rich with compounds that absorb at the maximum wavelength of p-nitrophenol. Pigmented extracts can often be clarified and effectively decolorized prior to assay by passage through a spin-column of Sepharose CL6B (see above).

3) The calculation given above derives from the Beer-Lambert law and assumes a path-length of 1 cm, as found in most spectrophotometers. However, if a microtiter dish and an ELISA reader are used, the path length will be different and the conversion factor must be recalculated. This can be done using commercially available liquid p-nitrophenol as a standard to calibrate the readings for specific volumes of solution assayed in the microtitre wells. Absorbance can also be measured at 415nm, at which wavelength the molar extinction coefficient of p-nitrophenol is 14,000.

Histochemical Assays of GUS

The most widely used substrate for histochemical assays of GUS is 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) (Holt and Withers 1958; Pearson *et al.* 1967; Jefferson *et al.* 1986). This substrate gives a blue precipitate, the very insoluble and highly colored indigo dye, 5,5'-dibromo 4,4'- dichloro indigo, at the site of GUS enzyme activity. It is important to understand that this dye is not the direct result of GUS activity, but is formed by oxidative dimerization of the colorless indoxyl derivative that results from GUS hydrolysis of X-Gluc.

This dimerization is stimulated by atmospheric oxygen, and it is thus rarely essential to use an oxidation catalyst for routine work. However, it is important to be aware that localized peroxidases will act as oxidation catalysts and thus may enhance the apparent localization of glucuronidase. While this will not generate false positives, it does mean that the relative degree of staining may not reflect the concentrations of glucuronidase. To overcome this effect and to ensure full color development, an oxidation catalyst such as a K+ ferricyanide / ferrocyanide mixture (Holt and Withers 1958; Lojda 1970; reviewed in Pearse, 1972) can be used.

There are numerous other variables that affect the quality of histochemical localization, many more important than the actual reaction itself. These include all aspects of tissue preparation and fixation. An excellent detailed description of laboratory methods for histological and microscopic manipulation of plants, including preparation of materials, sectioning tissues by hand, and microscopic analysis is given by O'Brien and McCully (1981). If anyone is seriously considering embarking on a study of cell-type or "tissue-specific" gene expression, then a thorough grounding in the methods for botanical microtechnique should be considered essential. There is a tendency for molecular biologists to be captivated by pretty pictures, and not to address sufficient rigor to the experiments leading up to the image, nor to the interpretation of the image. There are many important caveats that should be remembered when extrapolating from an image of a stained specimen to an assertion about gene action, and about the functioning of the living organism.

Procedures

Preparation of Samples

Fixation and Sectioning

Microscopic analysis of plant tissues generally involves the preparation of thin (or relatively thin) sections of material that can transmit a certain degree of light, and hence information about the specimen. When a live organism is killed and tissue sections are generated for microscopic analysis, many changes occur within the specimen. Cell contents leak out and mix with each other, ultrastructure is altered, degradative enzymes begin to destroy the macromolecular components, and chemical changes occur. Fixation -- the treatment of a tissue or tissue section with a chemical compound designed to cross-link or coagulate proteins and other macromolecules -- is the compromise that must be reached to minimize this perturbation. However, it clearly is a compromise for, while a heavily-fixed specimen may retain excellent morphology, it may have little, if any remaining biological or enzymatic activity. Therefore achieving a suitable balance between the preservation of morphology and the preservation of enzyme activity is the aim of a fixation protocol for histochemical assays of reporter gene activity. Clearly this will have to be determined empirically. However, a sound understanding of both the chemistry of fixation, and the properties of the specimen and enzyme (in our case, GUS) is invaluable. The exact order of fixation, embedding and sectioning varies with the requirements of the experiment. It is strongly recommended that the reader consult a specialist text to enter this byzantine realm.

Sectioning material - that is cutting plant tissue to achieve thin slices for analysis - usually requires embedding of the material (by infiltration or by providing some other means of support of the tissue) to maintain structure during sectioning.

Fresh tissues can be sliced by hand using a double-edged razor blade, or with a microtome, by supporting the tissue in a block of resilient yet soft material such as the two halves of a potato. Alternatively they can be quick frozen and sectioned on a cryostat.

Fixation conditions will vary with the fixative, the sample, the tissue, the cell-type, its permeability to the fixative and other variables of each experiment. The two most common fixatives are gluteraldehyde and formaldehyde. GUS is relatively sensitive to gluteraldehyde and so we recommend use of formaldehyde which penetrates plant tissues well.

One of the key problems in fixation is ensuring rapid and complete penetration of the fixatives to all parts of the tissue. In large pieces of tissues at least two problems can ensue. One is that, as outer cells are fixed and thereby killed, their cellular contents are released and therefore dilute the concentration of fixative reaching the inner cells. The second problem is that the outer, fixed cells, may actually become less permeable to the fixative and thus hinder penetration of fixative to inner cells.

These problems can be ameliorated by using small tissue slices and by gentle agitation during fixation. An additional technique that is useful for promoting penetration of fixative is vacuum infiltration of the fixatives into the intercellular airspaces. This can be accomplished by immersing the tissue in fixative solution in a small tissue culture dish, keeping the lid partially on and placing it in a vacuum chamber and evacuating slowly until air bubbles can be seen emerging from the tissue. When bubbles are no longer seen, release the vacuum very slowly. This will cause influx of the liquid into the interstices of the tissue that were formerly occupied by air.

Fixation Protocol

1) Submerge tissue in fixative. Vacuum infiltrate if desired. Incubate with gentle shaking at temperatures from 4 C up to room temperature for 30 - 60 minutes.

2) Wash several times for 10 - 15 minutes each, in phosphate buffer and/or osmoticum, to remove fixative.

Notes

1) Two different fixation solutions are suggested, one for protoplasts and one for other tissues. Protoplasts usually present very little permeability barrier to fixatives, and almost all starting GUS activity is maintained under the suggested conditions, although this should be measured with suitable controls for each new situation. By contrast, the permeability of intact plant tissues is very variable, and Triton X-100, Tween 20 or any other non-ionic detergent, serves to help wet the surface of the specimen, aiding in uptake of the fixative or substrate.

Solutions

Protoplast fixation buffer
- 1% formaldehyde
- 0.3 - 0.6 M mannitol
- 10 mM MES pH 5.6

General fixation buffer
- 1 - 2% formaldehyde
- 50 mM $NaHPO_4$ pH 7.0
- 0.05% Triton X-100 or 0.1% Tween 20

Histochemical Assay

1) Immerse the tissue in X-Gluc assay buffer (avoid deep solutions as O_2 is required for the development of the indigo color). Vacuum infiltration will aid in penetration of the substrate if it has not already been used in the fixation step.

2) Incubate at 25 - 37 C for times from a few minutes to overnight. The quality of the localization does not decrease much with lengthy assays, but assays longer than overnight are prone to uncover artifacts.

3) If the tissues being assayed have a tendency to darken with time, include 2 mM NaS_2O_5 (sodium meta bisulfite) which prevents oxidation and browning due to polyphenols (J. Callis and C. Gasser, Monsanto Co. and U. of California at Davis, pers. comm.). Use 1:100 dilution of a 200 mM stock.

4) For lengthy assays of tissues that may be contaminated with endophytic bacteria or fungi, include 0.02 % NaN_3 or **100 µg / ml chloramphenicol** to prevent induction of GUS activity in these microbes.

5) If an oxidation catalyst is used, try K+ ferricyanide at a final concentration of 1 mM in the staining mixture.

Notes

2) If live, unfixed material is to be assayed reduce the detergent concentration and include osmoticum if desired. Also reduce assay temperature to one compatible with the material. It has not yet been determined whether live blue-stained cells can be efficiently recovered.

Solutions

X-Gluc assay buffer
- 1-2 mM X-Gluc
- 50 mM $NaHPO_4$, pH 7.0
- 0.1% Triton X-100 or Tween 20

Notes

The usual commercially available form of X-Gluc is the cyclohexylammonium salt which one should dissolve first at 100 mM in dimethylsulfoxide or dimethylformamide before adding to the aqueous buffers. The Na^+ salt of X-Gluc is very water-soluble and is now sold by Biosynth AG.

Oxidation catalyst

Stock solutions of K+ ferricyanide and K+ ferrocyanide can be prepared separately at 200 mM in H_2O, and kept for several weeks at 4 C.

Clearing the Specimen

Clearing simply means elimination of colored material that masks the signal of interest. In plant tissues, most colored material is, of course, chlorophyll and related photosynthesis-associated pigments such as carotenoids. There are also numerous polyphenolics and lignins that can cause unacceptable darkening of a specimen. Prevention of their accumulation during staining and sectioning, and/or removal of these compounds after sectioning and staining can greatly enhance the resolution and sensitivity of the assay.

Many methods are available for clearing of plant tissues; a valuable section on clearing is included in O'Brien and McCully (1981). Successive washes in 70 - 100 % ethanol (EtOH) have been used with some success. However chlorophyll extraction by this method is slow, requiring several changes and overnight incubation in EtOH.

Interestingly, bleach makes a very good clearing agent for most purposes, and for eliminating liginification browning in roots, it is marvelous. Usually 50% household bleach for about 10 - 30 minutes give excellent clearing while retaining the full indigo stain.

The following protocol is very simple, with the advantage that the resulting material is extremely clear, permanently fixed and in a medium with a high refractive index for good optical microscopy. The fixed or unfixed sample is incubated with X-Gluc as described above until suitable reaction has occurred. The sample is then placed in an aqueous solution of 75% lactic acid (O'Brien and McCully, 1981) and autoclaved for 15 minutes at 1 bar (15 psi). The resulting sample is almost completely devoid of colored material except for the deposited indigo dye. The structure of the specimen is well preserved, and contrast is provided by a subtle off-white / beige tone to the specimen. Although this treatment seems drastic it is very simple, fast and effective.

Notes

Lactic acid is very effective for tobacco and potato leaf tissue, but its efficacy varies in other plant types and tissues. For example, cotton leaves treated with

lactic acid as above produce an intense crimson color in the tissue upon autoclaving (Baldwin Chipangura, U. of Zimbabwe, pers. comm.).

Synthesis in vivo

If the cost or availability of GUS substrates is a limitation, many substrates can be prepared biosynthetically by feeding a precursor - generally the aglycone - to laboratory animals, such as rabbits. These will be glucuronidated *in vivo* as part of the animal's general detoxification pathways, and the resultant GUS substrates can be purifed from the urine, often with very high yield (e.g. Mead *et al.* 1957).

Plasmids available for use in construction of GUS gene fusions

Older plasmids which enable GUS fusions to be constructed in all three reading frames are described in Jefferson (1987). However, many other GUS fusion plasmids have now been constructed in other laboratories that may be more suited to particular experimental requirements and the reader should consult the updates provided by the CAMBIA MGRS for information and sources of such plasmids.

Substrates and Suppliers

p-nitrophenyl-ß-D-glucuronide (PNPG) (Biosynth AG, Sigma Chemicals,)

4-methyl umbelliferyl ß-D-glucuronide (MUG) (Sigma Chemicals, Biosynth AG,)

4-trifluoromethyl umbelliferyl ß-D-glucuronide (TFMUG) (Molecular Probes Inc)

5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X GlcA Biosynth AG, Research Organics Inc,)

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pRAJ255

pRAJ255 has been described (Jefferson *et al.*, 1986). It consists of a 1.87 kb Pst I - Eco RI fragment containing the GUS gene inserted into the polylinker site of pEMBL9. This places *gusA* in-frame to the *lacZ* coding sequence. The Shine/Dalgarno translational initiation sequence has been removed from the *gusA* coding cassette, and the *gus* initiator codon is the first ATG on the cassette.

pRAJ275

pRAJ275 is a derivative of pRAJ255 in which the 5' sequences of GUS were removed by progressive BAL31 deletion and replaced with a synthetic oligonucleotide that has constructed a "consensus" translational initiator as described by M. Kozak (eg. Kozak, 1983). The fragment was then subcloned as a Sal I - Eco RI fragment into pUC19. This cassette has a very effective translational initiator in eukaryotes (Sleat *et al*, 1987), but only modest initiation in bacteria.

pRAJ294

pRAJ294 is a derivative of the genomic *Escherichia coli* sequence, with its complete and very active Shine/Dalgarno translational initiation context, fused to the tac promoter in the vector pTTQ18 (Stark, 1985). This vector has very good translational initiation in bacteria, and expression of GUS to levels of 50% of the soluble protein of *Escherichia coli* cells can be achieved by IPTG induction of the fusion. It is cloned into the polylinker as an EcoRI - Hind III fragment.

pBI101.1

pBI101 is a "promoter-less" GUS cassette in the *Agrobacterium* binary plasmid vector pBIN19 (Bevan, 1984; Jefferson *et al* 1987b). It consists of the GUS gene from pRAJ260 (Jefferson *et al.*, 1986) ligated into a filled-in Asp718 site of the pBIN19 polylinker but upstream of a 260 bp Sst I - Eco RI fragment containing the polyadenylation signal from the nopaline synthase gene of the *Agrobacterium tumefaciens* Ti plasmid (Bevan *et al.*, 1983). This allows plant promoters to be easily cloned upstream of GUS and transfered to plants. This vector has a low-copy RK2 origin of replication, but is relatively unstable, and confers kanamycin resistance, both in bacteria and in plants. It should be cautioned that while there are no "spurious" ATG codons on the GUS fragment of this vector, any promoters cloned into the Hind III site may have problems with the ATG from the polylinker's Sph I site (GCATGC). All pBIN19 derivatives (including the pBI 100 series) are unstable in the absence of selection, and must be propagated in the presence of kanamycin.

pBI101.2 & pBI101.3:

These are plasmids resulting from the pBI101 construction that provide the other two reading frames of GUS relative to the polylinker sites. Junction sequences of the pBI101 series, with the pUC19 polylinker sequence followed by the GUS coding sequence is as follows; the GUS initiator is boldface, the Bam HI site is underlined:

pBI101.1: AAG CTT GCA TGC CTG CAG GTC GAC TCT AGA GGA TCC CCG
GGT GGT CAG TCC CTT ATG TTA...

pBI101.2: A AGC TTG CAT GCC TGC AGG TCG ACT CTA GAG GAT CCC
CGG GTA GGT CAG TCC CTT ATG TTA...

pBI101.3: AA GCT TGC ATG CCT GCA GGT CGA CTC TAG AGG ATC CCC
GGG TAC GGT CAG TCC CTT ATG TTA...

pBI201.1
pBI201.2
pBI201.3

These are the polylinker, GUS and nopaline synthase polyadenylation sequences from the corresponding pBI101 series plasmids, subcloned into pUC19 to allow high yield DNA preparations and routine constructions.

pBI121.1:

This is pBI101.1 into which an 800 bp fragment containing the 35S promoter from Cauliflower Mosaic Virus has been cloned. This gives high levels of GUS upon transformation of tobacco (Jefferson *et al*, 1987a; Jefferson *et al*, 1987b). There are reports that differences in expression exist between different CaMV strains, and the promoter used in all of these constructions is one of the least active.

pBI121.2:

Similar to pBI121.1, but with the "consensus" initiator codon context of pRAJ275, including an Nco I site (not unique) at the initiator, and with the entire transcriptional cassette inverted with respect to the *lacZ* transcriptional unit in pBIN19 (Goldsbrough, Jefferson and Bevan, unpublished). This vector has greatly reduced GUS levels in E. coli and Agrobacterium, but gives excellent expression in tobacco and potato. Formerly called CG20.

EcoRI SstI Sma I Bam HI Xba I CaMV > > > >
.....CCCGGGGATCCTCTAGAGTCGAAATTGCC AGATTAGCCTTT...CaMV 35S Promoter >>>>CATTTGGAGAGAA CACG GGGGATTCGACC **ATG** GTC ....(GUS)...>

pBI221.2:

A CaMV-GUS fusion in pUC19 making use of the observation that the pRAJ275 initiator context gives enhanced translational yields, eg. a "consensus" CaMV-GUS-NOS cassette. This is simply the EcoRI - Hind III fragment of pBI121.2 subcloned into pUC19. This works well in transient expression in a variety of monocotyledonous and dicotyledonous plant protoplast systems. (See junction sequences above).

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GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants

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We have used the *Escherichia coli* β-glucuronidase gene (GUS) as a gene fusion marker for analysis of gene expression in transformed plants. Higher plants tested lack intrinsic β-glucuronidase activity, thus enhancing the sensitivity with which measurements can be made. We have constructed gene fusions using the cauliflower mosaic virus (CaMV) 35S promoter or the promoter from a gene encoding the small subunit of ribulose bisphosphate carboxylase (rbcS) to direct the expression of β-glucuronidase in transformed plants. Expression of GUS can be measured accurately using fluorometric assays of very small amounts of transformed plant tissue. Plants expressing GUS are normal, healthy and fertile. GUS is very stable, and tissue extracts continue to show high levels of GUS activity after prolonged storage. Histochemical analysis has been used to demonstrate the localization of gene activity in cells and tissues of transformed plants.

Key words: chimaeric genes/plant transformation/reporter gene/*Agrobacterium*

Introduction

Control of gene activity can be manifested at many levels, including the initiation of transcription or translation and the processing, transport or degradation of mRNA or protein. The use of precise gene fusions can simplify analysis of these complex processes and delineate the contribution of transcriptional control by eliminating the specific signals for post-transcriptional controls and replacing them with sequences from a readily assayed reporter gene. In addition, members of multi-gene families whose products are very similar can be regulated differentially during development. By using gene fusions to individual members of such families and introducing these fusions into the germline one can study the expression of individual genes separate from the background of the other members of the gene family. Analysis of mutationally altered genes in organisms accessible to transformation techniques is greatly facilitated by the use of sensitive reporter enzymes. By using a reporter gene that encodes an enzyme activity not found in the organism being studied, the sensitivity with which chimaeric gene activity can be measured is limited only by the properties of the reporter enzyme and the quality of the available assays for the enzyme.

To date, at least six reporter genes have been used in studies of gene expression in higher plants. Gene fusions using the *Escherichia coli* β-galactosidase (Helmer *et al.*, 1984) proved difficult to assay because of high endogenous β-galactosidase activity in plants. Use of the *Agrobacterium tumefaciens* Ti-plasmid-encoded genes nopaline synthase (Depicker *et al.*, 1982; Bevan *et al.*, 1983a) and octopine synthase (DeGreve *et al.*, 1982) promised to overcome problems associated with endogenous activity because the opines produced by these genes are not found in normal plant cells. However, these reporter genes are not widely used because the assays are cumbersome and difficult to quantitate, they cannot be used to demonstrate enzyme localization (Otten and Schilperoort, 1978), and octopine synthase cannot tolerate amino-terminal fusions (Jones *et al.*, 1985). The two most useful reporter genes to date have been the bacterial genes chloramphenicol acetyl transferase (CAT) and neomycin phosphotransferase (NPTII) which encode enzymes with specificities not normally found in plant tissues (Bevan *et al.*, 1983b; Fraley *et al.*, 1983; Herrera-Estrella *et al.*, 1983a,b). In addition, NPTII can tolerate amino-terminal fusions and remain enzymatically active, making it useful for studying organelle transport in plants (van den Broeck *et al.*, 1985). However, both CAT and NPTII are relatively difficult, tedious and expensive to assay (Gorman *et al.*, 1982; Reiss *et al.*, 1984). Competing reactions catalyzed by endogenous esterases, phosphatases, transferases and other enzymes also limit sensitivity and make quantitation of CAT or NPTII by enzyme kinetics very difficult. Recently, the firefly luciferase gene has been used as a marker in transgenic plants (Ow *et al.*, 1986), but the enzyme is labile and difficult to assay with accuracy (DeLuca and McElroy, 1978). The reaction is complex and there is little, if any, potential for routine histochemical analysis or fusion genetics.

We believe that future advances in the study of plant gene expression require the development of new gene fusion systems that are easy to quantitate and highly sensitive, thus allowing analysis of genes whose products are of moderate and low abundance. This is contingent on a complete absence of any intrinsic reporter enzyme activity in plants. Activity of the reporter enzyme should be maintained when fused to other proteins at its amino terminus to allow the study of translation and the processing events involved in protein transport. The reporter enzyme should be detectable with sensitive histochemical assays to localize gene activity in particular cell types. Finally, the reaction catalyzed by the reporter enzyme should be sufficiently specific to minimize interference with normal cellular metabolism and general enough to allow the use of a variety of novel substrates to maximize the potential for fusion genetics and *in vivo* analysis.

To meet these criteria, we have developed the *E.coli* β-glucuronidase gene as a reporter gene system for transformation of plants. β-Glucuronidase (GUS, EC 3.2.1.31), encoded by the *uidA* locus (Novel and Novel, 1973), is a hydrolase that catalyses the cleavage of a wide variety of β-glucuronides (Stoeber, 1961), many of which are available commercially as spectrophotometric, fluorometric and histochemical substrates. The β-glucuronidase gene has been cloned and sequenced, and encodes a stable enzyme that has desirable properties for the construction and analysis of gene fusions (Jefferson, 1985; Jefferson *et al.*, 1986; Jefferson *et al.*, 1987). In this paper we describe several useful features of GUS which make it a superior reporter gene system for plant studies. Many plants assayed to date lack

detectable glucuronidase activity, providing a null background in which to assay chimaeric gene expression. We show that glucuronidase is easily, sensitively and cheaply assayed *in vitro* and can also be assayed histochemically to localize GUS activity in cells and tissues.

Results

Many higher plants contain no detectable β-glucuronidase activity

Roots, stems and leaves from wheat, tobacco, tomato, potato, *Brassica napus* and *Arabidopsis thaliana*, potato tubers, and seed from wheat and tobacco were homogenized with GUS extraction buffer containing a variety of protease inhibitors such as PMSF and leupeptin. The plant extracts were incubated in a standard assay at 37°C for 4 to 16 h, and the fluorescence of 4-methyl umbelliferone (MU) was measured. Endogenous activity was below the limits of detection. Extremely lengthy assays occasionally gave low levels of MU fluorescence, but the kinetics of MU accumulation were consistent with a slow conversion of the glucuronide into another form, possibly a glucoside, that was subsequently cleaved by intrinsic glycosidases. β-Galactosidase assays performed under similar conditions on tobacco and potato extracts were off-scale (at least 10 000 times higher than the minimal detectable signal) within 30 min. Reconstruction experiments were performed with purified GUS added to tobacco and potato extracts to demonstrate the ability of these extracts to support β-glucuronidase activity (data not shown).

Construction of plasmids for transformation of plants with GUS fusions

A general purpose vector for constructing gene fusions was made by ligating the coding region of GUS (Jefferson *et al.*, 1986) 5′ of the nopaline synthase polyadenylation site (Bevan *et al.*, 1983a) in the polylinker site of pBIN19 (Bevan, 1984). This vector, pBI101 (Figure 1), contains unique restriction sites for *Hin*dIII, *Sac*I, *Xba*I, *Bam*HI and *Sma*I upstream of the AUG initiator codon of GUS, to which promoter DNA fragments can be conveniently ligated. The cauliflower mosaic virus (CaMV) 35S promoter (Odell *et al.*, 1985) as described in the expression vector pROK1 (Baulcombe *et al.*, 1986) was ligated into the *Hin*dIII and *Bam*HI sites to create pBI121. Similarly, the promoter from a tobacco gene encoding the small subunit of ribulose bisphosphate carboxylase (rbcS) Ntss23 (Mazur and Chui, 1985) deleted of rbcS coding sequences, was fused to pBI101 to make pBI131.

Chimaeric GUS genes are expressed in transformed plants

Nicotiana tabacum var. Samsun plants were transformed with *Agrobacterium* binary vectors (Bevan, 1984) containing transcriptional fusions of either the CaMV 35S promoter or the tobacco rbcS promoter with the coding region of GUS as shown in Figure 1. Several kanamycin resistant plants were regenerated from each transformation. Two rbcS−GUS transformants and two CaMV−GUS transformants were chosen for further study. We first assayed various organs of one plant from each transformation, axenically cultured in 3000 lux white light, 18 h day, 6 h night. The results of this analysis are shown in Figure 2, and tabulated in Table I using either of two normalization methods (see Discussion). The plant containing a rbcS−GUS fusion (rbcS−GUS 2) exhibited a pattern of gene expression consistent with earlier studies using heterologous rbcS gene fusions (e.g. Simpson *et al.*, 1986a). The highest sp. act., using either protein or DNA as a denominator, was found in older leaves (−8 cm long), with progressively less activity in very young leaves (<5 mm), stems and roots. The other rbcS−GUS fusion plant showed a similar pattern (data not shown).

The two plants transformed with the CaMV 35S−GUS fusion displayed a pattern of gene expression distinct from that of the rbcS−GUS fusion plants. The highest levels of activity were found in roots, with similar levels in stems. GUS activity was also high in leaves, consistent with previous observations that the CaMV 35S promoter is expressed in all plant organs (Odell *et al.*, 1985).

To verify that no significant rearrangements of the transforming DNA had occurred, a Southern blot analysis was conducted as shown in Figure 3. Digestion of DNA extracted from all of the tranformants with *Hin*dIII and *Eco*RI released a single internal fragment of T-DNA consisting of the nopaline synthase polyadenylation site, the GUS coding region and the promoter

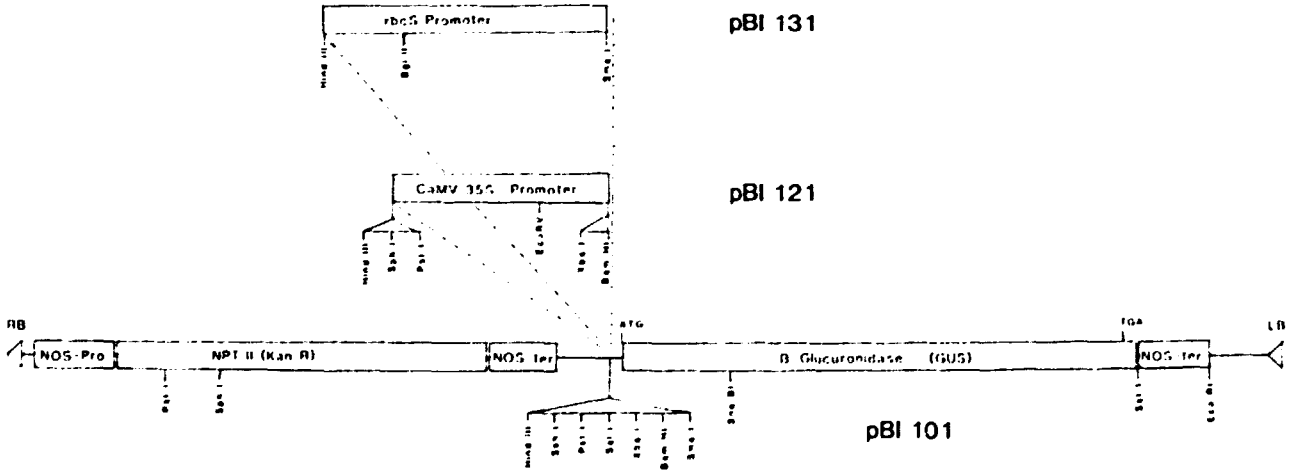

Fig. 1. Structure of expression vectors. **Bottom**: T-DNA region of pBI101, containing polylinker cloning sites upstream of the GUS, followed by the nopaline synthase polyadenylation site (NOS ter). *Pst*I and *Sph*I are not unique to the polylinker. The expression cassette is within pBIN19, giving pBI101 a total length of ~12 kb. **Middle**: Chimaeric CaMV 35S−GUS gene in pBI121. An 800 bp *Hin*dIII−*Bam*HI CaMV 35S promoter fragment (Guilley *et al.*, 1982) was ligated into the corresponding sites of PBI101. The mRNA initiation site is approximately 20 bp 5′ of the GUS initiator codon. **Top**: Chimaeric rbcS−GUS gene in pBI131. A 1020 bp *Hin*dIII−*Sma*I fragment containing the promoter of a tobacco rbcS was ligated into the corresponding sites of pBI101. The mRNA initiation site is ~55 bp 5′ of the GUS initiator codon, and contains nearly the entire untranslated leader of the rbcS gene.

(CaMV 35S or rbcS). RbcS–GUS transformants contained three copies (rbcS–GUS 2, Figure 3, lane 6) and about seven copies (rbcS–GUS 5, lane 8) of the predicted 3.1 kb *Hin*dIII–*Eco*RI fragment. Digestion with *Eco*RI revealed multiple border fragments (Figure 3, lanes 5 and 7), confirming the copy number estimates deduced from the double digestions. Similarly, CaMV 35S–GUS plants had multiple insertions as shown in Figure 3, lanes 1–4. CaMV–GUS 21 had three copies of the predicted 2.9 kb fragment, while CaMV–GUS 29 had two copies. No hybridization of the labelled GUS coding region to untransformed plant tissue was observed (lanes 9 and 10).

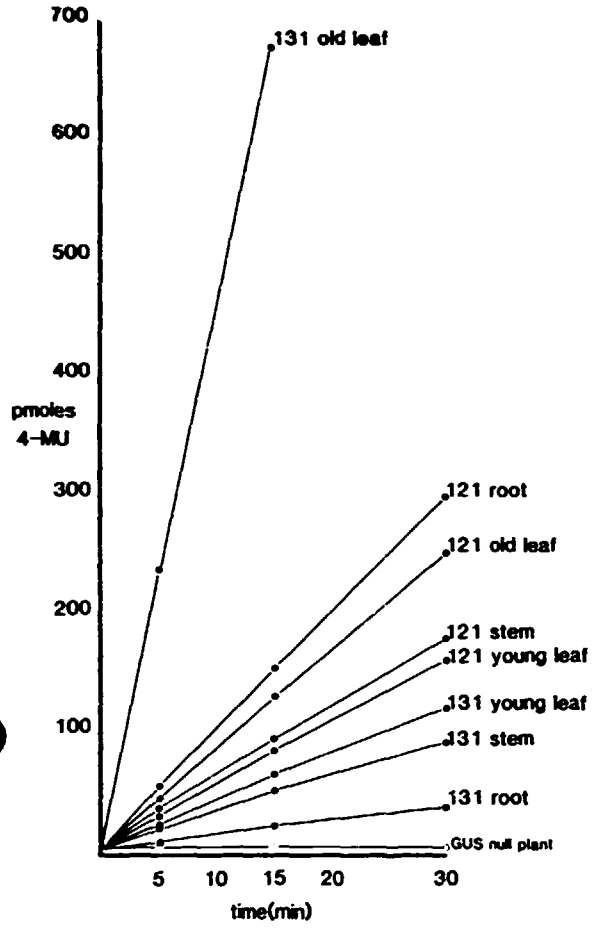

Fig. 2. β-Glucuronidase activity in extracts of different organs of transformed and non-transformed tobacco plants. Extracts were prepared from axenic tobacco plants using about 50 mg fresh weight of tissue ground in 500 μl extraction buffer. 5 μl of extract was assayed as described in Materials and methods. Mature leaves were lower, expanded leaves ~80 mm long, while young leaves were ~5 mm long, and were dissected from the shoot apex. All samples were taken from the same plant (either CaMV–GUS 21, rbcS–GUS 2 or non-transformed) at the same time. Leaf tissue was taken from a non-transformed plant for this assay, although all organs showed no GUS activity (data not shown).

GUS activity in plants can be visualized using histochemical methods

To determine whether we would be able to use histochemistry to investigate single-cell or tissue-specific expression of GUS gene fusions in plants, preliminary experiments were carried out on sections of stems of several independently transformed rbcS–GUS and CaMV–GUS plants. Typical results are shown in Figure 4. Stem sections were chosen both for their ease of manipulation and because most of the cell types of a mature plant are represented in stem. To illustrate the light-regulated nature of the rbsC–GUS fusion, the plants were illuminated from one side only for 1 week before sectioning. Sections from both plants stained intensely with the substrate while non-transformed tissue did not stain (Figure 4c). Stem sections of CaMV–GUS plants always show highest levels of activity in phloem tissues along the inside and outside of the vascular ring, most prominently in a punctate pattern that overlies the internal phloem and in the rays of the phloem parenchyma which join the internal and external phloem (Esau, 1977). There is also variable lighter staining throughout the parenchymal cells in the cortex and in the pith, and also in epidermal cells, including the trichomes (Figure 4a).

RbcS–GUS stem sections rarely if ever show intense staining in the trichomes, epidermis, vascular cells or pith, but tend to stain most intensely over the cortical parenchyma cells containing chloroplasts (chlorenchyma), with faint and variable staining in the pith. Although we most often see the strongest staining in a symmetrical ring around the vascular tissue just inside the epidermis, we sometimes observe an asymmetric distribution of staining in the cortical stem cells. Suspecting that this pattern was due to uneven lighting, we illuminated a plant from one side for 1 week before sectioning, and found that the staining was asymmetric, with intense staining in the chloroplast-containing cells proximal to the light source (Figure 4b). The staining patterns we observe for both the CaMV 35S–GUS and the rbcS–GUS transformants are consistent between several independent transformants. Untransformed plants never show staining with 5-bromo-4-chloro-3-indoyl β-D-glucuronide (X-Gluc), even after extended assays of several days (Figure 4c).

Discussion

We present here new methods for analysing gene expression in transformed plants that we feel will be of general utility. The β-glucuronidase gene from *E.coli* has been expressed at high levels in transformed tobacco plants with no obvious ill effects on plant growth or reproduction. The ability to quantitate gene expression through the routine use of enzyme kinetics greatly

Table I. GUS specific activity

Plant organ	pmole 4-MU/min/mg protein			pmol 4-MU/min/μg DNA	
	CaMV 35S–GUS	rbcS–GUS	Untransformed	CaMV 35S–GUS	rbcS–GUS
Leaf (5 mm)	283	205	<0.1	2530	4400
Leaf (70 mm)	321	1525	<0.1	5690	93 950
Stem	427	260	<0.1	13 510	2650
Root	577	62	<0.1	12 590	690

The rate data shown in Figure 2 were converted to sp. act. by measuring the protein concentration of the extracts using the Bradford reagent. The data are also presented as GUS activity/unit weight of DNA in the extract to account better for the differences in cell number between different tissues.

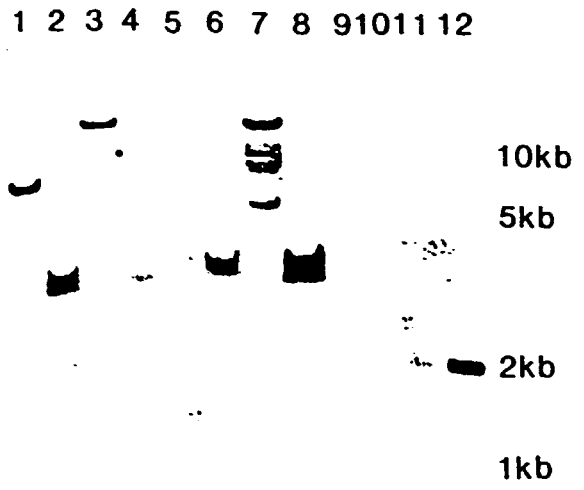

Fig. 3. Autoradiograph of a Southern blot of DNA extracted from transformed plants and digested with restriction endonucleases. The filter was hybridized with a ^{32}P labelled restriction fragment containing the coding region of the β-glucuronidase gene. Lane: (1) CaMV−GUS 21, *Eco*RI; (2) CaMV−GUS 21, *Eco*RI and *Hin*dIII; (3) CaMV−GUS 29, *Eco*RI; (4) CaMV−GUS 29, *Eco*RI and *Hin*dIII; (5) rbcS−GUS 2, *Eco*RI; (6) rbcS−GUS 2, *Eco*RI and *Hin*dIII; (7) rbcS−GUS 5, *Eco*RI; (8) rbcS−GUS 5, *Eco*RI and *Hin*dIII; (9) non-transformed, *Eco*RI; (10) non-transformed, *Eco*RI and *Hin*dIII; (11) single copy reconstruction of GUS coding region; (12) five copy reconstruction.

enhances the precision and resolution of the questions that we can ask. It should be emphasized that the determination of rates of enzyme activity eliminates the vagaries inherent in CAT, NPTII and luciferase assays, and allows accurate determination of a quantity of chimaeric gene product, even over an intrinsically fluorescent background. The fluorometric assay is very specific, extremely sensitive, inexpensive and rapid. Minute quantities of tissue can be assayed with confidence; recently we have measured GUS levels in isolated single cells of transformed plants (R.A. Jefferson *et al.*, in preparation).

β-Glucuronidase is very stable in extracts and in cells, with a half-life in living mesophyll protoplasts of ~50 h (unpublished data). Because of this, we feel it is reasonable to interpret GUS levels as indicative of the integral of transcription and translation, rather than the rate. In addition, GUS is not completely inactivated by SDS−PAGE, can tolerate large amino-terminal fusions without loss of enzyme activity (Jefferson *et al.*, 1986, 1987) and can be transported across chloroplast membranes with high efficiency (T.A.Kavanagh *et al.*, in preparation). We feel, therefore, that the system will also be very useful in studying the transport and targeting of proteins, not only in plants, but in other systems that lack intrinsic β-glucuronidase activity, such as *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Jefferson, 1985, 1986).

We have used a commercially available histochemical substrate to demonstrate GUS activity in transformed plant tissue. Other substrates are available and give excellent results (e.g. Jefferson *et al.*, 1987). We emphasize that meaningful interpretation of results of histological analysis in terms of extent of chimaeric gene activity, whether by *in situ* hybridization methods or by histochemistry, as presented here, is not a trivial or straightforward matter. There are numerous variables that must be dealt with (reviewed in Pearse, 1972). However, with these cautions, histochemical methods can be very powerful for resolving differences in gene expression between individual cells and cell-types within tissue.

We have observed a distinctly non-uniform distribution of GUS activity in stem sections of several CaMV−GUS transformed plants. Different cell-types within plants are expected to have differing metabolic activity with corresponding differences in rates of transcription and translation, and our results may reflect such a difference. Alternatively, since many of the cells of the phloem have very small cross-sectional areas, the intense dye deposition we see in these regions may simply reflect the greater cell number per unit area. The localization that we observe may also be due to a real difference in the level of expression of the CaMV 35S promoter between cell types. Recently, Nagata *et al.* (1987) have argued that the CaMV 35S promoter is preferentially active in cells during the S phase of the cell cycle. If this is true, then the pattern of GUS staining that we observe may reflect cell division activity in these cells. This observation is consistent with the proposed role of the 35S transcript of CaMV in viral replication (Pfeiffer and Hohn, 1983). It is also interesting that the other class of plant DNA viruses, the geminiviruses, replicates in the phloem parenchyma (Kim *et al.*, 1978). We conclude therefore that it is no longer adequate to describe the 35S promoter as 'constitutive' solely by the criteria of expression in all plant organs, when there may be a strong dependence of transcription on cell-type or cell cycle. This question is being investigated further.

The distribution of GUS activity in the stem sections of plants transformed with rbcS−GUS genes is consistent with data that indicate a requirement for mature chloroplasts for maximal transcription of chimaeric rbcS genes (e.g. Simpson *et al.*, 1986b). Cortical parenchymal cells in the stem contain varying numbers of chloroplasts, while those in the pith and epidermis of the stem rarely contain chloroplasts.

Different cell-types present in each organ contribute differently to the patterns of gene expression and each organ consists of different proportions of these cell-types. We have undertaken to minimize this effect on quantitative analysis of extracts by suitable choice of a denominator. The parameter that needs to be studied with gene fusions is most often the expression of the gene fusion in each cell. When preparing homogenates from plant organs, the number of cells that contribute to the extract will vary, as will the protein content of each cell and cell-type. The DNA content of the extract will reflect the number of cells that were lysed (Labarca and Paigen, 1980) whereas the traditional denominator, protein concentration, will not. For example, a single leaf mesophyll cell contains much more protein than a single epidermal cell or root cortical cell (R.A.Jefferson *et al.*, in preparation). However, each will have the same nucleus with the same potential to express the integrated gene fusion.

Using this approach, we find that the differential expression of the rbcS−GUS fusion is much more pronounced between immature and mature leaf when we express GUS activity/μg of DNA (see Table I). When protein concentration is used as a denominator, the massive induction of GUS activity during leaf maturation is masked by the concomitant induction of proteins involved in photosynthesis.

The observation that the sp. act. of GUS produced by CaMV−GUS fusions is the same in immature and mature leaves when expressed using a protein denominator indicates that the rate of GUS accumulation closely follows the rate of net protein accumulation. The two-fold difference in GUS sp. act. using a DNA denominator illustrates the accumulation of GUS per cell over time. This quantitative analysis, together with our histo-

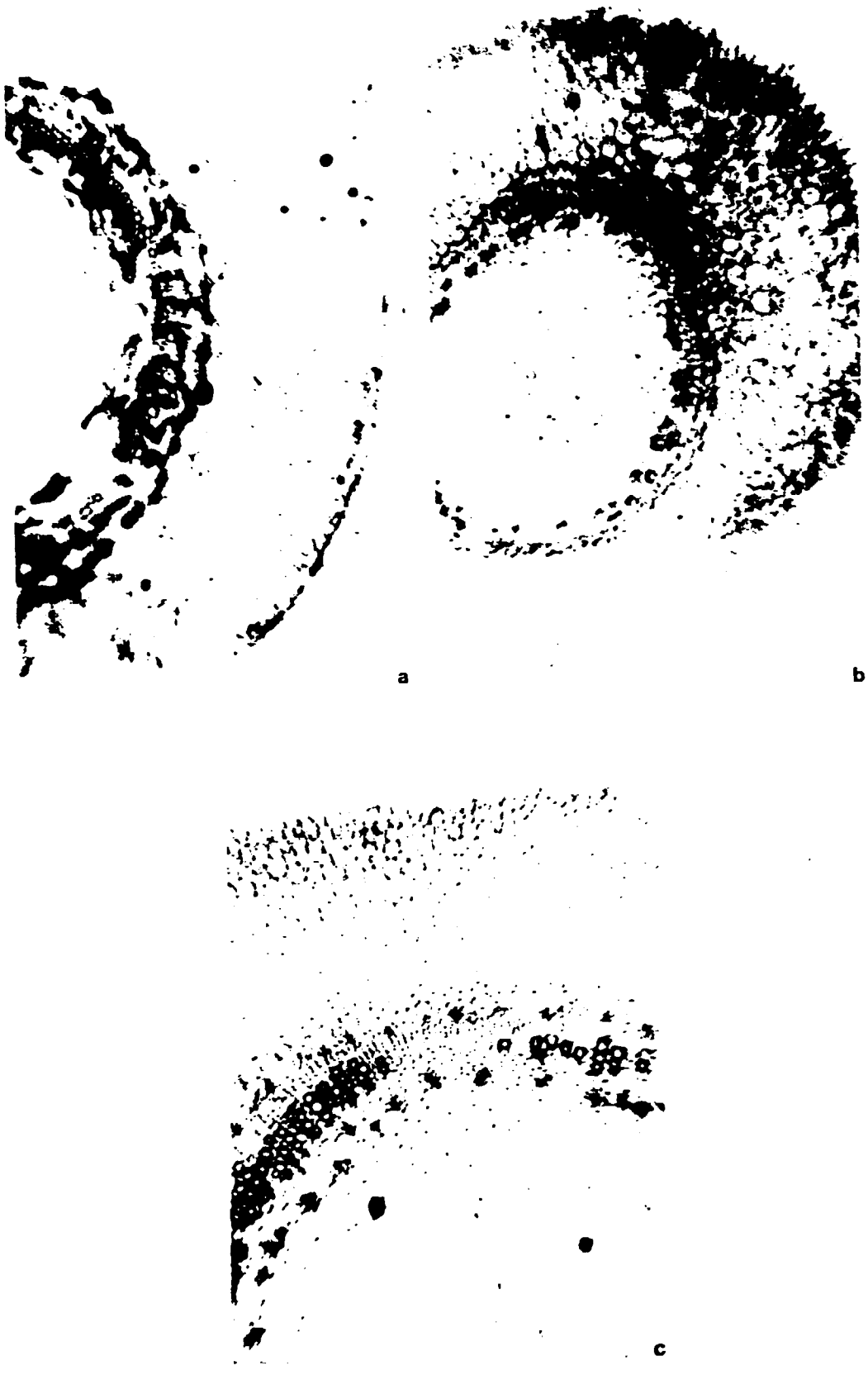

Fig. 4. Histochemical localization of GUS in transformed plant tissue. (a) Transverse stem section from CaMV −GUS-21, stained with 2 mg ml X-Gluc in NaH_2PO_4, pH 7.0, 1 h, 37°C. (b) Transverse stem section from rbcS −GUS-2, stained with X-Gluc, as above, for 3 h. (c) Transverse section of untransformed tobacco plant stained with X-Gluc for 16 h. All magnifications are ~ ×34.

chemical data, may indicate that the differences between GUS activity in the leaf, stem and root of CaMV–GUS fusion plants could reflect the larger proportion of phloem associated cells in roots and stems compared to leaves. We feel that the choice of a DNA denominator best reflects the expression per cell and hence is a more accurate reflection of the true regulation of the gene.

Prospects of further development of the GUS system

There are many important questions arising from the use of currently available gene-transfer techniques in plants that can be addressed with this new technology. Both *Agrobacterium*-mediated transformation and direct DNA uptake methods result in cells and plants transformed with varying numbers of integrated copies of the foreign DNA and with different sites of integration, resulting in plants expressing different amounts of chimaeric gene product (e.g. Jorgensen *et al.*, 1987; Jones *et al.*, 1987). Previously, analysis of gene expression in transformed plants has been sufficiently laborious to preclude quantitative assays of the large numbers of plants necessary to finally delineate the contributions of local integration sites and copy number to the expression of transformed genes. Using the methods described here, it will be feasible to quantitate the variation that is often ascribed to differing sites and copy numbers of integrations, and obtain statistically significant answers to these questions.

The availability of routine histochemical analysis will greatly facilitate studies of the mechanism of transformation both by *Agrobacterium* and by direct DNA methods, as well as permitting a more detailed study of developmental regulation. These methods will also allow very rapid and sensitive screening of transformed cells and tissues. Using the indigogenic substrate X-Gluc, we can easily resolve GUS activity from single cells and small cell clusters from suspension cultures (data not shown).

GUS assay systems lend themselves very well to automation. The existing spectrophotometric and fluorogenic assays, and new assays using fluorogenic substrates that fluoresce maximally at neutral pH (Jefferson, 1985), will allow the use of automatic microtitre plate analysis of very large numbers of samples. The activity of GUS in lysed single cells can be measured with accuracy; using new fluorogenic substrates, we are conducting an analysis of GUS expression in single cells of transformed plants using the fluorescence activated cell sorter (R.A.Jefferson *et al.*, in preparation).

We have also used the GUS fusion system successfully to monitor the transient expression of chimaeric genes introduced into plant cells via electroporation and/or polyethylene glycol treatment (data not shown). We find the sensitivity to be very high, allowing expression to be reliably measured from a very small number of cells (R.A.Jefferson *et al.*, in preparation).

Because of the lack of intrinsic β-glucuronidase activity in all plants thus far assayed in our laboratory, and because the synthesis of β-glucuronides can be relatively straightforward, we are pursuing the use of the GUS system to begin 'fusion genetics'. Due to the complex genomes and long generation times of higher plants, fine scale genetic analysis of complex processes is unfeasible by conventional means. However, by using the GUS system and novel substrates, we may be able to generate positive and negative selections for GUS activity, thereby selecting mutations in the activity of gene fusions, both *in planta* and in tissue culture.

Finally, new methods and substrates are being developed to allow the GUS system to be used quantitatively and reliably *in vivo* and *in situ*.

Materials and methods

Nucleic acid manipulation

DNA manipulations were performed essentially as described (Ma
1982). Enzymes were obtained from New England Biolabs, Boehrin

Plant transformation and regeneration

Binary vectors containing CaMV–GUS fusions and rbcS–GUS fus
MC1022 were mobilized into *A.tumefaciens* LBA4404 as described (B
The integrity of the vectors in *Agrobacterium* was verified by prep
In *Agrobacterium* immediately before plant transformation using
method of Holmes and Quigley (1981). Leaf discs of *N.tabacum*,
were transformed as described (Horsch *et al.*, 1984) and transformed
selected on MS medium (Murashige and Skoog, 1962) containing
kanamycin. Plants were maintained in axenic culture on MS basal
sucrose, 200 μg ml carbenicillin and 100 μg ml kanamycin, at ~20
day, 26°C.

Southern blot analysis

DNA was prepared from plants by phenol extraction and ethanol
of plant homogenates, followed by RNase digestion, phenol ex
isopropanol precipitation. DNA samples (10 μg) were digested wit
endonucleases, electrophoresed in an 0.8% agarose gel and
nitrocellulose (Maniatis *et al.*, 1982). Filters were hybridized wi
primed, ^{32}P-labelled GUS gene fragments (Feinberg and Vogelstein
washed with 0.2× SSC at 65°C.

Substrates

Substrates used included: 4-methyl umbelliferyl glucuronide (M
M-9130), X-Gluc (Research Organics Inc., Cleveland, OH, USA
glucuronide (ReG) (Jefferson, 1985; Molecular Probes Inc., Eugene

Lysis conditions

Tissues were lysed for assays in 50 mM NaH_2PO_4, pH 7.0, 10
0.1% Triton X-100, 0.1% sodium lauryl sarcosine, 10 mM β-mer
(extraction buffer) by freezing with liquid nitrogen and grinding wit
pestle with sand or glass beads. Disposable pestles that fit into Epp
(Kontes Glass) proved useful for homogenizing small bits of tissu
Extracts can be stored at −70°C with no loss of activity for at lea
Storage of extracts in this buffer at −20°C should be avoided, a
inactivate the enzyme.

Fluorometric assay

The fluorogenic reaction is carried out in 1 mM MUG extraction
a reaction volume of 1 ml. The reaction is incubated at 37°C, an
quots are removed at zero time and at subsequent times and the reacti
with the addition of 0.8 ml 0.2 M Na_2CO_3. The addition of Na_2CO
dual purposes of stopping the enzyme reaction and developing the
of MU, which is about seven times as intense at alkaline pH. Flu
then measured with excitation at 365 nm, emission at 455 nm on a
25 spectrofluorimeter, with slit widths set at 10 nm. The resulting
fluorescence versus time can therefore be measured independently
sic fluorescence of the extract. The fluorimeter should be calibrate
prepared MU standards of 100 nM and 1 μM MU in the s
Fluorescence is linear from nearly as low as the machine can mea
1 nM or less) up to 5–10 μM MU.

A convenient and sensitive qualitative assay can be done by pla
on a long-wave UV light box and observing the blue fluorescenc
can be scaled down easily to assay very small volumes (reaction v
terminated with 25 μl 1 M Na_2CO_3 in microtitre dishes or Epp

If the intrinsic fluorescence of the extract limits sensitivity, it
use other fluorogenic substrates. In particular, ReG has a very h
coefficient and quantum efficiency, and its excitation (560 nm)
(590 nm) are conveniently in a range where plant tissue does not abso
heavily. In addition, it fluoresces maximally at neutral pH, making
to stop the reaction.

Protein concentrations of plant extracts were determined by th
method of Bradford (1976) with a kit supplied by Bio-Rad Lab

DNA concentrations in extracts were determined by measuring th
enhancement of Hoechst 33258 dye as described by Labarca and
with the calibrations performed by addition of Lambda DNA stand
tract to eliminate quenching artefacts.

Histochemical assay

Sections were cut by hand from unfixed stems of plants grown
tially as described (O'Brien and McCully, 1981), and fixed in 0.3%
in 10 mM MES, pH 5.6, 0.3 M mannitol for 45 min at room tempe
ed by several washes in 50 mM NaH_2PO_4, pH 7.0. All fixatives

solution were introduced into sections with a brief (~1 min) vacuum infiltration.

Histochemical reactions with the indigogenic substrate, X-Gluc were performed with 1 mM substrate in 50 mM NaH_2PO_4, pH 7.0 at 37°C for times from 20 min to several hours. After staining, sections were rinsed in 70% ethanol for 5 min, then mounted for microscopy.

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GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression

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1 The *Escherichia coli gus* Operon: Induction and Expression of the *gus* Operon in *E. coli* and the Occurrence and Use of GUS in Other Bacteria

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The gene encoding β-glucuronidase, *gusA* (formerly *uidA*), which is now widely used as a reporter gene in plants and other organisms, was originally isolated from *Escherichia coli* (Jefferson *et al.*, 1986). In *E. coli*, *gusA* forms part of an operon. There are two genes downstream of *gusA*, one of which, *gusB*, encodes a glucuronide-specific permease; the function of the product of the third gene, *gusC*, is presently unknown. Upstream of *gusA*, and separately transcribed, is a gene, *gusR*, encoding a specific repressor of the *gus* operon. The primary focus of this chapter is to review the structure and functioning of the *gus* operon in *E. coli*, and to provide a protocol for the induction of the operon and

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GUS Protocols: Using the GUS Gene as a Reporter of Gene Expression

assay of its products. We also discuss the rare occurrence of other GUS$^+$ bacteria, and how the contribution of these to "background" GUS activity can be both assayed and prevented. In addition, current uses of GUS as a reporter gene in bacteria are reviewed.

Occurrence and Natural History of Bacterial β-Glucuronidase

One of the key features that has led to the widespread adoption of GUS as a reporter gene in plant molecular biology is the absence of background activity in higher plants (Jefferson *et al.*, 1986, 1987). GUS activity is also restricted among many other groups of organisms, including bacteria. In fact, GUS assays are routinely used as diagnostic assays for the specific detection of *E. coli* and *Shigella* species in clinical and environmental samples (e.g., Rice *et al.*, 1990; Cleuziat and Robert-Baudouy, 1990). There were earlier reports of some GUS$^+$ *Salmonella* species (Killian and Buellow, 1979) but these appear not to have been confirmed by later studies (e.g., Perez *et al.*, 1986; Cleuziat and Robert-Baudouy, 1990). The eighth edition of "Bergey's Manual" states that "it is taxonomically difficult to justify separate genera or even separate species status for (*E. coli* and *Shigella*)" (Brenner, 1984). Indeed *E. coli* and *Shigella* are serologically related and do exchange genetic information via intergeneric conjugation. Thus, at least among the Enterobacteriaceae, GUS activity can reasonably be said to be restricted to a single taxonomic group—that of *E. coli* and *Shigella* species.

The natural habitat of *E. coli* is the gut, and the GUS activity of *E. coli* plays a specific and very important role in its natural history. In vertebrates, one of the major pathways of detoxification of endogenous and xenobiotic organic compounds is by conjugation of these aglycones to glucuronic acid, a reaction carried out prominently in the liver, among other organs. The addition of the glucuronic acid group renders such hydrophobic compounds more water soluble, and enables them to be excreted in the bile or the urine (Dutton, 1966, 1980). Thus, the gut is a rich source of glucuronic acid compounds, providing a carbon source that can be efficiently exploited by *E. coli*. Glucuronide substrates are taken up by *E. coli* via a specific transporter, the glucuronide permease (see below), cleaved by β-glucuronidase, and the glucuronic acid residue thus released is used as a carbon source.

In general, the aglycone component of the glucuronide substrate is not used by *E. coli* and passes back across the bacterial membrane into

the gut and is reabsorbed into the bloodstream. This circulation of hydrophobic compounds resulting from the opposing processes of glucuronidation in the liver and deglucuronidation in the gut is termed enterohepatic circulation (Figure 1). This phenomenon is of great physiological importance because it means that, due in large part to the action of microbial β-glucuronidase, many compounds, including endogenous steroid hormones and exogenously administered drugs, are not eliminated from the body all at once. Rather, the levels of these compounds in the bloodstream oscillate due to this circulatory process. This process is of great significance in determining pharmaceutical dosages,

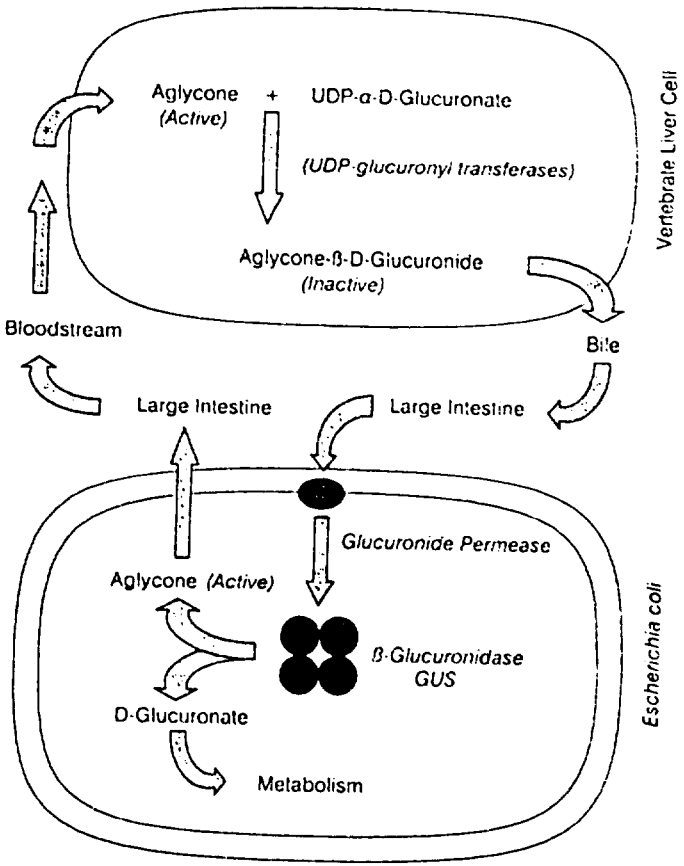

Fig. 1 Schematic representation of enterohepatic circulation.

and indeed some drugs are specifically administered as the glucuronide conjugate, relying on the action of β-glucuronidase to release the active aglycone (Draser and Hill, 1974). Enterohepatic circulation is also important in the day-to-day physiological state of the body, probably being a prime cause of the physiological impact of variations in diet or in gut flora (Goldin, 1986).

GUS activity is found in certain other bacterial species. In particular, it is found in other, nonenterobacterial, anaerobic residents of the gut, primarily in *Bacteroides* and *Clostridium* species (Hawkesworth *et al.*, 1971). Although these species exhibit lower β-glucuronidase activity per cell than *E. coli*, they are approximately 100-fold more abundant in the gut, and hence it was suggested that they might make a more significant contribution overall to enterohepatic circulation. However, it is difficult to judge the relative contributions of the different groups of bacteria based on a single set of measurements of their GUS activity with one glucuronide substrate. It is not known, for example, whether these organisms possess a glucuronide permease and whether their GUS activity, or any permease activity, possesses the same substrate versatility as those of *E. coli*.

There are reports of GUS activity in strains of *Streptococcus*, *Staphylococcus*, and *Corynebacteria* (Dutton, 1966), and we have found certain bacteria associated with plants that are GUS$^+$ (see below). However, GUS activity is not found in most of the bacterial species that are commonly studied because of their importance in agriculture, such as *Rhizobium*, *Bradyrhizobium*, *Agrobacterium*, and *Pseudomonas* species. Thus GUS is now being used as a reporter gene in these organisms, allowing studies of the spatial localization of gene activity of these bacteria in association with their plant hosts (see below).

The *gus* Operon in *E. coli*

The gene encoding β-glucuronidase, *gusA* (formerly *uidA*), maps at minute 36 on the *E. coli* chromosome, between the loci *add* (adenine deaminase) and *manA* (mannose-6-phosphate isomerase) (Novel and Novel, 1973). It has become clear that other genes involved in glucuronide metabolism and in regulation of β-glucuronidase activity map to the same region of the *E. coli* chromosome, forming the *gus* operon. The region has been studied extensively at a genetic and molecular-genetic level, and our current working model of the structure and functioning of the *gus* operon is summarized in Figure 2.

Regulation of GUS Activity in E. coli

β-Glucuronidase activity is not constitutively expressed in *E. coli*: rather, there appear to be three different factors regulating transcription of the operon. The primary mechanism of control is induction by glucuronide substrates. GUS activity is almost undetectable in cells that have been grown in the absence of glucuronides; however, incubation of *E. coli* in the presence of a glucuronide substrate leads to induction of high levels of GUS activity (Stoeber, 1961).

This regulation is due to the action of the product of the *gusR* (formerly *uidR*) gene, which encodes a repressor that is specific for the *gus* operon (Novel and Novel, 1976a). Inactivation or deletion of *gusR* leads to constitutive β-glucuronidase activity (Novel and Novel, 1976a; K. J. Wilson and R. A. Jefferson, unpublished results). The *gusR* gene maps to the same region of the chromosome as *gusA*, lying upstream of *gusA* and being separately transcribed. The direction of transcription is the same as *gusA* (Blanco *et al.*, 1985; K. J. Wilson and R. A. Jefferson, unpublished), and we have recently completed the DNA sequence of this region and have found that *gusR* is most likely encoded by an open reading frame of 195 amino acids.

GusR repression of β-glucuronidase activity has been shown by Northern analysis to be mediated by transcriptional regulation. RNA

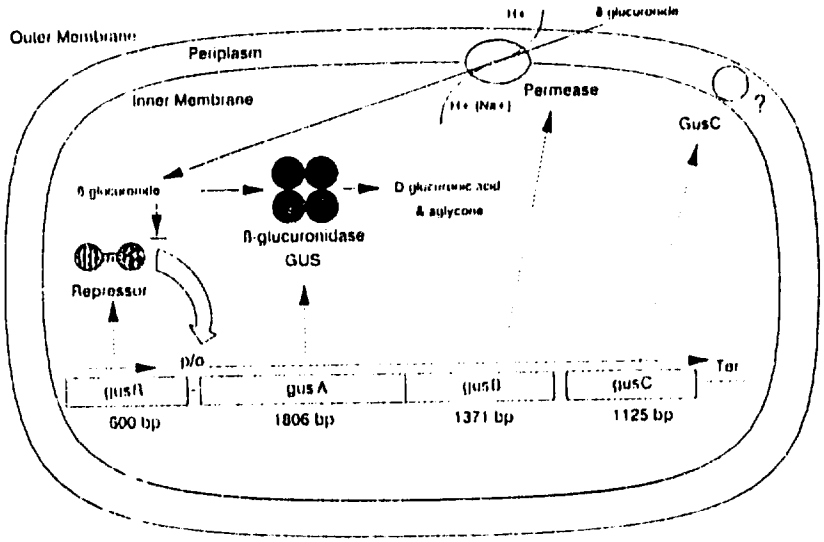

Fig. 2 The *gus* operon in *E. coli*.

from uninduced cultures of *E. coli* showed no hybridization to a *gusA* probe, in contrast to the strong hybridization observed to RNA extracted from cultures that had been induced with methyl β-D-glucuronide (Jefferson, 1985). Presumably, therefore, GusR acts by binding to *gusA* operator sequences so preventing transcription, this repression being relieved when a glucuronide substrate binds to the repressor and inactivates it. While the exact operator site remains to be defined, there are several candidate regions of dyad symmetry upstream of the *gusA* ATG (Jefferson *et al.*, 1986).

A second key level of control is that of catabolite repression. *Escherichia coli* grown in the presence of 1% glucose does not express β-glucuronidase activity even in the presence of a glucuronide inducer (Stoeber, 1961). A putative CAP binding site has been identified in the *gusA* upstream sequences (Jefferson *et al.*, 1986).

A third level of regulation of *gus* transcription appears to be exerted by the product of the *uxuR* gene. This gene, which maps elsewhere on the *E. coli* chromosome at minute 98, is primarily concerned with regulation of the *uxuAB* operon, which encodes enzymes involved in the further metabolism of glucuronic acid (Novel and Novel, 1976b). Repression of transcription of the *uxuA* and *B* genes is relieved by incubation with glucuronic acid, which presumably binds to and inactivates UxuR. In *E. coli* K-12, mutations in *uxuR* cause derepression of β-glucuronidase to only 1–4% of the full glucuronide-induced level (Novel and Novel, 1976b). As induction of transcription of a gene encoding an enzyme activity by the product of that enzyme activity is hard to understand, and as the level of regulation is <5% of that exerted by GusR and by CAP, it is not certain whether regulation by UxuR is of primary importance or is a secondary effect, perhaps resulting from some degree of homology between the two gene products. This latter possibility is supported by the observation that expression of *uxuR* on a multicopy plasmid can largely suppress the effect of a *gusR* mutation (Ritzenhaler *et al.*, 1983).

A Glucuronide-Specific Permease is Encoded by gusB

The existence of a glucuronide-specific permease was first demonstrated in the late 1950s by F. Stoeber working in the laboratory of Jacques Monod at the Pasteur Institute, Paris. Stoeber measured accumulation of [^{35}S]phenyl β-D-thioglucuronide, a glucuronide that is not hydrolyzed by GUS, in a fecal *E. coli* isolate and showed that this

compound could be accumulated from external concentrations as low as 5 μM, giving over 200-fold concentration in the cell. This accumulation was inhibited in the presence of sodium azide, indicating that it is an active transport process. Using uptake competition studies he demonstrated that different glucuronides have different affinities for the permease, and some, such as phenolphthalein β-D-glucuronide, are not taken up at all. Like GUS activity, the permease activity is induced by preincubation with a glucuronide substrate (Stoeber, 1961).

Interestingly, Stoeber found that *E. coli* K-12, in contrast to the fecal strain, had very poor uptake ability (Stoeber, 1961). We have repeated this observation with the cloned *gus* operon from a K-12 strain and from a fecal strain. When expressed in a host strain that is deleted for the *gus* operon, the cloned K-12 genes conferred almost no ability to accumulate phenyl thioglucuronide in the cells, in contrast to the strong accumulation observed on expression of the operon from the fecal isolate (W.-J. Liang, P. J. Henderson, K. J. Wilson, and R. A. Jefferson, unpublished results). This observation is of considerable importance in induction assays (see below).

Molecular-genetic evidence concerning the glucuronide permease was first obtained when 340 bp of an open reading frame, encoding a highly hydrophobic protein, was identified immediately downstream of *gusA*—in fact there is a four base-pair overlap (ATGA) between the two genes (Jefferson *et al.*, 1986). The sequence of *gusB* from *E. coli* K-12 has now been completed and indicates a protein with 12 membrane-spanning domains that has 25% identity at the amino acid level to the *E. coli* melibiose transporter encoded by *melB* (R. A. Jefferson and W.-J. Liang, unpublished data; Liang, 1989). From the homology to the melibiose transporter (a sodium symporter) and from the dependence on membrane potential, it is likely that the glucuronide permease also functions as a cation symporter coupled to the electrochemical gradient.

The Range of *gus* Operon Inducers

We now know that efficient induction of expression of β-glucuronidase and of the whole operon depends on two key steps:

1. The substrate must be taken up into the cell via the glucuronide permease.

2. The substrate must be able to alleviate repression by the *gus* repressor.

Stoeber tested the ability of a number of different glucuronides to induce GUS activity, and found that it varied greatly, methyl β-D-glucuronide at 1 mM concentration inducing a level of GUS activity approximately 15 times that of phenyl β-D-thioglucuronide (a gratuitous inducer). He also found that some GUS substrates, such as phenolphthalein β-D-glucuronide and aldobiuronic acid (galactosyl β-D-glucuronide), do not act as inducers of β-glucuronidase activity (Stoeber, 1961). In the case of phenolphthalein β-D-glucuronide, this is clearly due to the inability of this substrate to be transported by the permease.

Using the protocol given below, we have tested the ability of GUS substrates now commonly used in quantitative and spatial analysis of GUS activity to act as inducers of the *gus* operon in *E. coli*, and have found that 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-Gluc), *p*-nitrophenyl β-D-glucuronide (PNPG), 4-methylumbelliferyl β-D-glucuronide (MUG), and resorufin glucuronide all act as powerful inducers. In general, values of GUS activity measured after 90 min of induction, starting with 1 mM external concentrations of these glucuronides, are of the order of 1–50 nmol PNPG hydrolyzed per minute per OD_{600} unit of bacterial culture. We have also tested a number of glucuronides that would occur naturally in the body, including estrogen glucuronide and testosterone glucuronide, and found that they too appear to have low inducing power (Liang, 1989).

This range of inducers illustrates a remarkable fact about the glucuronide permease, namely, that it is able to recognize and actively transport an extraordinary range of glucuronides with different aglycone residues. Likewise, the *gus* repressor must be able to recognize the same range of glucuronides. Thus, for example, while X-Gal does not act to induce the *lac* operon in *E. coli*, presumably because it does not relieve repression by the *lac* repressor and perhaps also because it is poorly transported by the *lac* permease, X-Gluc is a powerful inducer of the *gus* operon, indicating that it is actively accumulated within the cell by the glucuronide permease and acts to relieve the repression by GusR.

All the measurements discussed above, carried out both by Stoeber and by ourselves, have been on fecal isolates of *E. coli*. In contrast, *E. coli* K-12 strains show only very low levels of GUS induction in similar conditions. This can readily be visualized by streaking a fecal sample on an LB plate containing 50 μg/ml X-Gluc: overnight, dark blue colonies will appear. In contrast, an *E. coli* K-12 strain streaked on the same plate will show no or very little blue coloration in the colonies even after several days incubation. This is because the K-12 permease fails to concentrate the X-Gluc from the low external concentration in the plate (about 120 μ*M*) and thus fails to induce the K-12 β-glucuronidase activity. To obtain high levels of K-12 β-glucuronidase induction it is necessary to use external concentrations of inducer of at least 10 m*M*.

Other Bacteria Show Inducible GUS Activity: Possible Contribution to "Background" Activity in GUS Assays

As discussed above, there are bacterial species other than *E. coli* that possess β-glucuronidase activity, particularly among the gram-positive genera *Staphylococcus* and *Streptococcus*. While the natural habitat of *Staphylococcus* is the skin and mucosal membranes of mammals, and that of *Streptococcus* is the intestinal tract and other organs also of mammals, it is important to remember that such microorganisms are not restricted in their distribution to these habitats but can be found elsewhere in stable and transient niches.

Solid rich media containing X-Gluc (50 μg/ml) provides a convenient means of screening for GUS-positive microorganisms. We have screened samples of soil, fecal matter, and some plant tissue in this way, and all, depending on provenance, have been shown to contain them. Here we give four examples of noncoliform GUS$^+$ bacteria that we have found in association with plants and that are illustrative of the false positive results that can occur in carrying out GUS assays with putatively transgenic plant material.

The first bacterium was found among the organisms that grew when pollen was shaken from the flowers of greenhouse-grown tobacco directly onto X-Gluc plates. It had the characteristic colonial morphology associated with highly mobile or gliding bacteria. At 30°C fingerlike projections advanced from the point of inoculation at the rate of about 2 cm per day. The mobile circular mound of cells at each end of the projection exhibited GUS (or GUS-like) activity. Of the recognized gliding bacteria, this organism most closely resembles the Myxobacteria. However, although spores were observed within the mounds, we did not observe the larger, sculpted fruiting bodies often found in this group. Of particular interest is the observation that when pollen grains of tobacco placed on X-Gluc plates are engulfed by these bacterial

swarms, an intense deposition of blue pigment characteristic of a GUS X-Gluc reaction appears within the grains (Color Plate 1). Whether or not this observation represents genuine GUS activity, the phenomenon clearly illustrates the capacity of microorganisms to confound histochemical procedures, and emphasizes the need for caution. It is certainly possible that some of the observations of GUS or GUS-like activity in control plants could be explained by the presence of this type of organism.

The second and third organisms were both isolated directly from plant tissues that had been subjected to particle bombardment. More specifically, they were recovered from blue zones that developed around the points of impact of shrapnel following histochemical staining with X-Gluc. Fatty acid profiles did not exactly match any characterized microorganisms, but one showed similarity to a *Micrococcus* or *Clavibacter* species and the second to a *Staphylococcus* or *Brevibacterium* species. These assignments are consistent with the results of fermentation and enzyme activity profiles. Both organisms could be cultured on the surface of potato tubers and gave blue zones when the slices were subjected to histochemical staining with X-Gluc.

The fourth organism was isolated from yam (*Dioscorea cayanenesis*), again from tissue that had been subjected to particle bombardment. Analysis of the fatty acid content of this organism identified it as a *Curtobacterium* species, a genus of bacteria that is commonly isolated from plants. The GUS activity of this bacterium was studied more carefully and it was found not to be constitutive but to be inducible by incubation with each of the GUS substrates X-Gluc, MUG, and PNPG— albeit to levels below those observed in *E. coli* (M. Tor and K. J. Wilson, unpublished results). This induction of GUS activity was completely repressible by inclusion of 100 μg/ml chloramphenicol in the medium.

The studies on the latter microorganism indicate that GUS activity is inducible not just in *E. coli* but also in other microorganisms. Thus, some of the "background" activity that is observed when samples of tissue are subjected to long incubation in GUS substrates—often overnight—may result from the induction and subsequent activity of GUS in plant-associated microorganisms. If the enzyme is inducible rather than constitutive then it is easy to overcome the problem by the inclusion of chloramphenicol in the incubation medium, as this specifically inhibits bacterial, but not eukaryotic, protein synthesis. Another approach that has recently been demonstrated to effectively suppress "endogenous" GUS activity in plants is the addition of 20% methanol to the assay buffer (Kosugi *et al.*, 1990; see also Martin *et al.*, Chapter 2, and Stomp, Chapter 7). It is possible that, in some cases, the background activity being suppressed is due to GUS$^+$ microorganisms.

Use of GUS as a Reporter Gene in Plant-Associated Bacteria

The use of GUS as a reporter gene in plant-associated bacteria has lagged far behind its use as a reporter gene in plants. Recently, however, its potential has begun to be realized.

Sharma and Signer constructed *gus* transposons based on transposon Tn*5* that create either transcriptional (Tn*5*-*gusA*1) or translational (Tn*5*-*gusA*2) fusions to genes adjacent to the site of insertion. They used these transposons to create *gus* fusions to *Rhizobium meliloti* genes required for nodulation (*nod*) and symbiotic nitrogen fixation (*nif* and *fix*), and demonstrated the different spatial patterns of expression of these two classes of genes within the legume root nodule (Sharma and Signer, 1990). A similar promotor-probe *gus* transposon based on the Tn*3*-HoHo *lacZ* transposon has been constructed and is being used to analyze *hrp* genes of *Pseudomonas syringeae* (D. Dahlbeck, R. Innes, and C. Boucher, personal communication).

GUS fusions are also being used to look at targeting of viral and microbial proteins within plant cells. Translational fusions to *gusA* that added the entire coding region of the tobacco etch potyvirus proteins Nla and Nlb (encoding respectively a 49-kDa proteinase and a 58-kDa RNA polymerase) were shown to target the hybrid GUS Nla/Nlb proteins to the nucleus of the plant cell, the location in which the native Nla and Nlb proteins are normally found. This system can now be used as a powerful means of identifying the signals within the Nla and Nlb protein sequences that target them to the nucleus (Restrepo *et al.*, 1990). A similar approach using GUS fusions is being taken to study targeting of the VirD2 protein of *Agrobacterium tumefaciens* within the plant cell (J. Zupan and P. Zambryski, personal communication).

Finally, we are developing the *gus* operon as a transgenic marker system for the detection and monitoring of bacteria in soil and in association with plants. In initial experiments expression of the *gus* operon has been demonstrated in *R. meliloti* and used to detect the presence of marked strains in alfalfa root nodules by simply infiltrating the roots with buffer containing X-Gluc and observing the development of blue-coloured root nodules (Color Plate 2). Likewise *gusA* expression in a

Bradyrhizobium strain has been used to detect infection threads and to visualize areas of the root surface densely colonized by marked bacteria (Wilson *et al.*, 1991).

Protocol for Induction and Assay of E. coli β-Glucuronidase

The following protocol can be used to examine the induction and expression of β-glucuronidase activity in *E. coli* or in any GUS$^+$ bacteria that might be found in association with plant material. The GUS assay described here can of course be used to measure GUS activity in any bacterial strain, including that of GUS fusions constructed to analyze the regulation of specific bacterial genes.

Reagents

Minimal medium for growth of bacteria, using glycerol or succinate as carbon source.

Chloramphenicol (Cm)10 mg/ml in methanol
GUS assay buffer:
50 m*M* $NaPO_4$, pH 7.0
5 m*M* DTT
1 m*M* EDTA
100 m*M* PNPG stock, dissolve PNPG at 35 mg/ml in water
100 m*M* stocks of substances to be assayed for inducing power, [e.g., 100 m*M* MUG: dissolve at 39 mg/ml in dimethylformamide; 100 m*M* X-Gluc: dissolve at 40 mg/ml in dimethylformamide (Na^+ salt) or dissolve at 52 mg/ml (cyclohexammonium salt)]
Chloroform
0.1% SDS
0.4 *M* Na_2CO_3

Induction of Strains

1. Grow overnight cultures of strains in minimal medium. The minimal medium used (e.g., M9 or M63) (Miller, 1972) should first be checked and modified as necessary with the addition of specific growth requirements to ensure that the strains do grow! Glycerol or succinate should be used as the carbon source, not glucose, to avoid any effects of catabolite repression.
2. Subculture strains into 1 ml minimal medium plus appropriate antibiotics and grow for at least 1 h to reach exponential phase.
3. Add 10 μl of 100 m*M* inducer. Keep one tube as a negative control without inducer. Grow for 1–3 h.
4. Transfer approximately 1.5 ml cells to an Eppendorf tube, spin 30 s, pour off supernatant and wash pellet by resuspending in 1 ml 1 × M9 salts + 100 μg/ml Cm. The washing removes all hydrolyzed glucuronide product, and the chloramphenicol prevents further protein synthesis. Centrifuge again, and resuspend pellet in 1.5 ml 1 × M9 salts + 100 μg/ml Cm.
5. Measure the OD of 0.5 ml at 600 nm. It is better if it is 0.5 or less. Keep remaining cells on ice or freeze at −70°C until ready for GUS assays.

GUS Assays

1. Thaw tubes on ice if necessary.
2. Prepare GUS assay buffer with 1.25 m*M* PNPG (add 12.5 μl 100 m*M* PNPG/ml). Prewarm to 37°C.
3. Permeabilize cells by vortexing with 1 drop 0.1% SDS and 2 drops chloroform for 10 s.
4. Prepare one Eppendorf tube per sample containing 800 μl GUS assay buffer with 1.25 m*M* PNPG. Add 200 μl permeabilized cell suspension (take care to avoid the chloroform which will be at the bottom of the tube). This gives a final concentration of PNPG of 1m*M*. Note approximate time.
5. Place reactions at 37°C. Keep an eye on the development of yellow color (*p*-nitrophenol). At at least three time points remove 100 μl into 800 μl 0.4 *M* Na_2CO_3. The time points do not have to be equally spaced, but note each time.
6. Measure absorbance at 405 nm for each time point against a substrate blank or a stopped blank reaction Under these conditions the molar extinction coefficient of *p*-nitrophenol is 18,000. Thus in the 0.9 ml final volume, an absorbance of 0.020 represents 1 nmol of product produced.
7. Calculate the rate of the reaction in nanomoles product per minute as outlined below. The use of several time points enables the rate of the reaction to be calculated from the linear enzyme kinetics (see Jefferson and Wilson, 1991, for further discussion). Alternatively, a single measurement could be taken at a given

time after the start of the reaction, as is normally done for β-galactosidase (Miller, 1972). Normalize to OD units, or, if preferred, to cell protein or to viable cell number.

Calculation

For each sample plot a graph of OD_{405} (Y-axis) versus time in minutes (X-axis). Calculate the slope S of the graph (which should be linear!) in OD_{405} units per minute.

Rate of reaction R in nanomoles product per minute per OD_{600} unit is then

$$R = S/(0.02 \times V \times OD_{600})$$

where V is the volume assayed in milliliters. In the protocol listed above, V = 0.02 ml because 0.2 ml sample is used in the initial reaction (step 4) and one-tenth of this (100 μl) is removed into stop buffer for each time point (step 5).

This measurement differs from the Miller units used to measure β-galactosidase in that Miller units are calculated as OD_{420} units × 1000 per min per ml of culture at OD_{600} measured in a final reaction volume of 1.7 ml; that is, they are not converted to actual nanomoles product produced per unit time per unit of culture (Miller, 1972).

Note: This assay can very easily be automated using commercially available ELISA plate readers and microtiter equipment. The calculation given above derives from the Beer-Lambert law and assumes a pathlength of 1 cm, as found in most spectrophotometers. However, if a microtiter dish and an ELISA reader are used, the path length will be different and the conversion factor must be recalculated. This can be done using commercially available liquid *p*-nitrophenol as a standard to calibrate the readings for specific volumes of solution assayed in the microtiter wells. Absorbance can also be measured at 415 nm, at which wavelength the molar extinction coefficient of *p*-nitrophenol is 14,000.

Acknowledgments

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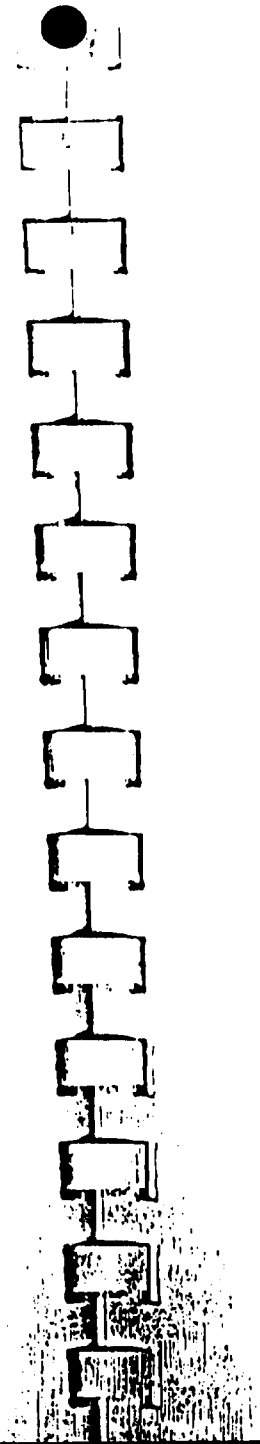

2 The GUS Reporter System as a Tool to Study Plant Gene Expression

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During the last few years the bacterial β-glucuronidase gene (*uidA*, *gusA*), commonly referred to as the GUS gene, combined with the increasing number of plant species accessible to molecular transformation, has become the major reporter gene used as a tool for the analysis of plant gene expression (Willmitzer, 1988; Walden and Schell, 1990, Table 2). The wide acceptance is mainly due to such advantages as fast and nonradioactive analysis. The assay is extremely sensitive, and it is possible to obtain both quantitative (i.e., level of expression) and qualitative (i.e., specificity of expression in tissues and organs) data with the same reporter gene. Quantitative assays are performed using fluorigenic substrates such as 4-MUG (4-methylumbelliferryl-β-glucuronide), whereas X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide), on the other hand, can qualitatively show cell- and tissue-specificity. Chemical modification of the substrate might even allow use of GUS as a selectable marker (Jefferson, 1989).

GUS is used for a wide range of applications. We and others have found promoter–GUS fusions very useful for promoter analysis and for dissecting gene families (Stockhaus *et al.*, 1989; Keil *et al.*, 1989; Rocha-Sosa *et al.*, 1989; Köster-Töpfer *et al.*, 1989, 1990; Liu *et al.*, 1990, 1991). For this purpose Jefferson (1987) has developed a set of vectors that are based on the binary plasmid pBIN 19 (Bevan, 1984) and

Mol Gen Genet (1990) 220:245-250

MGG

Construction of an intron-containing marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation

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Summary. *Agrobacterium tumefaciens* is a commonly used tool for transforming dicotyledonous plants. The underlying mechanism of transformation however is not very well understood. One problem complicating the analysis of this mechanism is the fact that most indicator genes are already active in *Agrobacterium*, thereby preventing the precise determination of timing and localisation of T-DNA transfer to plant cells. In order to overcome this obstacle a modified prokaryotic indicator gene was constructed. The expression of this indicator gene and its use in analysing early events in *Agrobacterium*-mediated plant transformation are described. A portable intron, derived from a plant intron, was introduced into the β-glucuronidase (GUS) gene. In transgenic plants containing this chimaeric gene the intron is spliced efficiently, giving rise to GUS enzymatic activity. Mapping of the splice junction indicates the exact removal of the intron. No GUS activity is detected in agrobacteria containing this construct due to the lack of a eukaryotic splicing apparatus in prokaryotes. Early phases after transformation of *Arabidopsis* cotyledon explants were analysed using this GUS-intron chimaeric gene showing that as early as 36 h after *Agrobacterium* infection significant GUS activity is detected. In vivo GUS staining of transformed cells clearly shows that quickly proliferating calli expressing GUS activity are formed, mainly at the cut surface. Minor transformation events occur however throughout the whole cotyledon. These data indicate that *Agrobacterium*-mediated T-DNA transfer to plants is much more efficient than has been judged from experiments where selection is applied immediately. The intron-containing GUS gene can be used as an optimised marker gene in transient and stable transformation experiments.

Key words: *Agrobacterium tumefaciens* β-glucuronidase - Portable intron - Splicing - Transformation

Introduction

The gram-negative soil bacterium *Agrobacterium tumefaciens* has been developed into a valuable tool for transferring genes to a number of higher plant species (Schell 1987). Despite its extensive use, little is understood about the early phase in transformation experiments (Zambrisky et al. 1989). Knowledge of the relative susceptibility of different cells and tissues to transformation and the amenability of the initially transformed tissue for regeneration would however be helpful in devising strategies for transformation experiments for recalcitrant plant species.

Various bacterial indicator genes have been developed which allow the selection of and/or screening for transformed cells (Schell 1987; Klee et al. 1987). One major hindrance however is the fact that despite the usage of eukaryotic promoters these genes are still well expressed in agrobacteria (unpublished observations). In order to overcome this problem we took advantage of the fact that prokaryotes are devoid of the eukaryotic splicing apparatus. To this end a portable intron derived from a plant gene was constructed and inserted into a reporter gene.

The intron to be used must fulfil the following conditions:

It should be "portable" in order to allow its easy insertion into different sites of any gene of interest.

It should contain stop codons in all three possible reading frames thus excluding expression in agrobacteria.

It should be a "typical" plant intron in order to increase the likelihood that it will be spliced efficiently.

Introns in plant genes have not been characterised in as much detail as their animal homologues. However some features common to all plant introns are known. Their average size is 250 nucleotides, their AT content is high and 5′ and 3′ splice junctions are similar to those of introns of animal genes (Hawkins 1988; Brown 1986; Wiebauer et al. 1988). The second intron (IV2) of the ST-LS1 gene (Eckes et al. 1986) comprises typical plant intron features: a length of 189 nucleotides, an AT content of 80% and typical splice junctions (Fig. 1). Moreover this intron also fulfils the second condition, i.e. it contains several stop codons in all reading frames.

The splicing mechanism in higher plants has not been investigated in great detail to date. The failure of a plant system to process mammalian introns (Bartha et al. 1986) raised the possibility that the mechanism of intron recognition differs in plants and animals. On the other hand several plant introns have been shown to be properly spliced in vitro in HeLa cell nuclear extracts (Brown et al. 1986; Harmuth and Bartha 1986). In this context an in vivo approach using a chimaeric intron-reporter gene could elucidate parameters involved in pre-mRNA processing.

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The indicator gene to be constructed should allow the sensitive detection of its encoded product both quantitatively in tissue homogenates and qualitatively in single plant cells. We therefore decided to use the *ß*-glucuronidase (GUS) gene from *Escherichia coli* and to modify it by inserting an intron in the protein-coding region, thus preventing its expression in a prokaryotic environment. The functionality of this portable intron, the accuracy of splicing and the effects on GUS enzymatic activity were analysed in transgenic plants. Furthermore the chimaeric intron-GUS gene was used as a tool in monitoring early events in *Agrobacterium*-mediated *Arabidopsis* transformation.

Materials and methods

Bacterial media and strains. All agrobacterial strains were grown in YEB medium (Vervliet et al. 1975). *E. coli* strains were grown in YT medium (Maniatis et al. 1982). Binary vectors were introduced into the GV 2260 agrobacterial strain (Deblaere et al. 1985).

Construction. The p35S GUS, kindly provided by J. Stockhaus, is a BIN 19 (Bevan 1984) derivative into which the GUS gene was introduced under the control of the 35S promoter from pRT 102 (Töpfer et al. 1987). The constructs were introduced into *Agrobacterium* via direct DNA transformation (Höfgen and Willmitzer 1988). Site-directed mutagenesis was performed according to the protocol of the supplier (Amersham), using synthetic oligonucleotides.

Plant transformation. Tobacco leaves (*Nicotiana tabacum* var. W38) were used for *Agrobacterium*-mediated leaf disc infection as described (Horsch et al. 1985). Transformants were selected on 100 µg/ml kanamycin.

Arabidopsis thaliana cotyledons were used as explants for transformation (Schmidt and Willmitzer 1988). The antibiotic G418 was used as the selective agent for transformants at a concentration of 20 µg/ml.

Root explants of *A. thaliana* were transformed according to Valvekens et al. (1988).

Northern analysis. Total RNA of leaves was isolated (Logemann et al. 1987), separated on 1% agarose gels in the presence of formaldehyde, blotted on Hybond nylon membranes and used for hybridisation as described by Amasino (1986). The complete GUS gene was used for probing after labelling using the multiprime approach (Amersham protocol).

Primer extension. Total leaf RNA (30 µg) was hybridised to an oligonucleotide (20-mer) overnight and extended using AMV reverse transcriptase according to Kingston (1987). The first strand cDNA was amplified using the polymerase chain reaction (PCR) according to the Perkin Elmer protocol. The denaturation was done at 92° C, the annealing at 50° C and the polymerase reaction at 65° C. Twenty cycles were done in a Perkin Elmer thermal cycler.

Assays for GUS activity. For the fluorometric GUS assay, explants were homogenised and incubated with the substrate 4-methylumbelliferyl-*ß*-D-glucuronide at 37° C. Quantification of the fluorescence was done according to Jefferson (1987) and Jefferson et al. (1987) and expressed as picomoles methylumbelliferrol per milligram protein per minute. Protein concentrations were determined according to Bradford (1979).

For in vivo staining, intact plant material was vacuum infiltrated with 1 mM X-gluc (5-bromo-4-chloro-3-indolyl-*ß*-D-glucuronic acid cyclohexylammonium) and incubated as reported by Jefferson (1987) and Jefferson et al. (1987).

Late logarithmic agrobacterial cultures were stained for 24 h in a similar way, omitting vacuum infiltration and ethanol treatment.

Results

Construction of a GUS gene containing a portable intron

The second intron (IV2) of the ST-LS1 gene was introduced into the bacterial GUS gene. In order to simplify the cloning the intron was slightly modified at the border sequences. In addition internal intron border sequences were optimised with respect to the consensus sequence for plant introns: AG//GTAAGT...TGCAG//G (Shapiro and Senepathy 1987). To this end IV2 was isolated from the ST-LS1 gene as a *StyI – HphI* fragment and cloned in an M13 intermediate vector. Modifications were performed by site-directed mutagenesis using synthetic oligonucleotides, changing the left border sequence AAG/GTT into TAC/GTA resulting

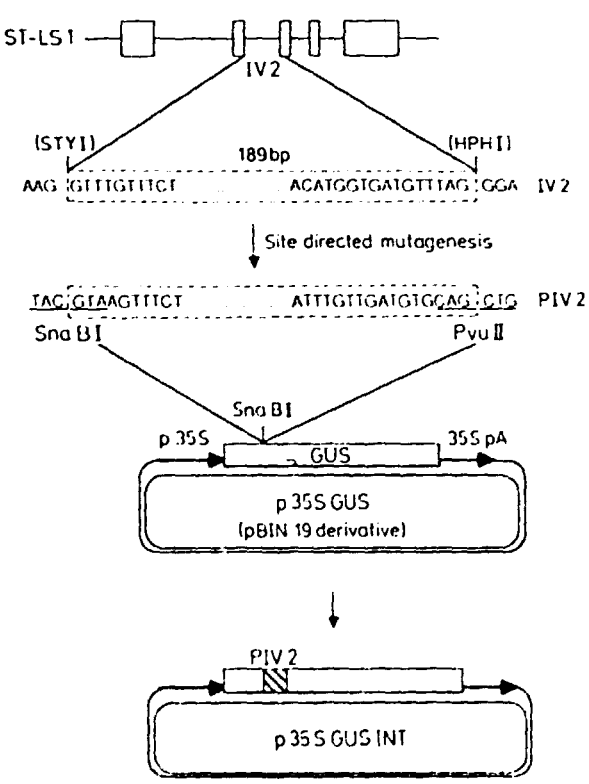

Fig. 1. Cloning of the portable intron in the *ß*-glucuronidase (GUS) gene. The upper drawing shows schematically a part of the structure of the ST-LS1 gene. *Boxes* represent exons and *solid lines* introns; in addition part of the nucleotide sequence of the second intron (IV2) is shown. The sequences in the boxes confined by the *dotted lines* represent the wild-type intron (IV2) and the mutated portable intron (PIV2). The lower drawing shows p35S GUS and its intron derivative p35S GUS INT, as described in Results

in a *Sna*BI restriction site and the right border TAG/GGA into CAG/CTG resulting in a *Pvu*II site (Fig. 1). The portable intron (PIV2) obtained was cloned into the single *Sna*BI restriction site of the GUS gene (Jefferson 1987; Jefferson et al. 1987) which had been inserted between the *Cauliflower Mosaic Virus* derived 35S promoter and polyadenylation signal in a derivative of the binary vector BIN 19 (Bevan 1984) resulting in plasmid p35S GUS INT (Fig. 1).

RNA analysis and mapping of the splice junction in transgenic tobacco plants

In order to analyse whether or not the intron was functional in the chimaeric construct, it was transferred into tobacco using *Agrobacterium*-mediated transformation (Horsch et al. 1985). Several independently transformed plants, containing intact copies of the 35S GUS INT gene, were scored for the presence of GUS INT-derived RNA. By Northern analysis a single RNA migrating at a molecular weight of approximately 1.8 kb was observed, which hybridised to the GUS gene (Fig. 2). This corresponds to the size of the mRNA of the GUS gene without intron (lane C in Fig. 2). The fact that no RNA species of higher molecular weight was observed, indicates that IV2 is spliced out efficiently. Moreover it is interesting to note that in the cases analysed the abundance of the GUS mRNA was similar to that of the intronless GUS gene construct (p35S GUS).

In order to map the splice junction in the GUS gene a combined primer extension-PCR was performed. Total leaf RNA of transgenic tobacco plants containing either the intact GUS gene (35S GUS; Fig. 3, lane A) or GUS gene interrupted by the intron (35S GUS INT; Fig. 3, lane B) was hybridised to a 20-mer oligonucleotide (Gol 1) localised 3′ of the splice junction and used for primer extension as shown in Fig. 3, (1). The first strand cDNA was consequently amplified using the PCR (Saiki et al. 1988). The second oligonucleotide (Gol 2, 25-mer) is localised 240 bp 5′ of Gol 1 (Fig. 3, (1)). After agarose gel electrophoresis a single amplified fragment with a size of 240 bp can be observed in both cases (Fig. 3, lanes A, B). A 430 bp fragment, which would result from an unspliced GUS mRNA, could not be detected. Digestion of the amplified fragments with *Sna*BI revealed a 190 bp fragment which is expected after the removal of the intron. However, some undigested DNA fragment was observed, which was most probably due to inefficient digestion by the *Sna*BI restriction enzyme. These data suggest that the portable is accurately spliced.

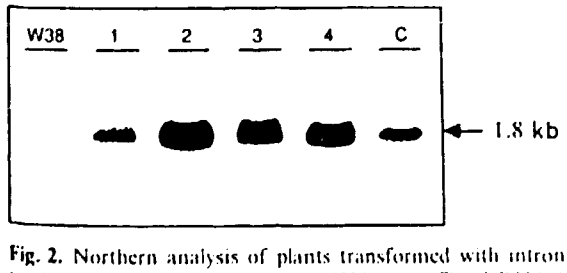

Fig. 2. Northern analysis of plants transformed with intron-containing and intron-free chimaeric GUS genes. Total RNA (50 µg each) isolated from leaves of four independent transgenic tobacco plants containing the 35S GUS INT gene (lanes 1–4) or the intron-free 35S GUS gene (lane C) were separated by gel electrophoresis, blotted and hybridised against the complete GUS gene. The size of the RNA is given in nucleotides on the right. Lane W38 contains RNA isolated from nontransformed tobacco plants

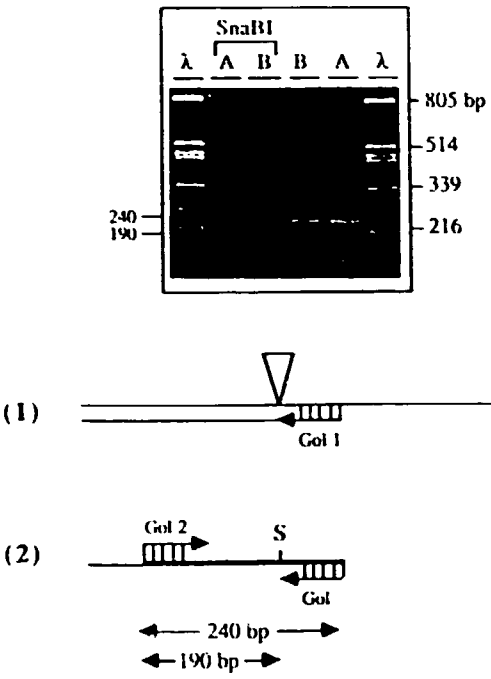

Fig. 3. Mapping of the splice site in the chimaeric GUS gene. Total leaf RNA (30 µg) of transgenic tobacco plants containing either p35S GUS (lane A) or p35S GUS INT (lane hybridised to a 20-mer oligonucleotide Gol 1 and used for extension (1). The first strand cDNA was amplified by the p ase chain reaction (PCR) using the 25-mer oligonucleotid (2). The molecular weight of the amplified fragment wa mined by 1.5% agarose gel electrophoresis as shown in th panel. Samples in the lanes marked SnaBI were digested restriction enzyme *Sna*BI. As a size marker λ DNA was with *Pst*I

Functional analysis of GUS activity in higher plants

The RNA analysis suggested in that the influence intron on the level of expression in transgenic plants was low. This was analysed in *Arabidopsis* detail by quantitative fluorescence assays (Jefferso Jefferson et al. 1987). Using the *Arabidopsis* co transformation procedure (Schmidt and Willmitze calli were obtained containing either 35S GUS or 3 INT. Quantitative measurements of GUS activity w formed and are summarised in Fig. 4. The GUS ranged from $5 \cdot 10^3$ 5×10^5 pmol methylumbellife protein per minute whether or not an intron was Hence both the Northern and GUS activity data that the chimeric intron is functional in different pl cies and does not dramatically influence expression

Expression of the chimaeric construct in Agrobacterium tumefaciens

To test whether or not all prokaryotic GUS enzym tivity is abolished by introduction of the portabl PIV2 into the GUS gene, expression in *Agrobacter* analysed. Overnight cultures of bacterial strains co

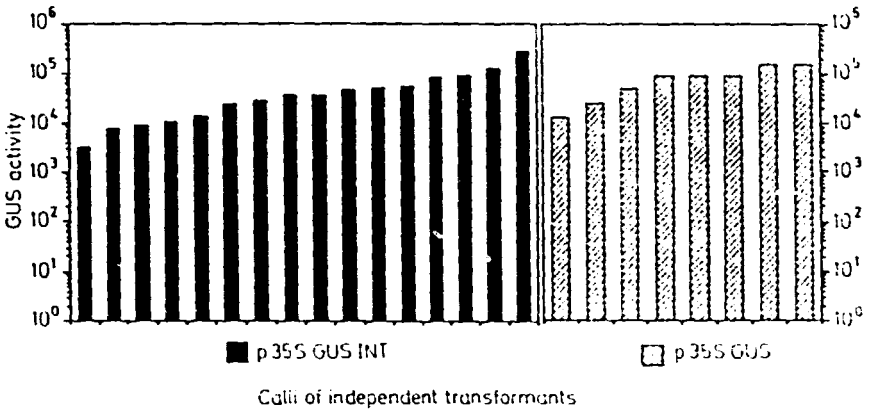

Fig. 4. Influence of the intron on GUS activity in stable transformants. Calli of independent transformants of *Arabidopsis thaliana* containing the GUS gene with (p35S GUS) or without (p35S GUS INT) the intron were analysed quantitatively for GUS activity which was expressed as picomoles methylumbelliferol per milligram protein per minute. The histogram indicates the activity on a logarithmic scale

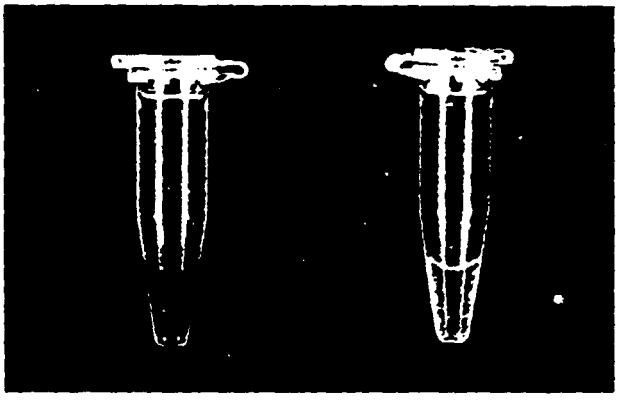

Fig. 5. In vivo staining of *Agrobacterium*. GUS activity displayed by *Agrobacterium* containing either the intron-containing (p35S GUS INT) (right) or the intron-free (p35S GUS) (left) chimaeric GUS gene. Late log cultures of *Agrobacterium* strains were incubated for 24 h with X-gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexyl ammonium)

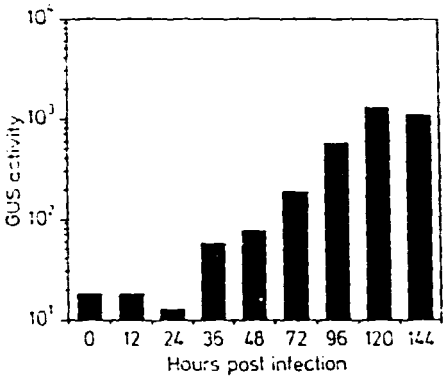

Fig. 6. Kinetics of total GUS activity after infection with an Agrobacterium strain containing the GUS-INT chimaeric gene. Cotyledon infections of *Arabidopsis* were performed with *Agrobacterium* (p35S GUS INT), omitting the G418 selection. After defined time intervals (hours post infection) ten explants were frozen at −70° C, and used for a quantitative fluorescence assay of cotyledon extracts. The GUS activity expressed as picomoles methylumbelliferol per milligram protein per minute, is indicated on a logarithmic scale

35S GUS or 35S GUS INT were incubated for 24 h with X-gluc a substrate for GUS. Agrobacteria containing the intronless construct showed significant GUS activity, whereas no activity was detected in agrobacteria containing the p35S GUS INT construct (Fig. 5). Thus the intron PIV2 allows efficient discrimination between agrobacterial and plant gene expression.

Detection of early transformation events after Agrobacterium *infection*

The results described above fulfil all requirements for the further use of the intron-containing construct for monitoring early events after *Agrobacterium*-mediated plant transformation. As a first experiment in this direction cotyledon explants of *A. thaliana* were incubated with the appropriate *Agrobacterium* strain and treated under the conditions described (Schmidt and Willmitzer 1988). After defined time intervals samples were frozen at −70° C and used for quantification of GUS activity. Figure 6 shows the kinetics of GUS activity obtained by [illegible] fluorescence measurements of cotyledon extracts. [illegible] GUS enzymatic activity can be observed as early as [illegible] after *Agrobacterium* infection. During the next few days an exponential increase in GUS activity is detectable. The initial increase most probably reflects the relative growth of the transformed tissue compared with that of the rest of the leaf explant. Similar kinetics were obtained when G418 selection was applied during the same time period (data not shown).

In simultaneous transformation experiments cotyledons and roots were stained by X-gluc at different stages after *Agrobacterium* (p35S GUS INT) infection. The high sensitivity of this staining procedure allows the detection of single cell transformation events. Blue staining was observed in proliferating calli indicating GUS activity especially in cells at the cut surface and in the neighbourhood of vascular tissue (Fig. 7A, B, C, E). Moreover in most of cases staining was observed in subepidermal cell layers (Fig. 7G, I), although epidermal events could also be observed (Fig. 7H, I). Most probably a high proportion of these cells would not proliferate in contrast to the callus tissue developing on the cut surface. Therefore it is unlikely that such transformation events would survive any continuous selection at the single cell stage. Hence these data support the idea that only rapidly dividing and/or regenerating tissue is selected for during transformation experiments.

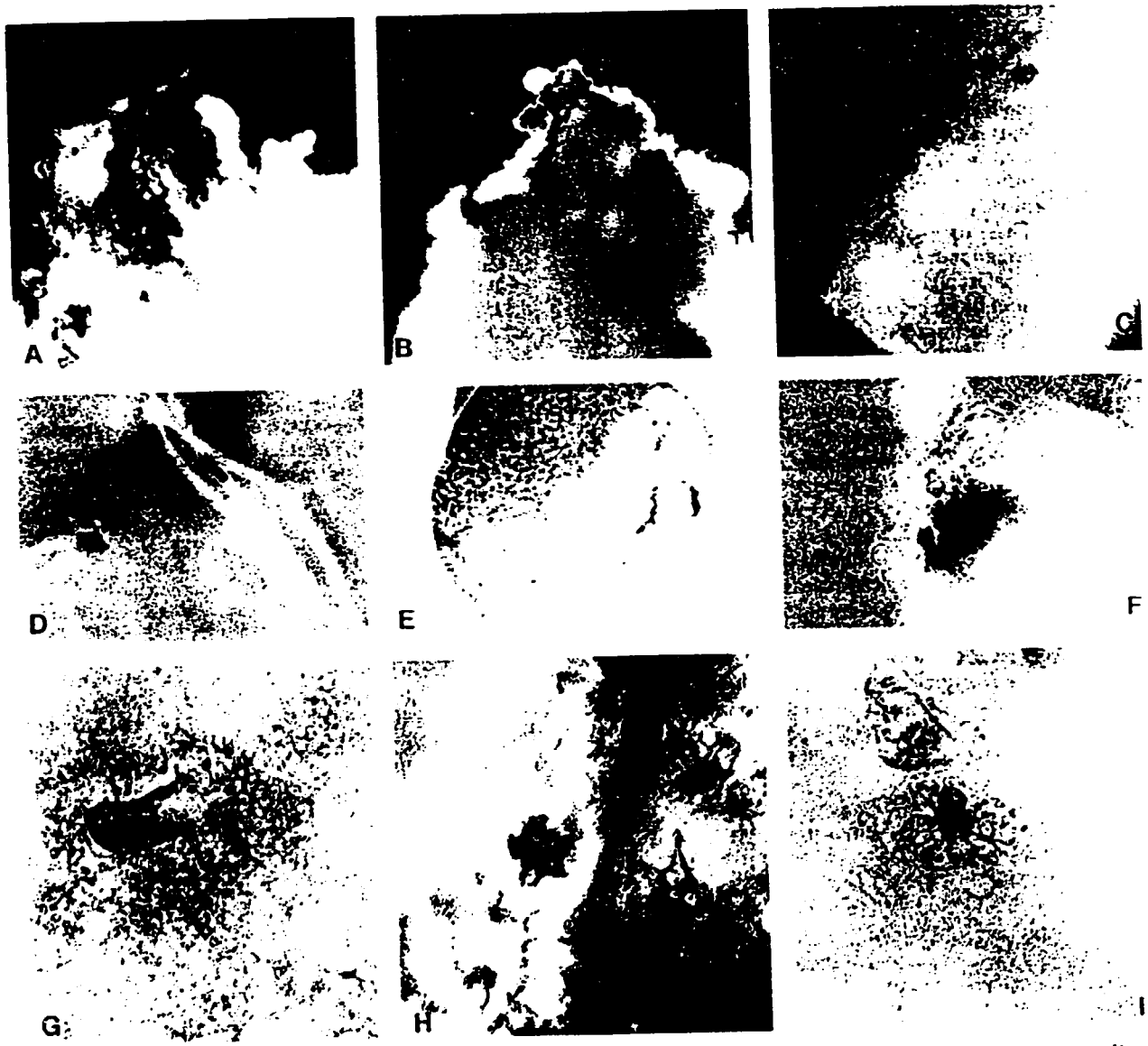

Fig. 7A–I. In vivo staining of *Agrobacterium*-infected cotyledon and root explants. **D–E** An overview of *Arabidopsis* roots (**D**) and cotyledons (**E**) 40 h post *Agrobacterium* (p35S GUS INT) infection (p.i.). Cut edges. **A–C** of cotyledons (**A**, **C**) 6 days p.i., (**B**) 15 days p.i., (**F**) of a root 40 h p.i. **G–I** Close ups of single transformation events: a subepidermal cell (**G**) 48 h p.i., an epidermal cell (**H**) 6 days p.i., around a stomatal opening (**I**) 40 h p.i. Magnification: **A** 20×, **B**, **C**, 15×, **D**, 2×, **E**, 10×, **F**, 20×, **G**, 400×, **H**, 200×, **I**, 300×

Discussion

In order to prevent expression of an indicator gene in prokaryotic organisms, such as *A. tumefaciens*, and to allow its expression in plants, we interrupted an indicator gene by a plant intron. A portable intron (PIV2) derived from the second intron of the ST-LS1 gene was constructed by modification of the splice junctions into appropriate restriction sites and introduced into the bacterial gene coding for GUS. In transgenic tobacco plants containing this chimaeric GUS-intron gene the portable intron is spliced out efficiently, thus resulting in a functional mRNA. Mapping of the splice junction suggested accurate removal of the intron. This indicates that the intron sequence itself comprises all information necessary for recognition by the splicing machinery and hence the intron is independent of the original neighbouring exon sequences. Using the described in vivo approach the elucidation of processing of pre-mRNAs in plants might be facilitated.

Moreover the inserted intron does not influence the production of GUS mRNA or the encoded enzymatic activity quantitatively, whereas the expression of the introduced GUS gene in *A. tumefaciens* is efficiently prevented by the interruption of the open reading frame by the portable intron. The chimaeric GUS-intron gene thus allows experiments aimed at the analysis of early events during *Agrobacterium*-mediated transformation.

As early as 36 h after *Agrobacterium* infection of *Arabidopsis* cotyledons significant GUS activity could be observed. This is in agreement with other experiments using an endogeneous *Agrobacterium* indicator gene coding for nopaline synthase after leaf disc infection of *Petunia hybrida*

(Horsch et al. 1986). However in the latter case endogenous *Agrobacterium* expression could not be excluded.

In contrast to nopaline synthetase, GUS furthermore allows the detection of single transformed cells by histochemical methods. After transformation single cells expressing GUS activity were observed throughout the cotyledon explants. In contrast to the tissues at the cut surface, these cells would probably not proliferate during the next few days and hence would not be likely to survive any kind of selection. These data indicate that by early selection the actual transformation frequencies after *Agrobacterium*-mediated gene transfer might be underestimated. However the possibility cannot be excluded that the observed GUS activity might be due to transient expression of the introduced GUS gene. Alternative regeneration protocols and a prolonged lag phase between *Agrobacterium* infection and the application of selection could result in the recovery of these minor transformation events, leading to an improved transformation efficiency. This approach should be helpful in analysing critical steps in transformation procedures and this in turn could result in new strategies to introduce novel features in recalcitrant plants.

The use of transgenic plants for the promoter analysis of genes which are specifically expressed at particular stages in plant development or induced by particular environmental conditions is time consuming due to the length of regeneration procedures. The use of the portable intron, allowing the discrimination of prokaryotic versus eukaryotic gene expression in a transient assay, could facilitate new experimental strategies for the analysis of plant genes using *Agrobacterium*-mediated transient expression in specific plant tissues.

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Received August 10, 1989

HELIUM GUN

Preparation of tungsten

1. Weigh out 100 mg tungsten in Eppendorf tube

2. Wash in 100% ethanol
   - Sonicate and spin
   - Repeat 3x
   - Remove supernatant

3. Wash in sterile distilled water
   - Mix and spin
   - Repeat 3x
   - Remove supernatant

4. Add 50% glycerol (filter sterilize) to give tungsten at 100 mg/ml

Preparation of tungsten particles.with DNA

Mix keeping everything cold:

- Sonicate Particles: 25µl tungsten particles in 50% glycerol (100mg/ml), well sonicated
- Add DNA: 2-6µl plasmid DNA (µg/µl)
- Add $CaCl_2$: 25µl$CaCl_2$(2.5M)
- Add SFB: 5µl spermindine free base (0.1M)

Mix for 4', prefereably at 4° (but room temperature is OK)
Vortex at 5 minute intervals for 20 minutes
Allow to settle
Remove 25-29µl supernatant
Keep on ice until shooting time
Briefly resuspend pellet with sonicator (setting 6) before use
Vortex each time before loading of gun

Getting ready to shoot:

Turn on vacuum pump, warm it up for about 10 minutes
Open big black tap on helium bottle
Set pressure to 690 Kpa(=100psi) with blue tap

Shooting:

Load 3-4μl of tungsten particles onto mesh of mesh holder unit
Twist unit gently into posistion in gun
Place tissue in petri dish on shelf 3 (~10cm from outlet)
this will shoot an area 2.5cm in diameter
Place mesh over petri dish
Open grey tap to vacuum pump
Close valve to gun chamber (turn lever to right)
Wait until vacuum is -25 inches Hg
Release safety catch by pressing blue button
Press red button
Gently release vacuum by turning lever to left
Close tap to pump
Remove mesh and cover with petri lid, seal with nescofilm
I get best results (when shooting leaves, petals and cotyledons) if the humidity in the dishes is kept high by putting damp filter paper in the lids of the tissue.
Incubate at 24° C for 24 hours
Stain with X-gluc overnight (some expression in pea tissue visible within one hour)

Closing up:

Shut off helium at big black tap
Release pressure at helium meter by shooting repeatedly until it goes from 690 to zero Kpa,
Leave grey tap to vacuum pump open
Leave lever to gun chamber open
Turn off vacuum pump
Wipe chamber with ethanol

(Notes Supplied by Richard Brettel CSIRO Division of Plant Industry)

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Xn

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S: 26-28-37

CAS [9002-93-1]

d. 1.06

n_D^{20} 1.4902

Code N°	Pack	
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Code N°	Pack	
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Trityl chloride

(see Chlorotriphenylmethane)

Tropaeolin 00

(see Orange IV)

Trypan blue R.A.L.

(1 23 650)

- Bleu trypan
- Trypanblau
- Azul trypan
- Blu tripano

$C_{34}H_{32}N_6Na_4O_{16}S_4$ CAS [72-57-1]
M.W. : 960.82

Code N°	Pack	
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Trypsin

- Trypsine
- Trypsin
- Tripsina
- Tripsina

CAS [9002-07-7]

Code N°	Pack	
122	10 g	GB

(L)-Tryptophan

- L(-)-Tryptophane
- L(-)-Tryptophan
- L(-)-Triptofano
- L(-)-Triptofano

$C_{11}H_{12}N_2O_2$ CAS [73-22-3]
M.W. : 204.23

M.P. 280 to 285 °C decomp

Code N°	Pack	
237	25 g	PB

DL-Tryptophan

- DL-Tryptophane
- DL-Tryptophan
- DL-Triptofano
- DL-Triptofano

$C_{11}H_{12}N_2O_2$ CAS [54-12-6]
M.W. : 204.23

M.P. 259 to 290 °C

Code N°	Pack	
28 824.131	25 g	PB

Tungsten 99,9 % wire

(ø 0.5 mm, length about 1,40 m)

- Tungstène
- Wolfram
- Tungsteno
- Tungsteno

W CAS [7440-33-7]
A.W. : 183.85

B.P. 5930 °C M.P. 3410 °C

Code N°	Pack	
28 826.104	ab. 5 g	BP

Tungsten 99,9 % powder

- Tungstène
- Wolfram
- Tungsteno
- Tungsteno

W CAS [7440-33-7]
A.W. : 183.85

B.P. 5900 °C M.P. 3410 °C

Code N°	Pack	
28 827.233	250 g	PB

TUNGSTEN (REAGENTS FOR THE ANALYSIS OF)

- Cinchonine for analysis
- Rhodamine B R.A.L.
- Tannic acid diethyl ether extracted

Tungsten trioxide

(see Tungsten (VI) oxide)

Tungsten (VI) oxide RECTAPUR™

(tungstic oxide)

- Tungstène (VI) oxyde
- Wolfram (VI)-oxid
- Tungsteno (VI) oxido
- Anidride tungstica

O_3W CAS [1314-35-8]
M.W. : 231.85

M.P. 1473 °C

WO_3 % ..... 99 min

Maximum % impurities

Loss by ignition at 800 °C	0.5
SiO_2	0.0200
Residue at thionyl chloride (as O)	0.0500

Code N°	Pack	
20 739.188	100 g	PB
20 739.292	1 kg	PB

Millipore Ordering Information:

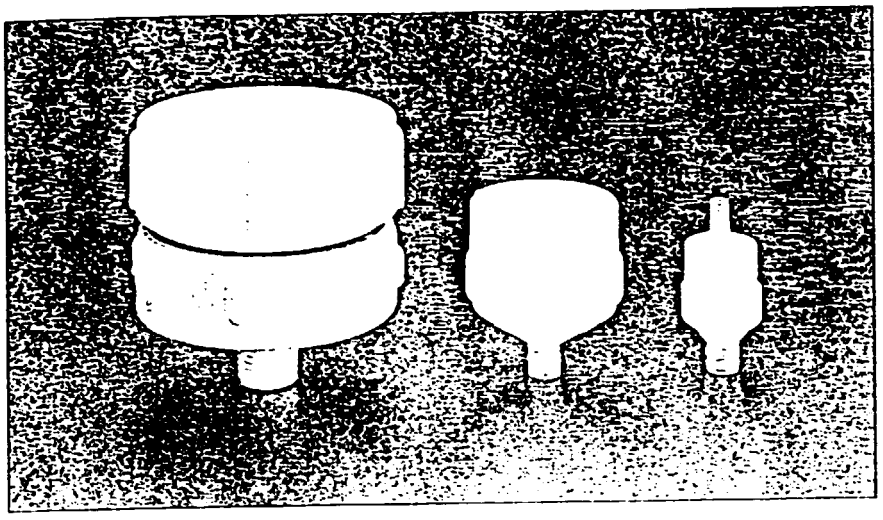
Description	Catalogue No:	Cost (AUS) $
Stainless Filter Support Screen	XX30 012 10	7.50
Swinnex 13mm (10/packet)	SX00 013 00	63.00

Rhône Poulenc

One for the most commonly used carriers for DNA in particle bombardment is tungsten. This can be purchased from Rhône Poulenc (Code No: 28 827.233, current price is (XXXXXXXXXXXXX per 250g.) It is critical that the particle size be between 1-3µm. This can be checked using electron microscopy.

Tungsten particles are prepared for particle bombardment as described overleaf.

Swinnex Disc Filter Holders



Function

Specifications

Materials

Filter Sizes

Filter Areas

Prefilter Sizes

Pressures

Connections

Dimensions

Typical Initial Flow Rates

Filter Type	Pore Size (µm)	ml/min SX13	ml/min SX25	ml/min SX47
Water				
Methanol				

Ordering Information

Description	Catalogue No.
Replacement Parts	
Accessories	

Function

...cleaning or sterilizing small volumes of ...ds as they are dispensed by ...podermic syringe. Autoclavable with filter in place. Wrench set supplied for tightening ...ilter.

Applications

This filter holder can be used for filtration of HPLC samples prior to injection. It is recommended to use the following membranes:

- Durapore hydrophilic 0.45 µm membrane with 13 mm diameter (HVLP 013 00) for filtration of aqueous or alcoholic solutions
- Fluoropore 0.5 µm membrane with 13 mm diameter (FHLP 013 00) for filtration of organic solutions

For filtration of samples heavily contaminated by particles, it is recommended to add a glass fiber prefilter (AP40 010 00) upstream of the membrane.

Specifications

Materials
Stainless steel, Teflon gasket and O-ring.

Filter Size
13 mm diameter.

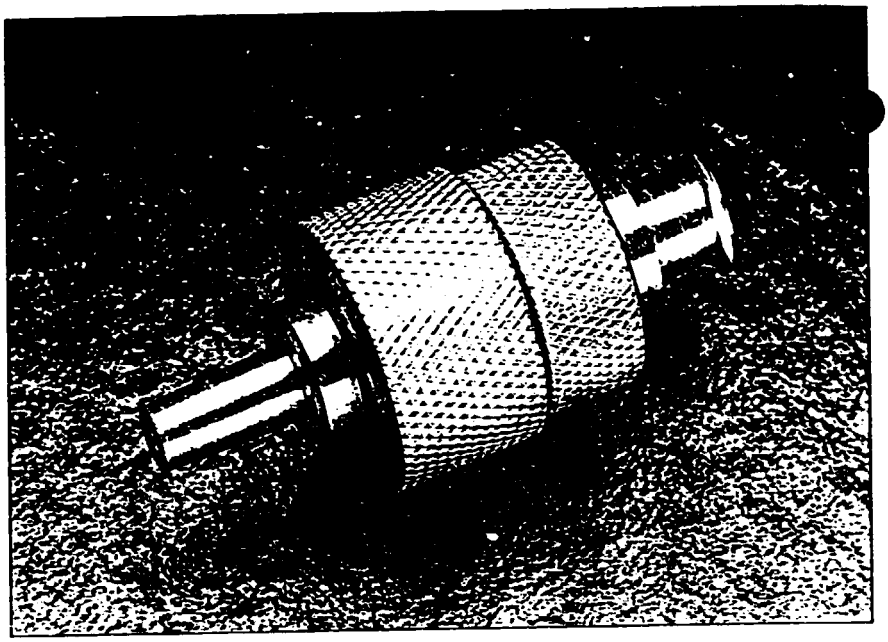
Filter Area
Approximately 0.8 cm².

Prefilter Size
Type AP depth prefilter, 10 mm diameter.

Pressures
7 bars (100 psi) inlet and differential.

Connections
Female Luer-Lok inlet, male luer slip outlet. With syringes of less than 10 ml capacity, locking inlet connection must be used to avoid leakage of high pressures generated.

Dimensions
33 mm (15/16") long, 16 mm (5/8") diameter.



Typical Initial Flow Rates

At 0.7 bar (10 psi) differential pressure and 20°C (68°F):

Filter Type	Pore Size (µm)	ml/min
Water		
GS/GV	0.22	6
HA/HV	0.45	13
RA	1.2	125
SM	5.0	320
Methanol		
FG	0.2	12
FH	0.5	32
FA	1.0	72

Significantly higher flow rates are produced by the greater pressures ordinarily developed with a syringe.

Ordering Information

Description		Catalogue No.
Swinny Stainless, 13 mm		XX30 012 00
Replacement Parts		
O-ring, Teflon (2-012)	5/pk.	XX30 012 01
Support screen gasket, Teflon	10/pk.	XX30 012 02
Stainless filter support screen		XX30 012 10
Support screen, O-ring & gasket	4 sets.	XX30 012 03
Wrench set		XX30 012 04
Accessories		
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Particle gun mediated transformation

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This review describes advances that have taken place in the area of gene transfer into plants by microprojectile bombardment over the last twelve months. Emphasis is placed on studies focusing on the mechanistic aspects of the particle bombardment process, the development of new instrumentation, and new crop varieties engineered using this technology. It is apparent that particle bombardment technology has become the method of choice for engineering crops of agronomic importance.

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Introduction

Following the initial description of the particle bombardment process for the introduction of foreign genes into plant cells five years ago [1], impressive breakthroughs leading to the engineering of almost all major crop species utilizing this technology have been reported [2•,3•]. Prior to its development, the application of recombinant DNA methodology to plant improvement was restricted to gene transfer methods dependent on cell-culture systems utilizing *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*, electroporation, or polyethylene glycol mediated transformation. The major advantage of particle bombardment is its ability to deliver biologically active DNA into intact cells and organized tissues. As a consequence, it is now possible to develop variety-independent gene transfer procedures that will undoubtedly accelerate the introduction of genetically improved plant strains to the market.

We are clearly approaching the point at which the introduction of foreign genes into plants will be routine. Problems remain of course, but gene transfer is rapidly becoming a tool at the disposal of all molecular biologists and breeders. The object of this review is to discuss experiments directed towards the elucidation of mechanistic and practical aspects of the technique, as well as to provide an update on additional plant species that have been engineered utilizing particle bombardment over the last twelve months. In the last section, advances in hardware design, new acceleration mechanisms and patents issued to control this technology will be discussed.

Mechanistic aspects

The principles and applications of particle bombardment have been described in detail [2•,4,5]. In most cases, DNA-coated tungsten or gold particles are accelerated into plant cells. A number of these cells can express the introduced gene(s) transiently, and some are capable of stably integrating the foreign DNA into the genome. In cases where intact plants can be regenerated from transformed cells, the plants exhibit an altered phenotype encoded by the novel gene(s). This phenotype will be passed on to the progeny of the plant in a predictable Mendelian fashion resulting in a new genotype. An assortment of accelerating mechanisms have been utilized. These include, amongst others, the original gunpowder device [1], electric discharge-based devices [6,7], pneumatic instruments [8], and a microtargetting apparatus [9].

A number of reports describing basic biological and physical aspects of particle bombardment have been published recently. Some of these reports provide the basis for a better understanding of the underlying mechanisms of the process and will be reviewed here.

When plasmid DNA encoding β-glucuronidase (GUS) was introduced into cultured cells, it was observed that more than 90% of GUS-expressing cells contained a bead in their nucleus. This was observed in the case of cultured tobacco cells [10•], as well as in soybean, cotton (DE McCabe and BJ Martinell, personal communication) and rice (P Christou, unpublished data). Iida *et al.* [11] described experiments in which the *gus* gene was delivered to synchronized cultured tobacco cells at different stages of the cell cycle. Cells bombarded at the M and G2 phases were 4–6 times more efficiently transformed, as measured by transient assays, than those bombarded at the S and G1 phases. An extension of these studies to stable transformation would have been very useful in order to assess whether this observation could smooth the way for increased overall stable transformation frequencies.

Russell *et al.* [12] used suspension cultures of tobacco as a model to study parameters affecting the transfor-

Abbreviations

GUS—β-glucuronidase; PAT—phosphinothricin acetyltransferase; PCR—polymerase chain reaction; PRV—papaya ringspot virus.

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mation of cells by particle bombardment. The *gus* and *nptII* genes were used to study transient expression and stable transformation. Important parameters included the promoter used to drive gene expression and osmotic preconditioning of cells before and after bombardment treatments. Results obtained in this study, coupled with observations by Ye *et al.* [13], who reported that treatment of cells with solutions under high osmotic pressure increases gene expression efficiency in higher plant protoplasts, will lead to a better understanding of parameters that may increase the conversion of transient to stable transformation events. Russell *et al.* [14] also compared results obtained using the original gunpowder device with an improved instrument utilizing compressed helium as the accelerating force. They determined that the lower transformation frequencies resulting from experiments in which the gunpowder device was used, were due to injury to the cells, caused primarily by physical trauma from the gas blast and acoustic shock generated by the device. They determined that the incorporation of baffles or a mesh screen in the case of the gunpowder device significantly increased the recovery of transformed kanamycin-resistant colonies. They also demonstrated that substituting tungsten with gold resulted in significantly higher transformation frequencies. This is consistent with our own choice of gold as the accelerating particle. Gold is chemically inert and is therefore not toxic to the plant cells; however, tungsten, being a third row transition element, can assume a number of different oxidation states resulting in the formation of various tungsten oxide moieties. These compounds will undoubtedly be toxic to plant cells because of the inhibition of essential enzyme functions or complexation with macromolecules and other cell components.

Novel uses of particle bombardment

Bidney *et al.* [15] demonstrated that bombardment of plant tissue with metal particles is an effective method of wounding to promote *Agrobacterium*-mediated transformation. When tobacco leaves were used in transformation experiments involving *Agrobacterium*, in which wounding was effected by particle bombardment, at least 100-fold more kanamycin-resistant colonies were obtained compared with the standard particle gun-transformation protocol. Similarly, in experiments in which sunflower apical meristems were infected with *Agrobacterium* following wounding by bombardment with gold particles, GUS expression patterns indicative of meristem transformation were observed in the regenerated shoots.

Following the report by Svab *et al.* [16] describing chloroplast transformation in tobacco utilizing particle bombardment, Staub and Maliga [17] found that long regions of homologous DNA were incorporated into the plastid genome. They obtained transplastomic lines after introducing a suitable plasmid into tobacco leaf cells via particle bombardment following selection for the spectinomycin-resistance marker. These studies resulted in the generation of a plasmid containing engineered cloning sites between two selectable markers, suitable for use as a plastid insertion vector.

Kanevski *et al.* [18] bombarded tobacco cells in suspension culture with one of the two tomato golden mosaic virus genome components encoding viral proteins required for the replication and encapsidation of viral DNA. Replicating, unit-length viral DNA of up to 1000 copies per cell was found in approximately 10% of the kanamycin-resistant clones selected following bombardment. The authors suggest that geminiviruses may serve as useful multicopy vectors in plant cells; however, subsequent studies to confirm this premise have not yet been reported.

New plant species engineered by particle bombardment

Following early reports of the transformation of soybean [7,19], tobacco [20], maize [21,22] and papaya [23] by particle bombardment, reports describing the recovery of additional transgenic plants (dicotyledons, monocotyledons, and tree species including gymnosperms) have been published during the last twelve months. This section will provide a brief outline of the methodology and parameters that made the recovery of transgenic plants from these additional species possible. Only species for which transgenic plants were fully characterized by molecular analyses and showed Mendelian segregation in subsequent generations (where appropriate) are included here.

Dicotyledonous species

Soybean and other legumes

The first report describing the recovery of an intact transgenic plant using particle bombardment was published in 1988, and used soybean (*Glycine max*) as an example [19]. Two years later, the same group reported significant improvements in the process resulting in a procedure that is now being used commercially [7]. Later, Finer and McMullen [24] reported the recovery of transgenic soybean plants from an embryogenic suspension culture following particle bombardment. They took advantage of a rapidly proliferating suspension culture to generate a number of transgenic soybean plants. Unfortunately, such embryogenic cultures could only be derived from a single soybean variety, limiting the applicability of this potential alternative soybean transformation system. In addition, problems associated with somaclonal variation and other culture-induced mutations did not permit the wider utilization of this system for soybean engineering.

The method originally developed for soybean engineering was found to be applicable to the transfor-

mation of *Phaseolus* [25] and peanut [26]. It appears, therefore, that particle bombardment into intact meristems is the method of choice for introducing genes into most leguminous crops, especially those that can be regenerated from a *de novo* organogenic pathway.

Cotton

Transgenic cotton (*Gossypium hirsutum* L.) plants were recovered following bombardment of embryogenic suspension cultures using a modified bullet gun [27]. Such cultures, however, could only be generated from very few varieties and only transgenic cotton plants from the Cocker 310 variety have been recovered. As with other systems involving regeneration from embryogenic suspension cultures, somaclonal variation and sterility in regenerated plants severely limited the usefulness of suspension culture cells as target tissue. McCabe and Martinell [28], utilizing a modification of the soybean transformation procedure, were able to recover transgenic cotton plants from all major elite varieties, including Pima S6, Delta Pine and Sea Island lines. Whereas in the soybean case transgenic plants are recovered through an organogenic process, in cotton almost all transgenic plants recovered have been the result of direct germination of the bombarded explant followed by selective pruning of non-transformed sectors on the plants to allow development of axils that had been clonally transformed.

Cranberry

Genetic transformation of the American cranberry (*Vaccinium macrocarpon* Ait.) was accomplished by electric discharge particle bombardment [29]. Stem sections derived from *in vitro* cultures were bombarded with *gus*, *nptII* and *bt* genes. Following bombardment, stem sections were cultured on solid bud-inducing medium containing kanamycin. A thin overlay of water containing kanamycin was added to inhibit the growth of non-transformed cells. Within seven weeks, green shoots emerged, most of which were demonstrated to contain all three genes, as shown by polymerase chain reaction (PCR) and Southern blot analysis.

Arabidopsis

Stable transformation of *Arabidopsis thaliana* with plasmid DNA containing the *nptII* gene was achieved using a pneumatic particle gun driven by compressed air [30]. Transgenic plants were regenerated from root sections bombarded with DNA-coated gold particles and the kanamycin-resistant phenotype was passed to the next generation, indicating stable integration of the introduced trait.

Monocotyledonous species

Rice

A simple and effective procedure, using electric discharge particle acceleration to introduce foreign genes into scutellar tissue of rice with subsequent recovery of transgenic plants from both *indica* and *japonica* varieties, has been described [31]. Subsequently, the same group reported refinements in the procedure that led to the development of a variety-independent gene-transfer method for rice, capable of introducing any gene into any variety at very high frequency [32•]. This system allows direct DNA transfer into organized tissues of additional cereals and represents the best method, at present, for achieving truly genotype-independent transformation of cereals. The advantages of such an approach have been discussed [33] and provide a theoretical framework for extending this technology to additional cereals.

Wheat

Bombardment of regenerable embryogenic callus of wheat resulted in the recovery of fertile transgenic plants from two cultivars [34•]. A plasmid encoding phosphinothricin and GUS was delivered into type C long-term regenerable embryogenic callus. Phosphinothricin acetyltransferase (PAT) activity, encoded by the *bar* gene, was demonstrated in four independently transformed callus lines selected on BASTA®. Although somatic embryos and shoots were formed in each of the four clones, plants were recovered from only two of the lines. More than 100 green R0 plants were regenerated from the first callus line. PAT activity was shown in each of 28 R0 plants tested. Transgenic R0, R1 and R2 plants were resistant to topical applications of the herbicide and the *bar* gene segregated as a dominant Mendelian trait in the progeny. Despite this success, however, routine gene transfer into wheat still remains elusive, as only wheat cultivars from which embryogenic callus lines can be derived are potential targets. Transgenic R0 plants had to be pollinated with wild-type pollen in order to recover transgenic progeny. This requirement vividly illustrates the advantages of methods capable of by-passing complex and long-term tissue culture dependent procedures. Recovery of transgenic wheat, however, is a major accomplishment and there is little doubt that optimization of the procedure will result in improved efficiency and increased fertility.

As a result of the low efficiency of DNA delivery and the reduced regeneration capability of microprojectile-bombarded wheat tissue, Perl *et al.* [35] developed a regeneration system that appears to be more amenable to bombardment. They determined that scutellar calli of wheat could be regenerated at higher efficiencies in a liquid culture phase. In addition, they determined that expression of a reporter gene following DNA de-

livery could be improved by maintaining the scutellar calli in 0.25 M mannitol before and after bombardment. Replacing calcium chloride with calcium nitrate and eliminating spermidine from the DNA/microprojectile mixture resulted in a several fold increase in transient expression of the reporter gene, as compared to previously published procedures. It is possible that by incorporating these modifications, the recovery of transgenic wheat will become easier.

Sugarcane

Bower and Birch [36•] reported recovery of intact transgenic sugarcane (*Saccharum* spp.) plants. Optimal bombardment conditions for embryogenic callus required microprojectile velocities higher than those that were effective previously in sugarcane suspension culture cells [37]. Stable transformants were obtained following bombardment with the *nptII* gene under the control of the *Emu* promoter. NPTII levels in transformed plants were 20–50 times the background levels in control plants as shown by enzyme-linked immunosorbent assays. DNA analysis showed that transgenic plants had 1–3 copies of the introduced gene integrated into the sugarcane genome. The procedure afforded 1–3 transgenic plants per treated plate within 16 weeks of bombardment.

Dendrobium orchid

Transformed *Dendrobium* orchids (*Dendrobium* x Jaquelyn Thomas hybrids) were recovered from protocorms bombarded with particles coated with plasmid DNA encoding kanamycin resistance and papaya ringspot virus (PRV) coat protein genes [38]. Following selection on kanamycin-enriched regeneration medium, putative transformants were subjected to DNA analyses utilizing PCR and Southern blots. This is the first report of the application of particle bombardment technology to the engineering of orchids.

Tree species

Incorporation of foreign DNA into trees and the subsequent regeneration of transformed plants has been reported for a very limited number of genera primarily utilizing the *Agrobacterium* vector system. As with other crops, however, complex host–plant interactions and other factors have significantly limited the scope of *Agrobacterium*-based vectors in the engineering of woody species.

Poplar

Particle bombardment technology was utilized to extend the range of transgenic *Populus* species that could not be regenerated from protoplasts. McCown *et al.* [39] utilized three different target tissues, including protoplast-derived callus, nodules, and stems, to generate transgenic plants through electric discharge particle bombardment. Pretreatment of explants, fine-tuning of bombardment parameters, and the use of a selection technique employing flooding of the target tissue were found to be important for reliable recovery of transformed plants.

Single cells and small cell clusters isolated from embryogenic suspension cultures of yellow poplar (*Liriodendron tulipifera* L.) were bombarded with genes encoding GUS and NPTII [40]. Between 3–30 copies of the intact *gus* gene were detected in independently transformed callus lines. Somatic embryos induced from transformed cell cultures were found to be uniformly positive for GUS expression. Transgenic plants expressed both genes in the roots and leaves.

White spruce

Gymnosperms have been particularly difficult to engineer. The only report on the recovery of transgenic plants from conifers describes the recovery of transgenic white spruce (*Picea glauca*) following electric discharge particle acceleration of plasmid DNA into regenerable embryogenic callus [41•]. Successful transformation was dependent on three factors: the ability to form embryogenic callus, the ability of the putatively transgenic callus to survive selection, and the competence of transformed tissue to express the foreign DNA.

Papaya

Following the original report describing recovery of transgenic papaya plants via particle bombardment of embryogenic tissue [23], Fitch *et al.* [42] characterized papaya plants engineered with the coat protein gene of PRV. By utilizing 2,4D-treated immature zygotic embryos as targets, and following regeneration of transgenic plants on kanamycin-containing media, they obtained plants that exhibited varying degrees of resistance to PRV. One line appeared to be completely resistant when challenged with the severe Hawaiian strain, PRV HA. Tests to recover PRV from the inoculated resistant plants by means of transferring leaf extracts to a local lesion host were negative, indicating complete resistance, presumably as a result of inhibition of PRV replication. A number of virus-resistant lines are currently being tested in the field.

Advances in hardware design and new acceleration mechanisms

The most important advance in hardware design is the development of second generation instruments in which the accelerating force was changed from the original gunpowder discharge to compressed helium [43••]. The new system results in less damage to the target cells and provides more flexibility and higher

efficiencies. This is a direct result of higher velocities with less tissue damage, and also a more uniform particle distribution. Hand-held devices using helium as well as the electric discharge mechanisms have also been described (see patent section below). Hand-held devices now extend the application of particle bombardment to intact organisms, including whole plants and live animals.

Takeuchi *et al.* [44] and Finer *et al.* [45] described a simple and inexpensive device based on the acceleration of particles directly in a helium stream under vacuum. Bombardments utilizing this device resulted in high levels of transient activity in embryogenic suspensions of corn and soybean and in the leaf tissue of cowpea. More recently the device was used to obtain stable transformants following bombardment of an embryogenic soybean cell culture. Additional data are required to evaluate whether this device will transfer enough momentum to the metal particles to achieve penetration into germline cells or their progenitors.

A modified version of the electric discharge gun was constructed by Rech *et al.* [46] and was used to demonstrate transient activity in zygotic embryos of soybean and *Phaseolus*. However, as no data on stable transformation were reported, it is difficult to compare the performance of this instrument with others based on alternative acceleration mechanisms.

Patents

A number of patents controlling particle bombardment technology have been issued in the United States, Europe and elsewhere. Of interest is a US patent entitled "Method for transporting substances into living cells and tissues and apparatus therefor" by Sanford *et al.* at Cornell [P1••], which controls the concept of particle bombardment and also describes various acceleration mechanisms in detail, including the original gunpowder device. US patent number 5,066,587, entitled "Gas driven microprojectile accelerator and method of use" assigned to the Upjohn Company, Kalamazoo, Michigan [P2] is also of interest, as it may control part of the technology not covered in other patents. The patents assigned to Agracetus Inc. [P3•,P4] describe the electric discharge acceleration device and a hand-held version of the instrument, respectively. US patent number 5,015,580, also assigned to Agracetus Inc., describes the particle-mediated transformation of soybean plants and cell lines [P5]. The patent assigned to North Carolina State University [P6] describes ballistic transformation of conifers. The patent application by Dekalb Plant Genetics claims transgenic maize plants recovered through particle bombardment technology [P7].

A large number of patent applications have been published in Europe; primarily, these cover various acceleration mechanisms and instruments, and in some cases specific cell-culture systems compatible with particle bombardment.

Conclusion

Dramatic improvements in gene transfer technology have taken place over the past twelve months. Particle gun technology is primarily responsible for bringing on-line plant species that were previously recalcitrant to conventional gene transfer methods. Most of the major agronomic crops can be engineered successfully using particle bombardment, in some cases in a variety-independent fashion. Of course, particle bombardment is not a panacea. Problems still remain, but with new instruments and alternative acceleration mechanisms becoming widely available, we are beginning to see studies directed towards fundamental aspects and mechanisms underlying gene transfer using this technology. Such experiments will undoubtedly lead to improved transformation protocols and a better understanding of critical parameters that affect the stable integration of foreign DNA into plant cells.

New methods of gene transfer into plants are continuing to be developed and it is unlikely that one method alone will dominate. It is probable that by the time gene transfer becomes routine for all plants, particular methods of gene transfer will be shown to be optimal for specific species. Studies focusing on conventional, as well as novel, gene transfer methodologies should not be abandoned. Such studies can provide valuable information for achieving routine and efficient gene transfer into plants by elucidating fundamental principles governing transfer, integration and expression of exogenous DNA in cells and tissues, irrespective of the gene transfer method used.

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- •• of outstanding interest

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Patents

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P1. •• CORNELL RESEARCH FOUNDATION: **Method for Transporting Substances into Living Cells and Tissues and Apparatus Therefor.** 7/30/92 US 5,036,006.

Ground floor patent controlling the concept of introducing DNA into cells utilizing microparticles. The patent also covers instrumentation based on the biolistics concept.

P2. THE UPJOHN CO: **Gas Driven Microprojectile Accelerator and Method of Use.** 11/19/91 US 5,066,587.

P3. • AGRACETUS INC: **Apparatus for Genetic Transformation.** 6/9/92 US 5,120,657.

This patent covers an acceleration mechanism based on electric discharge which has distinct advantages over alternative devices. Experiments utilizing the instrument have resulted in genetic transformation of plants from organized tissue in most cases in a variety-independent fashion.

P4. AGRACETUS INC: **Apparatus for Genetic Transformation.** 9/22/92 US 5,149,655.

P5. AGRACETUS INC: **Particle-Mediated Transformation of Soybean Plants and Lines.** 5/14/92 US 5,015,580.

P6. NORTH CAROLINA STATE UNIVERSITY (RALEIGH): **Ballistic Transformation of Conifers.** 6/16/92 US 5,122,466.

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RESEARCH

PRODUCTION OF TRANSGENIC RICE (*ORYZA SATIVA* L.) PLANTS FROM AGRONOMICALLY IMPORTANT INDICA AND JAPONICA VARIETIES VIA ELECTRIC DISCHARGE PARTICLE ACCELERATION OF EXOGENOUS DNA INTO IMMATURE ZYGOTIC EMBRYOS

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We have recovered transgenic rice plants from a number of commercially important cultivars, including until now recalcitrant Indica varieties, using electric discharge particle acceleration. Immature embryos from greenhouse-grown plants were bombarded with gold particles carrying DNA, and transgenic plants were recovered following a simple culture protocol. Mendelian segregation of foreign genes was observed in R1 progeny and stable integration was demonstrated by Southern blot analysis of genomic DNA isolated from progeny plants. Alternative transformation protocols that are dependent on the development of protoplast and suspension culture systems are no longer necessary as we have shown that a wide variety of diverse cultivars can be transformed. Transgenic plants expressing agronomically useful traits such as herbicide resistance have been obtained and are currently undergoing further evaluation. This report also demonstrates that it is possible to produce transgenic monocotyledonous plants by transforming scutellar tissue of immature embryos.

The genetic modification of crop plants has undergone a revolution in the past few years. Development of new *in vitro* culture systems and novel transformation technologies have opened the way for the engineering of even the most recalcitrant of crops. The engineering of maize[1], cotton[2], and soybean[3] represent examples of different methods that have been successfully used to introduce exogenous DNA into crop plants.

However, in order to develop a practical transformation system for any crop, a number of criteria need to be fulfilled: (1) Transformation systems have to be independent of genotype or cultivar in order to facilitate introduction of useful genes into elite varieties. (2) Large numbers of transgenic plants should be recovered in order to assess useful levels of gene expression and (3) extensive tissue culture manipulations, involving time-consuming and labor-intensive operations such as protoplast and embryogenic suspension cultures, should be minimized, or if possible eliminated, in order to avoid culture-induced mutations and somaclonal variation.

It is apparent, that promising reported gene transfer methods for cereal crops suffer from some or all of the above drawbacks. A recent example is the engineering of maize, which requires development of regenerable embryogenic suspension cultures[1,4] amenable to transformation, a not so trivial task. The requirement for development of embryogenic suspension cultures compatible with current transformation technologies makes practical and efficient engineering of elite cultivars of maize elusive. Until now, recovery of transgenic rice plants was only possible using direct DNA transfer methods such as electroporation[5–7] or PEG-mediated transformation[8,9] of protoplasts. Transgenic rice tissues have been recovered from immature rice embryos using *Agrobacterium*, but regeneration of plants was not reported from these cultures[11]. A report describing a simple method using pollen tubes as a vehicle to introduce exogenous DNA into rice plants has not been confirmed[12]. Molecular data was not presented to convincingly show integration of the foreign gene into high molecular weight genomic DNA; neither was progeny analysis, the ultimate proof of transformation reported.

The development of a genotype-independent transformation system for soybean followed by commercial application[13], led us to believe that development of similar genotype-independent gene transfer systems for monocots using organized tissue should be possible. We have initially focussed on rice as a model to address fundamental problems that touch on many key areas of plant sciences[14,15].

Rice *in vitro* culture has a very strong genotype- and culture-dependent component. Even though genetic engineering of rice has been reported, only cultivars from japonica varieties can be transformed using protoplast-dependent methods; although recently, transformation and subsequent regeneration of transgenic indica rice

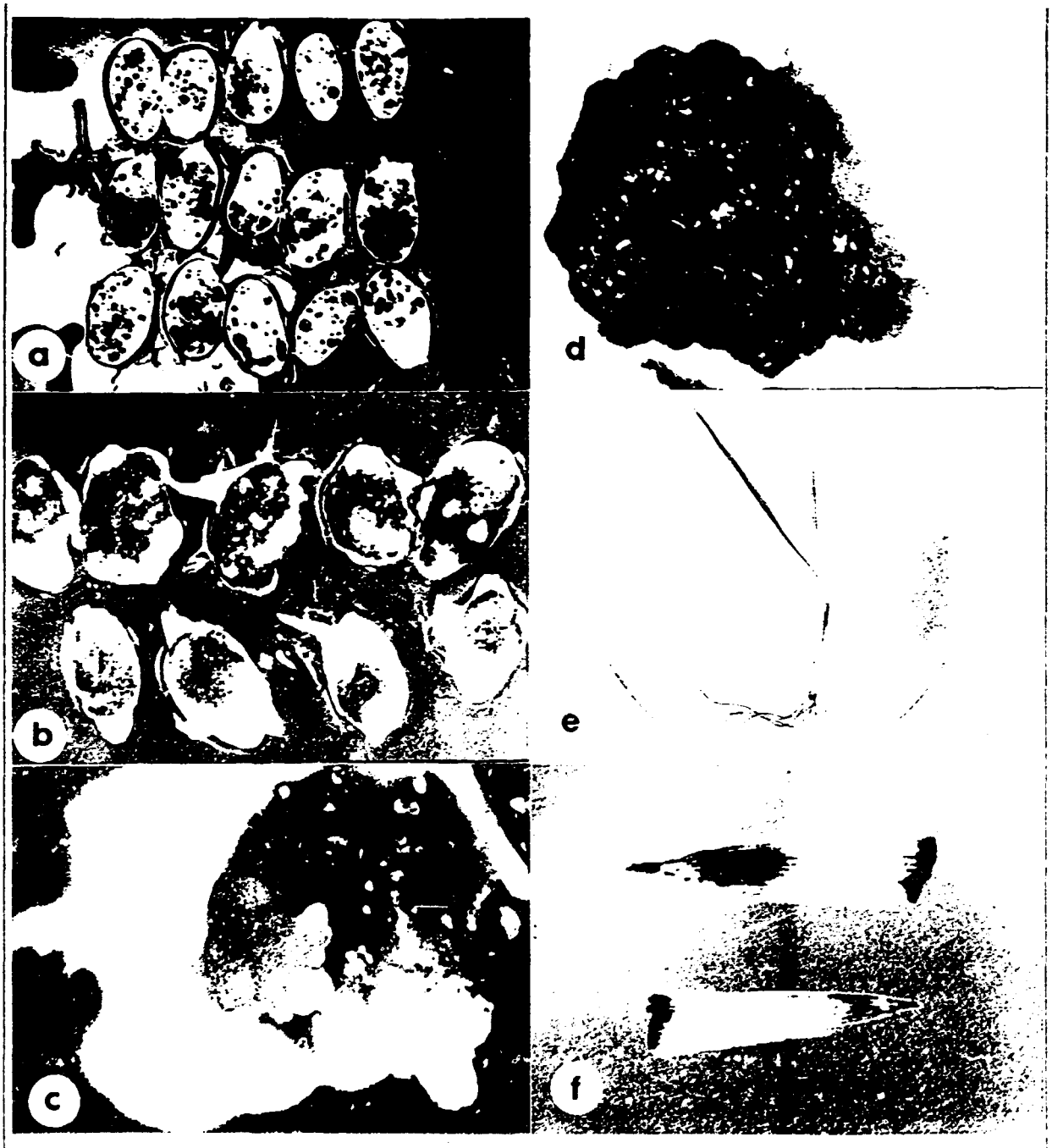

FIGURE 1 (**a**) Rice immature embryos exhibiting transient GUS activity, 24 hours following particle bombardment at 10 kv. (**b**) Top row, immature rice embryos bombarded with a gene that confers resistance to the antibiotic hygromycin B showing callus proliferation on media supplemented with 50 mg/l Hgm B. Bottom row, embryos bombarded with a bialaphos-resistance gene, plated on Hgm-containing media. (**c**) Embryogenic callus expressing GUS activity growing under hygromycin selection conditions. (**d**) Transformed callus expressing GUS from an unselected explant. (**e**) Transgenic IR54 plant expressing GUS; this plant was incubated in the appropriate solution for 30 minutes and then rescued. It is currently growing in the greenhouse. (**f**) GUS activity in leaf tissue from plant in panel e following transfer to the greenhouse.

plants, from a specific cultivar, was reported utilizing protoplasts[27]. Indica rice varieties provide the staple food for more than two billion people worldwide, including Indochina and the Indian subcontinent. Eighty percent of cultivated rice worldwide is of the indica type[28,29].

A second, and perhaps more compelling, reason for testing this system pertains to the fundamental question of whether meristematic, or even potentially meristematic cells, are amenable to transformation[7]. Genetic modification of any cereal crop, using organized tissue or other than protoplast- or suspension culture-based systems, has not been documented and success in this field would be a substantial contribution to the development of commercial, genotype-independent transformation systems for cereals.

In this report, we describe a simple and effective pro-

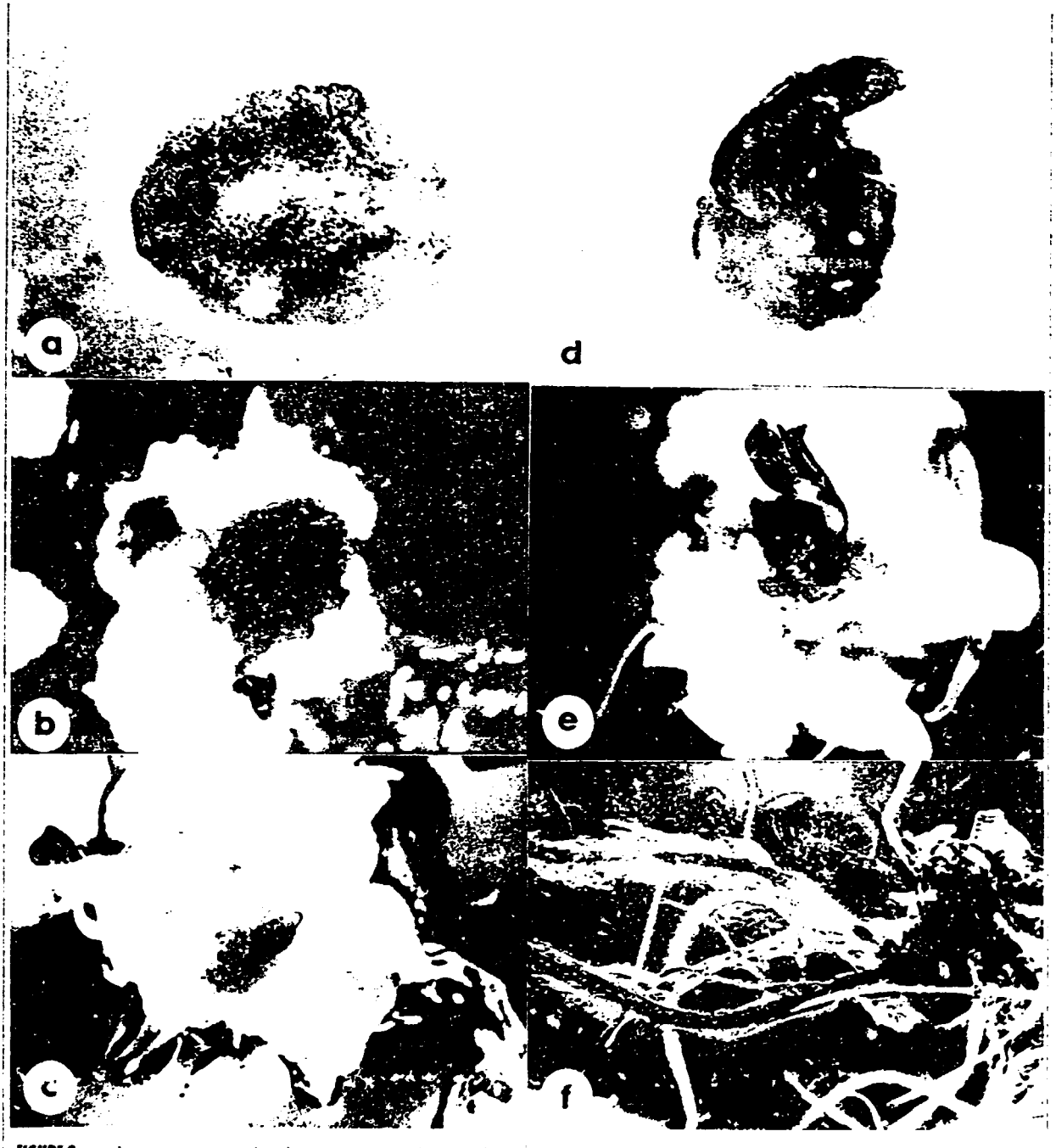

FIGURE 2 a GUS-expressing callus following transformation of immature rice embryo under hygromycin selection. b Embryogenic callus obtained forming under non-selective conditions. The chimeric nature of the callus is apparent. c Transformed rice embryos developing under selection conditions. d Rice shoot developing from selected embryogenic callus. e Transformed shoots forming on selected tissue. f Transformed roots under selection conditions.

culture, using electric discharge particle acceleration, to introduce DNA-coated gold particles into scutellar tissue of rice, with subsequent recovery of transgenic plants from both indica and japonica varieties.

RESULTS AND DISCUSSION

Transformation of immature embryos. Twelve- to fifteen-day old rice immature embryos were isolated from greenhouse grown plants and subjected to electric discharge particle mediated transformation using plasmids encoding either β-glucotonidase (gus) and bialaphos resistance (bar) or GUS and hygromycin resistance (hpt). In the particular experiments described in this report, seven diverse cultivars were used. Table 1 illustrates frequencies of transformation events in these cultivars. We focussed our attention to two of these cultivars in order to accelerate the development of an efficient and reproducible protocol for rice transformation, amenable to commercialization. Taipei 309 is one of the most important commercial US cultivars with both japonica and indica characteristics in its

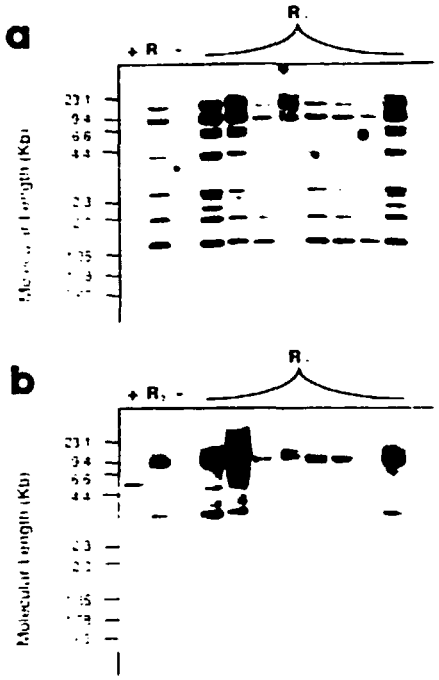

FIGURE 3 Southern blot analysis of R0 and R1 transgenic rice plants, expressing BAR (a) and GUS (b). + and − lanes indicate positive and negative controls respectively. Ten μg of DNA from transformed and control plants (leaf tissue) were isolated as described in the Experimental Protocol. The position of molecular weight length markers is indicated on the left. Plant DNA was digested with restriction endonuclease XbaI which cuts the intact 35S-*bar* coding sequence. The enzyme also cuts the entire 35S-*gus*-vector sequences. The resulting fragments were resolved by electrophoresis and transferred to nylon membranes. Membranes were hybridized with a ^{32}P-labeled RNA probe corresponding to the minus strand of the *bar* or *gus* coding regions. Hybridizing fragments were detected by autoradiography after 12 hours.

TABLE 1 Frequencies of transformation events in diverse rice varieties.

Variety	Explants used	Explants with embryogenic callus	Number of transformed plants
Gulfmont	100	85	3 (1*)
Gulfmont	100	90	6
Gulfmont	100	83	8 (1*)
IR54	100	85	3 (1*)
IR54	100	67	5
IR26	100	75	2
IR36	100	78	2
IR72	100	80	1

*Refers to plants that have been analyzed through at least the R1 generation. The other plants were either destructively assayed or are awaiting genetic analyses.

pedigree; IR54 is a true indica variety[19]. The scutellar region of the embryo was bombarded under previously described conditions[20], and transient activity for the introduced genes was observed 24 hours following the experiment (Fig. 1a). Levels of transient activity could be altered by varying DNA and bead loading rates, accelerating voltage, and depth of penetration. In general, transient GUS activity was found to increase with increasing amounts of DNA and decreasing voltages. An accelerating voltage of 10 Kv was found to be optimal for maximizing recovery of stably transformed embryogenic callus.

Induction of embryogenic callus and plantlet regeneration. Bombarded tissue was plated on regeneration media supplemented with appropriate selective agents, in this case the antibiotic hygromycin B (Hgm) or the herbicide bialaphos. Appearance of callus expressing the introduced genes could be seen 5–7 days following particle bombardment (Fig. 1b, top row). Tissues bombarded with alternative plasmids, not containing the antibiotic- or herbicide-resistance gene(s), or with naked gold particles (no DNA) did not proliferate any callus and died within a few days following plating on selective media (Fig. 1b, bottom row). Continuous selection of the proliferating tissue on Hgm-containing media (Hgm 50 mg/l), resulted in the appearance of transformed embryogenic callus (Fig. 1c). However, transformed embryogenic callus and somatic embryos also appear in the absence of any selection pressure (Figs. 1d and 2b,c). Subsequent transfer of this embryogenic callus to appropriate media[21,22], resulted in the development of plantlets (Fig. 2d,e) and plants (Fig. 1e,f) expressing marker genes, in addition to antibiotic/herbicide-resistance genes. Not all plants recovered from antibiotic-resistant tissues were transformed. It appears that transformed tissue on a particular explant is capable of detoxifying the selective agent so efficiently that non-transformed tissue in close proximity can also survive and regenerate plants. This was found to be the case whether the herbicide bialaphos or the antibiotic hygromycin were used as selective agents. Dekeyser et al., evaluated a number of selectable markers for rice transformation[23]. Rice calli, recovered from electroporation of protoplasts were used to determine those markers most suitable for selection. Their results indicated that phosphinothricin, conferring resistance to the herbicide bialaphos, was very effective as a selective agent. However, caution should be exercised in extrapolating results based on protoplast-derived callus to selection of organized tissue such as immature zygotic embryos. Our results demonstrated that physiological and other differences between dedifferentiated and organized tissue make such extrapolations unreliable. Following particle delivery and initial selection, transformed callus expressing antibiotic resistance and GUS activity develops from the scutellum of bombarded explants (Fig. 2a). This callus is chimeric, with both transformed and non-transformed sectors proliferating together. Consequently, large numbers of plants regenerated from this tissue will not be transformed. We found selection at this later stage to be ineffective. In a number of experiments, we have observed that up to 50% of bombarded explants were capable of developing transformed embryogenic callus which could subsequently result in the recovery of transformed embryos and other transgenic organized tissues, such as shoots and roots (Fig. 2e, f, respectively), in addition to transformed plants. However, non-transformed cells proliferating as a result of effective detoxification of the selective agent by transformed cells on these explants retained and expressed their regeneration potential to the same extent as transformed cells.

DNA analysis of transgenic rice plants and their progeny. Southern blot analyses of R0 plants and their progeny demonstrated stable integration of exogenous genes into the rice genome (Fig. 3). Plants exhibiting GUS activity and/or antibiotic or herbicide resistance, upon restriction endonuclease digestion of genomic DNA, were shown to contain fragments corresponding to the intact coding region of the enzymes. Digestion with other restriction endonucleases, which do not cut within the *gus*, *bar*, or *hgm* genes, resulted in high molecular weight fragments that hybridized to the probe(s) (data not shown). Molecular analysis of a number of independently-

derived transgenic rice plants indicated that the DNA profile and integration patterns in rice are very similar to those of soybean, which we have previously analyzed extensively[24].

Figure 4a, illustrates a fertile R0 rice plant. The introduced genes in R1 progeny from this and additional plants were found to segregate in a Mendelian ratio (3:1).

Introduction of agronomic traits into diverse rice varieties. When progeny from transgenic rice plants, carrying the *bar* gene, were sprayed with the herbicide bialaphos they were shown to express total resistance to the herbicide at levels of 500 ppm. In fact, a number of transgenic plants grew more vigorously than unsprayed controls (Fig. 4b).

Transgenic rice tissues or plants from the varieties Gulfmont, Lemont, IR26, IR36, IR54 and IR72 (which is the latest indica variety developed by the International Rice Research Institute [IRRI; G. Toenniessen, The Rockefeller Foundation, per. com.].) have been recovered. Experiments are under way to introduce useful traits such as herbicide and insect resistance into additional elite rice varieties including the true japonicas CM101 and S201.

We believe that results presented in this report demonstrate conclusively that engineering of rice through transformation of organized tissue (with frequencies similar to those of other crops, e.g. soybean[25].) can be accomplished via particle bombardment. Extension of these results to other cereals, such as maize, is in our opinion a feasible goal.

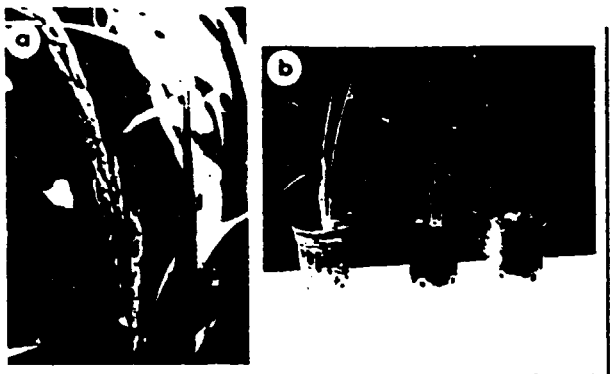

FIGURE 4 (a) Fertile R0 plant which gave rise to transformed progeny. The cultivar is Gulfmont, one of the top US commercial varieties. Progeny from this plant segregated in a 3:1 ratio for the transgenes (GUS expression and bialaphos resistance). (b) Demonstration of herbicide resistance in R1 progeny of transgenic rice plants: Left to right, transgenic R1 plant sprayed with 500 ppm bialaphos, non-sprayed wild-type control plant, control plant sprayed at 500 ppm bialaphos. The photograph was taken one week following spraying.

EXPERIMENTAL PROTOCOL

Plasmids. Plasmid WRG2114 contains the pUC19 backbone with the *gus* and *bar* genes arranged to give convergent transcription. Both the *gus*[26] and *bar*[27] genes were fused to a 436 bp CaMV 35S promoter and a 5′ untranslated leader sequence. The *gus* gene has a 271 bp 3′ poly A addition site from the *Agrobacterium nos* gene while the *bar* gene has a 3′ poly A addition site from a soybean *ssu* gene[28]. Plasmid WRG1515, containing both the *gus* and hygromycin-resistance genes, each controlled by its own 35S promoter and *nos* polyadenylation region was constructed using several intermediate plasmids. The orientation of the genes in this plasmid has the *gus* gene 5′ to the *hmr* gene, with transcription in a clockwise direction.

DNA preparation. DNA-coated gold particles were prepared by mixing gold particles (10 mg) with a solution of the DNA (20 µg) in 100 µl of buffer (150 mM sodium chloride, 10 mM Tris-HCl, pH 8.0) and vortexed gently for 5–10 seconds. Spermidine (100 µl of 0.1 M solution) and 100 µl of a 25% PEG solution (MW 1300–1600) were added with vortexing, followed by the dropwise addition of 100 µl calcium chloride (2.5M). The mixture was allowed to stand at room temperature for 10 minutes and then spun in a microfuge. The supernatant was removed and the precipitated gold with the DNA complex resuspended in 10 ml of 100% ethanol. The resulting suspension was then coated onto an 18 × 18 mm carrier sheet at a rate of 163 µl per carrier sheet, or a calculated rate of 0.05 mg/cm^2.

Isolation of immature embryos and preparation for particle bombardment. Twelve to fifteen-day old rice immature embryos were harvested from expanded panicles and sterilized with 2% sodium hypochlorite for five minutes. They were subsequently rinsed repeatedly with sterile distilled water and the glumes were removed under a dissecting microscope. The embryos were then aseptically removed and plated on a water-agar plate with the adaxial side in contact with the medium.

Particle bombardment. The carrier sheet bearing the beads was loaded onto the particle accelerator, which uses the discharge of a high voltage capacitor through a small water droplet as the motive force. A 100 mesh retaining screen was placed between the sheet and the target tissue suspended above the machine. The assembly was then evacuated to 500 mm Hg to reduce aerodynamic drag. Ten to sixteen Kv from a 2 µF capacitor was discharged through a 10 µl water droplet inside the expansion chamber. The sheet was thus blown against the retaining screen permitting the gold particles to continue onward to impact the target tissue suspended above the screen. The targeted immature embryos were positioned on a water-agar plate so that, when the plate was inverted over the screen, the scutellar region of the embryos would be in the direct path of the accelerated particles.

Plant regeneration. Following particle bombardment, embryos were plated on MS or CC media supplemented with 2,4D at 0.5 or 2 mg/l and embryogenic callus and plantlets were recovered as described[21,22].

Recovery of transformed embryogenic callus and plants. Transformed callus and plants were recovered under both selective and non-selective conditions. In experiments in which selection was incorporated in the transformation/regeneration protocol, hygromycin at 50 mg/l or bialaphos at 10 mg/l were used. Regenerating plants were screened for GUS activity by sampling small segments of their leaves[26]. Putative transformants thus identified, were subjected to molecular and genetic analysis to confirm stable integration and inheritance of the introduced gene.

Southern blot analysis. DNA was prepared from young leaf tissue harvested from primary plants and their progeny using the method of Dellaporta[29]. DNA, digested with restriction endonucleases under the conditions recommended by the supplier, was resolved by electrophoresis on a 0.8% agarose gel, and then transferred to nylon membranes (Biodyne membranes, Pal, Irvine, CA) as described by Southern[30]. ^{32}P-labeled RNA hybridization probes were synthesized *in vitro* using an SP6 transcription system (Promega Biotec., Madison, Wisconsin) and [α-^{32}P CTP] (300 Ci/mmol; Amersham; 1 Ci = 37GBq). Hybridization and washing conditions were described by Church and Gilbert[31]. Filters were analyzed by autoradiography, using X-Omat AR5 film (Kodak) at −80 °C with two intensifying screens (Cronex Lightning plus, Du Pont).

Herbicide applications. R1 plants shown to contain the *bar* gene were sprayed with glufosinate-ammonium (Hoechst AG, FRG) at a rate of 500 ppm. The chemical was mixed at a ratio of 2.5 ml/l of water, and the actual spray rate used was 0.25 ml/100 ml of water.

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MINI-REVIEW

Genetic transformation of crop plants using microprojectile bombardment

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Introduction

Development of procedures in cell biology to regenerate plants from single cells and organized tissue, and the discovery of novel techniques to transfer genes to plant cells provided the prerequisite for the practical use of genetic engineering in crop improvement. These advances have given us the opportunity to create, characterize and select plant cultivars which could not be obtained by traditional breeding methods. Genetic engineering of such recalcitrant crops as maize (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990), rice (Christou *et al.*, 1991; Datta *et al.*, 1990; Toriyama *et al.*, 1988), cotton (McCabe and Martinell, 1991; Umbeck *et al.*, 1987), and soybean (Christou *et al.*, 1990; McCabe *et al.*, 1988) is now possible and in some cases routine. Soybean and cotton plants, highly resistant to commercial herbicides and insect pests, will be some of the first agricultural commercial products of recombinant DNA technology. These plants are expected to be in the market well before the end of this decade (Cutler, 1991).

Potrykus (1990) developed a model in an attempt to explain why some species are more recalcitrant to *in-vitro* manipulation and transformation than others. He postulated that the relative ease with which *Agrobacterium* may transform certain dicotyledonous plants is likely due to the wound response these species exhibit. Such a response is absent from most monocotyledonous plants, making the latter very difficult to infect. It is important that any given DNA delivery method should be able to target as many competent cells as possible; in addition, it would be advantageous to develop ways to maximize the numbers of such cells. Commonly used transformation vectors, e.g. *Agrobacterium tumefaciens*, suffer from severe host specificity which limit the scope of their use. Selectable markers developed to permit preferential growth of engineered cells are, unfortunately, only effective in systems involving fully dedifferentiated tissue. Attempts to select organized tissue have not met with much success, with the notable exception of leaf-disk transformation of certain Solanaceous plants (Horsch *et al.*, 1985).

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Regeneration of intact plants from transformed tissue is not always an easy task. In a number of systems it is quite straightforward to engineer tissue that is not competent for regeneration (Christou *et al.*, 1987). Additional barriers include tissue culture-induced variation, time factors for the recovery of transformants, labor intensive protocols, and limitations in regenerating plants from protoplast, callus and suspension cultures. It would be advantageous therefore, to develop efficient transformation methodology which would allow recovery of transgenic plants without the above constraints.

Microprojectile bombardment

Microprojectile bombardment employs high velocity metal particles to deliver biologically active DNA into plant cells. The concept has been described in detail by Sanford (1988). Following the original observation by Klein *et al.* (1987) that tungsten particles could be used to introduce macromolecules such as RNA and DNA into epidermal cells of onion with subsequent transient expression of enzymes encoded by these compounds, Christou *et al.* (1988) demonstrated that the process could be used to deliver biologically active DNA into living cells and result in the recovery of stable transformants. The ability to deliver foreign DNA into regenerable cells, tissues, or organs appears to provide the best method, at present, for achieving truly genotype-independent transformation in many agronomic crops, bypassing *Agrobacterium* host-specificity and tissue culture-related regeneration difficulties. Due to the physical nature of the technique there is no biological limitation to the actual DNA delivery process, thus genotype is not a limiting factor. Combining the relative ease of DNA introduction into plant cells with an efficient regeneration protocol, avoiding protoplast or suspension culture, we appear to have the optimum system in place for transformation. Important advances and refinements in the process described subsequently, using soybean (Christou *et al.*, 1990; McCabe *et al.*, 1988) and rice (Christou *et al.*, 1991) as model systems for dicotyledonous and monocotyledonous species, respectively, demonstrated the power and versatility of the technique. The list below illustrates major advantages that make microprojectile bombardment the method of choice for engineering various crops:

- transformation of organized tissue;
- rapid recovery of transformed R_1 seed;

- universal delivery system;
- transformation of recalcitrant species;
- study of basic plant developmental processes.

A number of different methods can be used to accelerate particles into living cells (Sanford *et al.*, 1987). These include pneumatic devices, instruments utilizing mechanical impulse or a macroprojectile, centripetal, magnetic or electrostatic forces, spray or vaccination guns, or apparatus based on acceleration by shock wave, such as electric discharge, etc.

Metal particles may be coated with DNA or RNA, or they may be used to carry the genetic material into a cell from a solution of DNA or RNA surrounding the cell. Alternatively, micro-encapsulated material can be transported into the cells and released immediately upon entry into the cytoplasm or nucleus, or made available through a slow release mechanism.

A number of critical variables have been identified and need to be considered very carefully in experiments involving transformation utilizing particle bombardment.

Physical parameters. These include (i) the nature, chemical and physical properties of the metal particles used to carry the foreign DNA into the cells, (ii) nature, preparation and binding of DNA onto the particles, (iii) target tissue. Metal particles should be of high enough mass in order to possess adequate momentum to penetrate into the appropriate tissue. Suitable metal particles include gold, tungsten, palladium, rhodium, platinum, iridium and possibly other second and third row transition metals; they should be chemically inert, to prevent adverse reactions with the DNA or cell components and they should also be able to form organometallic complexes with the DNA possessing the correct stereochemistry that will allow optimal dissociation of the metal–DNA entity once the coated particle enters the target cell. Additional desirable properties for the metal include size and shape, as well as agglomeration and dispersion properties. The nature, form and concentration of the DNA need also be carefully considered. In the process of coating the metal particles with exogenous DNA, certain additives such as spermidine and $CaCl_2$ appear to be useful. The nature of the DNA, e.g. single- versus double-stranded, may also be important under some conditions, even though this was shown not to be a significant variable in specific cases (e.g. the soybean transformation system). Finally, it is very important to target the appropriate cells that are competent for both transformation and regeneration. It is apparent that different tissues have different requirements; extensive histology needs to be performed in order to ascertain the origin of regenerating tissue in a particular transformation study. Depth of penetration thus becomes one of the most important variables and the ability to tune a system to achieve particle delivery to specific cell layers may be the difference between success and failure in recovering transgenic plants from a number of different tissues.

Environmental variables. These include such parameters as temperature, photoperiod and humidity of donor plants, explants and bombarded tissues. These parameters have a direct effect on the physiological state of tissues. Environmental factors will influence the receptiveness of target tissue to foreign DNA delivery and also affect its susceptibility to damage and injury that may adversely affect the outcome of the transformation process. Some explants may require a 'healing' period following bombardment, under special regimens of light, temperature, humidity, etc.

Biological factors. These include choice and nature of explant, pre- and post-bombardment culture conditions, and interactions between the introduced DNA and cytoplasmic or nuclear components.

Particle bombardment using biolistics

The first acceleration device was reported by Sanford *et al.* (1987). They described a mechanism via which an apparatus based on transferred mechanical impulse, and utilizing a macroprojectile and a stopping plate, was used to accelerate small tungsten particles to high velocities (328–656 m sec^{-1}). These particles could penetrate cell walls and membranes and enter cells in a non-lethal manner. Klein *et al.* (1987) subsequently demonstrated transient activity of introduced genes in epidermal onion tissue. Modifications of this device in subsequent years initially permitted recovery of transgenic tobacco and maize plants. Subsequently, additional plants, listed in Table 1, could be engineered using technology based on this apparatus or second generation instruments utilizing compressed gasses to accelerate particles coated with DNA into plant cells. However, one of the most important limitations of this and other devices based on the principle of acceleration through transferred impulse, is the fact that only embryogenic cultures can be utilized in transformation experiments. Therefore, unless an embryogenic culture can be derived from a specific plant or a particular cultivar, recovery of transgenic plants is very difficult and problematic. The uncontrolled accelerating force resulting from such devices compounds the problem by causing excessive injury and death to target tissues. Random depth penetration of DNA-coated particles into bombarded explants restricts the use of organized tissue such as embryos and meristems.

Table 1. Contribution of particle bombardment to transformation technology

Crop	Result	Reference
Soybean	Only commercial genotype-independent process for the generation of transgenic plants	Christou *et al.* (1990)
Cotton	Only genotype-independent transformation method	McCabe and Martinell (1991)
Phaseolus	Only method for recovering transgenic plants	McCabe (personal communication)
Poplar	Extends transformation to varieties non-susceptible to *Agrobacterium* infection, or to those that cannot be regenerated from protoplasts	McCown *et al.* (1991)
Spruce	No other method available. Transformed somatic embryos, embryogenic lines and plants recovered	Ellis *et al.* (1991)
Papaya	No other transformation method available	Fitch *et al.* (1990)
Cranberry	Only method available	Serres *et al.* (1990)
Maize	Only method available. Bombardment of embryogenic suspension cultures	Fromm *et al.* (1990). Gordon-Kamm *et al.* (1990)
Rice	Transformation of elite indica and japonica varieties that cannot be regenerated from protoplasts	Christou *et al.* (1991)
Sugarcane	Only method available	Bower and Birch (1992)
Wheat	Recovery of transformed callus (and plants)	Vasil *et al.* (1991), Vasil (personal communication)
Sorghum	Transformed callus	Hagio *et al.* (1991)

Particle bombardment using electric discharge

In order to address as many of the critical variables identified as being crucial to the transformation process as possible, a mechanism was developed that permitted maximum flexibility for tuneability, targeting and cell penetration. The apparatus is thus finely tuneable and by varying the intensity of the electric discharge, penetration of the target tissue can be controlled very accurately, directing the majority of the particles carrying the DNA to a specific cell layer. This capability is extremely important because even identical explants from different genotypes of the same species may require different acceleration conditions for optimum particle penetration.

Transformation of dicotyledonous species

The versatility and usefulness of particle bombardment is illustrated by the development of a genotype-independent transformation protocol for soybean (Christou *et al.*, 1990; McCabe *et al.*, 1988). Starting with isolated immature embryonic axes a simple protocol permitting recovery of clonal plants from elite varieties was developed. The overall transformation frequency can be as high as 15% with germline transformation frequencies approximating 0.25% based on the number of bombarded explants.

In this process, meristematic regions from either mature or immature embryo axes were exposed, and gold particles coated with plasmid DNA were introduced into the general area of the meristem. Plant regeneration proceeded through organogenesis under the influence of BAP which induced multiple shoots from the general area of both the primary and axillary meristems (McCabe *et al.*, 1988). On average, eight to ten shoots were recovered per explant. Regenerated shoots were harvested and transferred to the greenhouse after an appropriate acclimation period. Conversion of shoots to plants was greater than 95%. Expression of the GUS gene was originally used to visualize transformed plant tissues (Jefferson *et al.*, 1987). Due to the nature of the transformation process, most of the recovered plants were chimeric; however, clonal plants were also obtained at a significant frequency (Christou *et al.*, 1989, 1990). These plants were found to express foreign genes in essentially all their tissues and usually passed the transgenes to their progeny in a manner consistent with Mendelian inheritance of a single dominant locus. Though germ-line transformation frequency appears to be somewhat low, it is relatively straightforward to

identify transformed plants due to the ease of the histochemical GUS assay. Consequently, production of reasonably large numbers of transformed plants is practical by this method which does not utilize selection to identify transgenic plants. In parallel experiments in which co-transformation frequencies were evaluated using two genes, either linked on the same plasmid or unlinked on separate plasmids, it was determined that co-transformation frequencies between the screenable and the agronomic or non-selected genes were as high as 95–100%, in the former case. The frequency for co-transformation was significantly reduced (to approximately 25%) when two genes were introduced on two separate plasmids. All co-transformed families found to express one gene, also expressed the other indicating that the two genes were genetically linked, even when they were introduced into the plant tissue on two separate plasmids (Christou and Swain, 1990). Similar results were obtained in cell lines derived from protoplast transformation experiments, involving either electroporation or particle acceleration, and in regenerated transgenic plants from experiments involving particle bombardment into soybean meristems. Both chimeric and clonal plants gave rise to transformed progeny, with the majority of transgenic families segregating in a Mendelian fashion in the R_1 and R_2 generations (Christou *et al.*, 1990).

Hundreds of independently derived soybean plants transformed by this method have maintained the foreign genes for many generations. Elite soybean varieties expressing resistance to the herbicides Basta® and Round-up®, engineered through particle bombardment using electric discharge, are currently undergoing large-scale field evaluation. In a parallel series of experiments, similar approaches resulted in the development of variety-independent transformation protocols for cotton (McCabe and Martinell, 1991) and *Phaseolus* (McCabe and Martinell, personal communication). The single most important factor in recovering stably transformed, germ-line transgenic plants using this technology is the capacity to modulate the intensity of the shock wave, and ultimately the depth of particle penetration. A very fine balance exists between particle penetration, DNA delivery and cell damage. Unless such a capability exists, it will be very difficult to apply this technology to the successful engineering of agronomic crops.

Transformation of monocotyledonous plants

Monocotyledonous plants which include some of the world's most important food crops, e.g. cereals such as wheat, rice, maize, barley, etc., have until recently been extremely recalcitrant to genetic manipulation *in vitro*. Particle bombardment resulted in the recovery of transgenic plants and progeny for such important crops as maize (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990) and rice (Christou *et al.*, 1991). It is only a matter of time before transformation of additional cereals is reported.

Until recently, recovery of transgenic rice plants was only possible using direct DNA transfer methods such as electroporation (Shimamoto *et al.*, 1989; Tada *et al.*, 1990; Toriyama *et al.*, 1988; Zhang *et al.*, 1988) or PEG-mediated transformation (Datta *et al.*, 1990; Hayashimoto *et al.*, 1990) of protoplasts. Rice *in-vitro* culture has a very strong genotype- and culture-dependent component. Even though genetic engineering of rice has been reported, only cultivars from japonica varieties could be transformed using protoplast-dependent methods; transformation and subsequent regeneration of transgenic indica rice plants, from a specific cultivar, has been reported utilizing PEG-mediated transformation of protoplasts (Datta *et al.*, 1990). The same group recently extended this technology to an additional elite indica variety, IR72 (Datta, personal communication). Indica rice varieties provide the staple food for more than two billion people world-wide, including Indo-China and the Indian sub-continent.

Successful genetic modification of any cereal crop using organized tissue (i.e. other than protoplast- or suspension culture-based systems) had not been reported; success in this field would certainly contribute significantly to the development of commercial, genotype-independent transformation systems for cereals.

Twelve- to fifteen-day-old rice immature embryos were isolated from greenhouse-grown plants and subjected to electric discharge particle-mediated transformation (Christou *et al.*, 1991). Bombarded tissue was plated on regeneration medium supplemented with appropriate selective agents. Continuous selection of the proliferating tissue resulted in the appearance of transformed embryogenic callus. However, transformed embryogenic callus and somatic embryos also appeared in the absence of any selection pressure. Subsequent transfer of this embryogenic callus to appropriate media, resulted in the development of plantlets and plants expressing marker genes in addition to antibiotic, herbicide, and insect resistance genes. Not all plants recovered from selected tissues were transformed. It appears that transformed tissue on a particular explant is capable of detoxifying the selective agent so effectively that non-transformed tissue in close proximity can also survive and regenerate plants.

Southern blot analyses of R_0 plants and their progeny demonstrated stable integration of exogenous genes into the rice genome. Molecular analysis of a number of independently derived transgenic rice plants indicated that the DNA profile and integration patterns in rice were very similar to those of soybean, which was analyzed extensively in the past (Christou *et al.*, 1989).

When progeny from transgenic rice plants carrying the *bar* gene were sprayed with the herbicide bialaphos they

Table 2. Technical parameters for recovering transgenic soybean and rice plants via electric discharge particle bombardment

Parameter	Soybean	Rice
Explant	Embryonic axes	Immature embryo scutellum
Accelerating voltage	14–18 kV	10 kV
Origin of regenerating plants	2nd–4th cell layer	Epidermis
Regeneration pathway	*De-novo* shoot formation	Somatic embryogenesis
R_0 transgenic plant to greenhouse	4–6 weeks post-bombardment	8–10 weeks post-bombardment
Selection	No	Yes
Screenable marker	Essential	Unnecessary
Hormonal requirements for regeneration	Cytokinin (BAP)	Auxin (2,4-D)
Chimerism	Likely	No
Germ-line transformation frequency	0.1–0.5%	1–2%
Number of transformation events per explant	One	One

were shown to express total resistance to the herbicide at levels of 2000 p.p.m. (non-transgenic plants were effectively killed at levels of 250–500 p.p.m.).

Comparing parameters identified as crucial to the engineering of soybean and rice (Table 2) leads to conclusions that can be used as guidelines for the wider application of this technology to successful gene transfer into other crops:

- a prolific regeneration system is a necessary prerequisite for the successful recovery of transformation events at practical frequencies;
- flexibility for delivering DNA-coated particles to cells in specific layers that are competent for both transformation and regeneration is very important;
- development of selection procedures greatly enhances transformation frequencies;
- nature of the regeneration pathway determines likelihood and frequency of chimerism in transgenic plants.

Molecular analysis of plants derived through particle bombardment indicates that DNA profiles and integration patterns are very similar to other direct DNA transfer methods (Table 3). Transgenic plants typically include at least one copy of the foreign gene, although sometimes more complicated patterns are observed. In both crops all copies of the transgene are inherited as a unit. Approximately 10% of transgenic rice plants recovered were infertile, whereas no such infertility was observed in soybean. This is a reflection of the different regeneration pathways for the two species.

Table 3. Summary of molecular and genetic data for transgenic soybean and rice plants recovered through electric discharge particle bombardment

	Soybean	Rice
Plants with 1 copy (%)	30	45
Plants with 2–5 copies (%)	60	40
Plants with multiple (> 5) copies (%)	10	15
Fertile plants segregating in Mendelian fashion in the R_1 generation (%)	75	100
Non-fertile plants (%)	0	10

Limitations and problems

It is apparent that the conversion frequency of transient to stable transformation events is very low. This makes recovery of large numbers of independently derived transformation events labor intensive and rather expensive. More attention needs to be paid to the biology of explants prior to, and following bombardment. We need to identify how more cells can be induced to become competent for stable DNA uptake and regeneration. Optimization of biological interactions between physical parameters and target tissue needs to be better studied and understood. Not much is known about the fate of DNA from the time particles are introduced into plant cells. Recipient tissue variation and variability due to bombardment conditions complicate the picture even further. Additional issues

such as irregular particle size and uniformity need to be addressed. Finally, improvements in hardware design and particle gun availability will certainly improve efficiencies and extend the list of crops that can be transformed using this technology. An example of such improvements in hardware design has recently been described by Sautter and Potrykus (1991). They have developed an instrument which can deliver DNA-coated particles into the apical meristem of plants very precisely.

Conclusion

Particle bombardment is certainly not a panacea. There are still major technical and scientific barriers that need to be overcome in order to bring the technology to its full potential. It is clear, however, that utilization of this technology opens the way for the transformation of tissues and species that are not otherwise accessible to genetic modification using recombinant DNA techniques. A number of additional applications of particle bombardment have been recognized and are currently in use. These include transient expression studies (Ellis *et al.*, 1990; Morikawa *et al.*, 1989), mechanical viral infections (Gilbertson *et al.*, 1990), gene deletion and promoter analysis (Ellis *et al.*, 1990; McCormick *et al.*, 1991), organelle (Day *et al.*, 1990; Newman *et al.*, 1990; Svab *et al.*, 1990; Ye *et al.*, 1990) and micro-organism (Johnston *et al.*, 1988; Shark *et al.*, 1991) transformation, studies pertaining to basic plant development (Christou, 1990; Christou and McCabe, 1992), introduction of multiple genes into plants, RNA delivery (Walker *et al.*, 1990), study of biosynthetic pathways in plants (Roth *et al.*, 1990), and mammalian cell and organ transformation (Johnston *et al.*, 1991; Yang *et al.*, 1990).

It is clear that this technology has impacted significantly on agricultural biotechnology. It is not unreasonable to expect that additional major crops will be engineered using this technology and before too long transformation technology will become a tool in the wider application of molecular and genetic approaches to crop improvement.

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TABLE I
Saccharomyces cerevisiae STRAINS AND EPISOMAL PLASMIDS USED FOR ELECTROPORATION[a]

Strain	Transforming plasmid	Selectable marker
M12B	pCTΔ[b]	URA3
M12B	YRp7	TRP1
M12B	YCp50[c]	URA3
M12B	pMC1790[d]	TRP1
JT-26A	YEp24	URA3
JT-26A	pMC1790	TRP1
JT-26A	pSZ214	LEU2
AH22	pSZ214	LEU2
SUB60	pMCCEN4	TRP1
SUB60	YCp50	URA3

[a] Adapted and reprinted with permission of Life Technologies (Gaithersburg, MD).
[b] A. R. Buchman and R. D. Kornberg, *Mol. Cell. Biol.* **10**, 887 (1990).
[c] M. D. Rose, P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink, *Gene* **60**, 237 (1987).
[d] M. J. Casadaban, A. Martinez-Arias, S. K. Shapira, and J. Chou, this series, Vol. 100, p. 293.

up to 20 kb (pSZ214),[17] little difference in the transformation frequencies obtained with each has been detected.

Concluding Remarks

As stated above, using this method we typically achieve transformation frequencies as high as 3×10^3 transformants per microgram of episomal plasmid DNA. This method is most appropriate for rapid transformations using low concentrations of DNA when extremely high transformation frequencies are not required. This method may not be appropriate for those transformation experiments using high concentrations of DNA where very high numbers of transformants are desired, because, as noted above, the transformation frequency reaches a plateau value at approximately 100 ng of transforming DNA. However, it is remarkably versatile in that it can be used to transform yeast cells with CsCl density gradient-purified DNA, with linear DNA, and with crude minipreparation DNA prepared from *E.*

[17] S. W. Ruby, J. W. Szostak, and A. W. Murray, this series, Vol. 101, p. 253.

coli as well as from *S. cerevisiae*. Due to the ease of this method, it should be possible to complete 20–25 transformations in 1 hr.

Acknowledgments

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[36] Optimizing the Biolistic Process for Different Biological Applications

By J. C. SANFORD, F. D. SMITH, and J. A. RUSSELL

Introduction

The biolistic process employs high-velocity microprojectiles to deliver nucleic acids and other substances into intact cells and tissues.[1-4] This process has also been called the microprojectile bombardment method, the gene gun method, the particle acceleration method, and so on. Diverse applications for the biolistic process are rapidly being found for both basic research and genetic engineering.

The biolistic process was originally developed as a means to deliver foreign genes into the nuclear genome of higher plants.[1,2] This is where most efforts have been focused, resulting in successful biolistic transformation of a wide range of tissues in a wide range of plant species.[3-28]

[1] J. C. Sanford, T. M. Klein, E. D. Wolf, and N. Allen, *J. Part. Sci. Technol.* **5**, 27 (1987).
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Transformed plant tissues include cell suspensions, calli, immature embryos, mature embryo parts, meristems, leaf pieces, microspores, and pollen. Transformed species include those that were otherwise impossible or very difficult to transform.[12-14]

The biolistic process has proved to be effective even in very small cell types, and has therefore been useful in transforming diverse microbial species. These include microbial eukaryotes such as yeast and filamentous fungi[29] and algae[30]; prokaryotes such as *Bacillus megaterium*,[31] *Pseudomonas syringae*, *Agrobacterium tumefaciens*, *Erwinia amylovora*, *Erwinia stewartii*, and *Escherichia coli*[32]; and obligate fungal pathogens such as *Uncinula necator*.[33]

The biolistic process first made possible the transformation of organelle

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[33] F. D. Smith, D. M. Gadoury, P. R. Harpending, and J. C. Sanford, manuscript in preparation (1992).

genomes. Chloroplasts of *Chlamydomonas* can now be routinely transformed,[34,35] yeast and *Chlamydomonas* mitochondria can be biolistically transformed,[36,37] and higher plant chloroplasts can be either transiently[38,39] or stably[40] transformed using the biolistic process.

Most recently, the biolistic process has proved useful in transforming animal cell lines,[41] primary animal cells,[42] and intact animals.[43,44]

While the biolistic process clearly has value, we are still learning how to make the process optimally effective within its diverse fields of application. In the last 2 years we have learned a great deal about how to make the process more effective. This chapter is meant to communicate what we have learned, and to help elucidate for others how they might best go about optimizing the process for their own particular applications. The basic features that must be considered by anyone using the biolistic process are (1) particle accelerator parameters, (2) microprojectile parameters, (3) biological parameters, and (4) experimental design.

Particle Accelerator Parameters

There are several ways of accelerating microscopic particles to supersonic speeds, as is required by the biolistic process. These were outlined by Sanford *et al.*[1] Of these various acceleration methods, the only method that has proved to be of general value thus far is acceleration of microprojectiles on the face of a macroscopic carrier, or "macroprojectile." The macroprojectile is in all cases driven by a gas shock. The gas shock can be derived by use of a chemical explosion (gunpowder),[1] an electric explosion of a water droplet,[11,12] a discharge of compressed air,[45] or by a

[34] J. E. Boynton, N. W. Gillham, E. H. Harris, J. P. Hosler, A. M. Johnson, A. R. Jones, B. L. Randolph-Anderson, D. Robertson, T. M. Klein, K. B. Shark, and J. C. Sanford, *Science* **240**, 1534 (1988).
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helium shock[45] generated by a rupture-membrane mechanism. The macroprojectile may be any lightweight object that has a front surface that can carry microprojectiles, a back surface that can receive the energy of the gas shock, and sufficient cohesive integrity to withstand the gas shock, sudden acceleration, and violent deacceleration.

While there are now numerous particle accelerator designs in use, we will limit our discussion to the gunpowder-driven PDS-1000 accelerator [previously distributed by Du Pont (Wilmington, DE)] and its helium shock driven retrofit (now distributed by Bio-Rad, Richmond, CA). This focus is due to our familiarity with these acceleration systems, both of which were developed in our laboratory. In addition they are the systems most widely used and are the only ones that are commercially available. Researchers using other accelerator designs can still benefit from the information gained through our experience with these types of devices.

Some people may choose to continue to use the gunpowder-driven apparatus, so we include some discussion of it. However, as the new helium-driven apparatus is dramatically superior,[45] most of our discussion will relate to this improved biolistic system.

Power Source

Gunpowder-driven designs employ standard nail-gun cartridges, as are used in the construction industry. Until now this has been the established biolistic power source, but it has the disadvantages of being somewhat dangerous, uncontrolled, and messy in terms of generating dirty gases and debris within the apparatus.

The new helium-driven apparatus has the advantage that the power source is safer and cleaner, and the power output can be regulated. We have observed that helium is clearly superior to other gases such as compressed air or nitrogen. This was as we expected, because helium is a light gas and expands much faster than other conventional bottled gases, imparting higher velocities to lightweight macroprojectiles. We believe the velocities achieved by the gunpowder and helium power sources are not fundamentally different, based on depth of penetration studies. The gunpowder-driven system seems to have higher velocity in the "epicenter" of a target region, but this is usually associated with a zone of cell death roughly 1 cm in diameter.[19] The helium-driven system does not generally produce a zone of death, and apparently produces higher velocities over a wider target area combined with better dispersal of particles, resulting in a more uniform field of transformation. In all biological systems we

[45] J. C. Sanford, M. J. DeVit, J. A. Russell, F. D. Smith, P. R. Harpending, M. K. Roy, and S. A. Johnston, *Technique* 3, 3 (1991).

have tested, the helium system has proved dramatically superior to the gunpowder system in effectiveness.[45]

Macroprojectile

The gunpowder system requires a cylindrically shaped, high-density polyethylene macroprojectile, a tight-fitting, relatively long, heavily-armored acceleration barrel, and a special Lexan (Dupont, Wilmington, DE) stopping plate with a very small central aperture.

The helium system employs a 2.54-cm circular Kapton (Dupont) membrane (only 0.06 mm in thickness) as a macroprojectile, which has the following important benefits. Only a short flight distance is needed, as the membrane requires very little time to come up to speed. The less massive membrane can be stopped with a screen, rather than a Lexan disk. Therefore more particles can be delivered without any associated high-velocity "debris," which can be generated from the macroprojectile or stopping plate. In addition, the microprojectiles are accelerated in a dried-down form over a larger surface of the wider macroprojectile, and are subsequently dispersed much more widely and uniformly on impact against the stopping screen.

Vacuum/Residual Gas

Regardless of the apparatus used, the gas overlying the target sample usually must be modified. Most commonly, as much of the overlying air is removed with a vacuum pump as is practical, such that a standard vacuum gauge will read 28–29 in. Hg (about 710–740 mmHg). Higher vacuums are not generally practical because of residual water vapor pressure from the biological sample itself. The strength of the vacuum must be reduced for certain applications. For example, bombardment of mouse skin *in situ* requires a reduced vacuum of approximately 20 in. Hg (about 510 mmHg), or the suction on the tissue can damage cells and reduce expression levels.[44] Likewise, mouse liver tissue will not tolerate a vacuum at any level.[44]

The efficiency of transformation of certain biological targets can be enhanced by flushing the chamber with helium prior to pulling a vacuum, such that the residual gas is helium instead of air. This advantage is quite dramatic in microbial systems, and helium flush can increase bacterial transformation by five- to sixfold,[32] and yeast transformation fourfold (J. C. Sanford, unpublished observation, 1992). However, this advantage is not universal. In tobacco cell suspensions the benefit is small or absent (J. C. Sanford, unpublished observation, 1992).

There are two reasons why the gas overlying the biological sample can

affect biolistic transformtion. The principal reason is that microprojectiles are rapidly deaccelerated as they pass through any gas. By removing most of the overlying gas, the amount of deacceleration can be significantly reduced. Likewise, by using a light gas such as helium, the drag can also be reduced. The smaller the microprojectile, the more dramatic the deacceleration problem. Because bacterial transformation involves a subset of microprojectiles that are extremely small, a helium flush and a strong vacuum become especially important factors affecting biolistic efficiency. A second reason why the gas overlying the sample can be important is that this gas can transmit a potentially damaging shock wave. By reducing the density of the overlying gas, or by using a low molecular weight gas such as helium as an overlay, the severity of such a shock wave can be reduced.

Baffles/Meshes

The acoustic shock/gas blast that is generated during the supersonic acceleration of the macroprojectile can kill cells, especially those cells that are multiply traumatized by also being penetrated by the microprojectiles, and which may also be exposed to a selective medium. Even when such shock is not lethal, it may impair subsequent cell division, growth, and regeneration. Modification of the overlying gas can reduce the intensity of this shock, but only slightly. We have, therefore, attempted to reduce the shock wave further. Very fine meshes placed between the microprojectile launch site and the biological sample have been reported to improve gunpowder-driven biolistic transformation dramatically.[14] We have found that such meshes are indeed effective with gunpowder-driven systems, but are less critical with the helium system. We have also tested a single-aperture postlaunch baffle with the gunpowder system, and a prelaunch baffle with the helium system.[19] To evaluate the severity of the shock to the sample, we developed a "shave cream" assay. This assay simply measures the degree to which a layer of shave cream is disrupted by bombardment. We find that the mesh is more effective than a prelaunch baffle, which in turn is more effective in reducing shock than a postlaunch baffle. In addition, a mesh plus a prelaunch baffle is better than either alone. We believe that the benefit of the mesh with the gunpowder system is in reducing shock-generated trauma to cells, in addition to improving disaggregation, as has been proposed previously.[14] While these results show that mechanical shock trauma to target samples can be reduced by meshes and baffles, biological experiments with the helium system show these devices generally have little or no benefit in terms of increasing the number of stable transformants. We conclude that when suitable settings

and distances are used with the helium system, shock injury to cells is not a principal limiting factor for transformation efficiency. This also supports our belief that the overlying gas is more important in maintaining microprojectile velocity than in reducing shock wave intensity.

Determinants of Velocity

There are several particle gun parameters that affect velocity and that interact. These include power load (pressure), gap distance (distance from power source to macroprojectile), macroprojectile flight distance, and target distance (distance from microprojectile launch site to biological target). Obviously, higher power loads, shorter gap distances, and longer macroflight distances give higher launch velocities. Longer microflight distance reduces impact velocity, but improves particle dispersion and minimizes gas shock to cells. Use of smaller microprojectiles, reduced vacuum, or baffle/meshes also results in reduced impact velocity.

The helium-driven system is functional from 600 to 2400 psi. We have observed that 600 psi is sufficient for some degree of biolistic transformation, but is suboptimal for most applications. For most applications, 1000 psi is optimal or nearly optimal. As pressure increases toward 2400 psi velocities also increase, but the higher pressures do not necessarily result in significantly higher transformation rates. There appear to be negative effects at higher pressures, largely balancing out the advantages of higher velocities. Our "shave cream" assay indicates that the gas shock impacting the biological target increases markedly as pressures increase above 1000 psi.

In the helium system, we have tested gap distances ranging from 1 to 20 mm, and have found that shorter gap distances increase velocity, but also increase variability and off-centered macroprojectile flight. Generally, a 6- to 12-mm gap distance seems optimal in terms of effective energy transfer with minimal variability.

We know that in the gunpowder system, shortening the barrel length (acceleration distance of the macroprojectile) reduces the final velocity of the macroprojectile. In the helium system, we have tested macroprojectile flight distances of 0–20 mm. As distance increases, velocity increases as expected; however, so does variability and off-centered hits. Generally a 10-mm flight distance gives near optimal velocities, without serious variation in flight orientation or transformation frequency.

Distance to target is not very critical when using larger microprojectiles. However, when small microprojectiles are used, as is the case in bacterial transformation,[31,32] microprojectile flight distance is critical, and must be minimized to maintain adequate impact velocity.

The factors that affect velocity interact. For example, an increase in power load can compensate for increased gap distance, decreased macroflight distance, or increased microflight distance. Introduction of a baffle-mesh, or reduced vacuum, may need to be offset by shorter microflight distance or higher power load.

Safety

People who choose to build their own biolistic devices should be aware of certain hazards, and should incorporate features into their devices that make them inherently safer. All devices that have electrical components should include a ground fault interrupter mechanism, because users typically are working, often with wet hands, in a metallic, grounded environment (a laminar flow hood)! The hazards associated with gunpowder charges include premature firing due to heat or impact, exploding acceleration barrels due to barrel blockage, and ejection of high-velocity macroprojectile, cartridge, or other debris from a system that has not been fully sealed before firing. High-pressure helium can be hazardous at the tank source, or from rupture of fittings or tubing. Firing of the rupture membrane of a helium system without the benefit of enclosure or vacuum is extremely loud. Such premature firing could conceivably generate small pieces of high-velocity membrane material, or cause injury to the hearing of the user.

Microprojectile Parameters

Choice of Microprojectiles

Tungsten. Tungsten particles can be obtained in various size ranges from Sylvania (Sylvania Chemicals/Metals, GTE Products Corp., Towanda, PA). These particles are extremely irregular in shape and heterogeneous in size. Although different mean sizes range from 0.5 to 2.0+ μm, their distributions overlap extensively. The advantages of tungsten are that it is very inexpensive, it is available in numerous sizes, each size class represents a broad spectrum of particle diameters, it is easy to coat with DNA, and we have more experience with it than with other particle types. The disadvantages are that it is potentially toxic to certain cell types, it is subject to surface oxidation that can alter DNA binding, it catalytically degrades the DNA bound to it over time, and it is highly heterogeneous in shape and size, which prevents optimization of size for a particular cell type.

Gold. Gold particles are available in a very limited range of sizes from either Aesar (Johnson Matthey Aesar Group, Seabrook, NH), or from Bio-Rad. Aesar particles tend to be 2 μm or larger, while Bio-Rad particles are smaller. Currently available gold particles are much rounder and more uniform in size than tungsten. A principal advantage of gold particles is their uniformity, which allows for optimization of size relative to a given cell type—assuming one of the few available sizes happens to be optimal. An even more important advantage of gold is that it is biologically inert. Gold is not toxic to any cells we have tested, and is already approved by the Food and Drug Administration (FDA) as a human therapeutic agent. Unlike tungsten, gold does not catalytically attack DNA bound to it. A major disadvantage of gold is that it is relatively expensive. Surprisingly, gold is not stable in sterile aqueous suspensions, and over a period of time it agglomerates irreversibly; therefore it is best to prepare gold particles the day they are to be used. The uniformity of gold is undesirable in the sense that if the correct specific particle size for a certain cell type is not available, then transformation rates may approach zero. Last, while DNA can be bound to gold as well as to tungsten, from our experience gold coating is more subject to variation, associated with slight perturbations of precipitation conditions.

Other Particles. We have tried other high-density particles for use as microprojectiles. In our hands, platinum and iridium particles both yield very poor results. We do not know if the problem is that these particles are suboptimal in terms of their diameter, or if they do not coat well with DNA. We have also tried lower density particles as microprojectiles. Glass needles (~1 mm in diameter 3–30 mm long) can penetrate cell walls and enter into onion epidermal cells. Likewise, dried cells of *E. coli* and *A. tumefaciens* can be shot into living onion epidermal cells. While these lower density particles have the ability to penetrate cell walls, their reduced momentum dramatically reduces the efficiency (rate) of such penetration.

While lower density particles do not penetrate cells efficiently, they provide intriguing possible advantages. For example, dried cells such as *E. coli*, *A. tumefaciens*, and yeast make ideal biological capsules, which should be capable of delivering plasmids or minichromosomes in a naturally encapsulated form. Such encapsulation could completely eliminate problems with particle size heterogeneity, irregular DNA coating, particle agglomeration, and shearing or abrasion of DNA. Experiments indicate that dried cells of *E. coli* harboring a plant expression vector can be used to bombard tobacco or maize cell suspension cells, resulting in β-glucuronidase (GUS)-expressing tobacco cells.[46] Likewise, intact λ

[46] J. L. Rasmussen, J. A. Russell, and J. C. Sanford, manuscript in preparation (1993).

phage bearing a common yeast transformation vector in its "stuffer" region, used as projectiles to bombard yeast, yield moderate to good rates of yeast transformants.[47] Ideally, such biological capsules could be modified to increase their density so that they would penetrate the target cells more effectively.

When cells that lack cell walls or other types of outer sheath material are used as targets (such as animal cell cultures), high rates of transformation can be obtained even when using low-density particles such as silica particles (GlassMilk; Bio 101, San Diego, CA) (J. L. Rasmussen and J. C. Sanford, unpublished observations).

Particle Size. The size of the particles chosen for biolistic transformation is generally based on the size of the target cells. As a rule of thumb, particles should be roughly one-tenth the diameter of the cell. However, there are examples where this is not true. For example, for intact mouse epidermal transformation, surprisingly large particles (3.9 μm) are effective, for cells less than 20 μm in diameter. On the other hand, particles as small as 1.0 μm are very effective on primary cell cultures of myotubes (40 × 100 μm in length). A summary of available particles and their attributes and uses is given in Table I.

Coating Particles

Microprojectile coating is one of the most important sources of variation affecting biolistic efficiency. Apparently each time DNA is precipitated, its pattern of precipitation and aggregation is unique and nonreproducible. The precipitation occurs so rapidly that it is nearly impossible to obtain a uniform reaction mixture--especially because gold or tungsten particles are difficult to keep in suspension. Thus, even when we do our best to hold conditions constant we see important differences in transformation efficiency from one microcentrifuge tube (precipitation event) to another. Furthermore, we still experience fluctuations from day to day and month to month that we cannot explain (transformation rates seem to go down consistently in the summer, perhaps relating to humidity). Hopefully, superior and more reproducible coating procedures will be developed. Until then, users should strive to make the precipitation reaction mixture as homogeneous and reproducible as possible.

Various DNA-coating protocols have been published, and the essential components of these protocols are given in Table II. Of these protocols, we can best describe and critically evaluate the protocol we currently use, which is distinctly superior to our previously published protocols.

[47] J. L. Rasmussen and J. C. Sanford, manuscript in preparation (1993).

TABLE I
DIFFERENT PROJECTILES AND THEIR USES

Projectile	Diameter (μm)	Attributes	Proven applications
M5 tungsten (Sylvania)	0.1–1.0	Size heterogeneity, irregular shapes	Bacteria, yeast, possibly meristems, with high velocities
M10 tungsten (Sylvania)	0.2–1.5	Size heterogeneity, irregular shapes	*Chlamydomonas*, yeast, plant cells, animal cell cultures
1-μm gold (BioRad)	~1	Uniform in size, round	Plant cells, animal cell cultures, yeast, approximately the same as M10
1.7-μm gold (BioRad)	~1.7	Uniform in size, round	Larger plant cells, mouse skin
1- to 3-μm gold (Aesar)	1–3	Fairly uniform in size, round	Larger plant cells, mouse skin
2- to 5-μm gold (Aesar)	2–5	Fairly uniform in size, round	Mouse liver, muscle, spleen, intestine
Dried *Escherichia coli*, bearing plant vectors	~1	Very uniform in size, symmetrical	Large plant cells
λ phage with markers	~0.1	Very uniform in size, polyhedral	Yeast, plant cells
Glass fragments	~1 × 3–30	Heterogeneous, glass crystals vary in length	Large plant cells

1. To begin, 60 mg of particles is weighed out, placed in a microcentrifuge tube, and vortexed vigorously in 1 ml 70 or 100% (v/v) ethanol. Surprisingly, the brand of microcentrifuge tube can be important. Tungsten and apparently DNA can stick to the surfaces of some types of tubes. "Treff" microcentrifuge tubes (Tekmar, Cincinnati, OH) work very well. Twice we have switched to less expensive brands, resulting in a dramatic loss in efficiency that was not diagnosed until many experiments were ruined. At room temperature the particles are then soaked in ethanol for 15 min, pelleted by a 15-min centrifugation (15,000 rpm), decanted, washed three times with sterile distilled water, and brought up to a final volume of 1000 μl in a 50% (v/v) glycerol solution. These particles can be stored at room temperature for 1–2 weeks (prolonged storage can lead to oxidation of the surfaces of the particles). It was previously recommended that particles be extensively sonicated while in ethanol. We no longer feel this is beneficial, and under certain conditions can make particle agglomeration worse rather than better, especially when gold particles are used.

TABLE II
VARIOUS PROTOCOLS FOR COATING DNA ONTO MICROPROJECTILES

Protocol	Particles	DNA	$CaCl_2$ (M)[a]	Spermidine (M)[a]	Notes	Ref.
Original method (per shot)	18 μl–0.18 mg tungsten (0.008 mg)	4 μg (0.077 μg)	7.5 μl (0.25 M)	None	First method (obsolete)	2
An improved method (per shot)	25 μl–1.25 mg tungsten (0.125 mg)	5 μg (0.5 μg)	25 μl (2.5 M)	10 μl (0.1 M)	Our early method	6
Current method (per shot)	25 μl–1.5 mg tungsten (0.5 mg)	2.5 μg (0.8 μg)	25 μl (2.5 M)	10 μl (0.1 M)	Our best current method	19, 29–39, 52
DeKalb method (per shot)	Pellet—2.1 mg tungsten (0.7 mg)	245 μl/25 μg (8.3 μg)	250 μl (2.5 M)	50 μl (0.1 M)	A clear improvement over that described in Ref. 6	14
Agracetus method (per shot)	Powder—3.5 mg gold (0.324 mg)	70 μg (6.5 μg)			Involves drying DNA onto gold using a nitrogen gas stream	11

[a] The volume and molar concentration (M) of the stock solutions used to coat tungsten for three shots.

2. For convenience, sterile aliquots of 2.5 M $CaCl_2$ and 0.1 M spermidine (free-base) are stored at 4 and −20°, respectively. Surprisingly, after several months the frozen spermidine goes bad in the freezer, which on several occasions has lead to a ruinous loss of transformation efficiency, which went undiagnosed for considerable periods of time. Therefore, frozen spermidine aliquots should be made fresh at least once a month.

3. We prepare DNA at a concentration of 1 μg/μl. It appears that contaminating protein is a principal cause of particle agglomeration during coating, and has limited how much DNA can be used effectively. Ideally, DNA to be used for biolistic experiments should be put through several additional phenol extraction steps to remove all traces of protein. If the DNA is very pure and in abundant supply, the amount used for coating can be increased severalfold over what we otherwise recommend, increasing transformation rates. However, when transforming bacteria with M5 particles (effective particle size, ~0.1 μm), we find DNA concentrations should be *reduced* fourfold to 0.25 μg/μl.[32]

4. There are different views on how large an aliquot of particles should be coated at one time. For a long time, our laboratory only prepared aliquots large enough for three bombardments (see Table II). However, we now more typically use double aliquots (sufficient for six bombardments). Other laboratories seem to prepare enough particles in a single vessel for an entire experiment. It is not clear to us yet if larger aliquots yield a more or a less uniform reaction mixture. We describe our "traditional" small aliquot (three bombardment) procedure, which obviously can be increased for larger scale reactions.

5. We begin by aliquoting 25 μl of the tungsten suspension into microcentrifuge tubes. It is important to vortex *continuously* while removing aliquots of the tungsten suspension, to avoid nonuniform sampling.

6. We then add 2.5 μl of the DNA stock, 25 μl of $CaCl_2$ stock, and 10 μl of the spermidine stock, in that order, while the microcentrifuge tube is continuously being vortexed. Continuous vortexing is important to ensure a uniform reaction mixture.

7. The mixture should be allowed to react and to coat the particles for several minutes during continuous vortexing. The coated particles should then be gently pelleted by pulse centrifugation (early protocols called for hard pelleting, which leads to more agglomeration).

Loading Particles

Once particles have been coated with DNA they should be used as soon as possible. This is particularly true when tungsten particles are used, because the tungsten can degrade the DNA. If a full day of bombardment

is planned, we recommend coating particles as they are needed, two to four times during the day.

For the gunpowder-driven system, 50 μl of supernatant is then removed, leaving enough for three bombardments of 2 μl to be loaded onto each macroprojectile. Effort is made to divide the particles accurately—one-third per aliquot (which is difficult), and to place the aliquot in the exact center of the macroprojectile (which takes practice).

For the helium-driven system, *all* of the supernatant is removed, and the pellet is washed in 70 μl of 70% (v/v) ethanol. A second wash in 100% ethanol is optional. The particles are then gently pelleted and brought up in 24 μl of 100% ethanol. The resulting suspension is mixed by dipping the microcentrifuge tube in an ultrasonic cleaner (Branson 1200), and then aliquoted (6 μl) onto Kapton flying disks, again using care to take equal amounts of particles per aliquot, and to place the aliquot in the exact center of the disk. It is important that the disks have been washed in 70% ethanol before use, and are free from grease, fingerprints, and so on, to ensure uniform coating and drying. The suspension can be spread over an area 1 cm in diameter in the center of the disk, using the pipette tip. It is *crucial* that, immediately after loading, the disk be placed in a desiccator until thoroughly dry (~60 sec) and that it is kept there until immediately before use. Exposure to humidity during or after drying *dramatically* reduces transformation rates, apparently due to hygroscopic clumping and agglomeration.[32] For certain applications, the amount of particles loaded can be substantially increased.

We believe that the coated particles are relatively stable while in ethanol (at least for half a day), but that once dried they are unstable. Therefore, we dry particles onto disks as needed, and use them within 1–2 hr.

Biological Parameters

There are several biological parameters that are important for successful biolistic transformation. First, one must have an appropriate gene construct with a promoter that is strong and that will express in desired target tissue. Second, the target cells must be in a state receptive to transformation. Third, there must be high rates of particle penetration and cell survival and growth after bombardment. A detailed discussion of the biological factors that have been important in optimizing biolistic transformation of various species in our laboratory is given below. Most of this information has come from our experiments with bacteria, yeast, and plant cell suspension cultures.

Vector Constructs

Obviously, it is important that appropriate vectors be utilized in biolistic experiments. The vectors must have appropriate reporter or selective genes with appropriate promoters, and may be either replicating or integrative. The size and form of the transforming DNA should also be considered.

In plants, we routinely employ the β-glucuronidase (GUS) gene[48] as a reporter gene for evaluating transient expression. Other laboratories use luciferase or anthocyanin genes as reporter genes. For determination of stable transformation rates we routinely use the neomycin phosphotransferase (NPTII) gene,[49] which confers resistance to kanamycin. Herbicide resistance genes can also be used as selective markers.[13,14]

For dicot plants we usually use the plasmid pBI426 (obtained from W. Crosby, Plant Biotechnology Institute, Saskatoon, Canada), which has a GUS–NPTII gene fusion,[50] driven by a double 35S cauliflower mosaic virus (CaMV) promoter plus a leader sequence from alfalfa mosaic virus. This plasmid yields 10- to 100-fold more transiently transformed tobacco cells than does pBI121 (Clonetech, Palo Alto, CA), which has a GUS gene driven by a single 35S promoter and the NPTII gene driven by the nopaline synthase promoter.

In monocot species, constructs with the alcohol dehydrogenase promoter and intron[5] or the rice actin promoter[51] yield much higher numbers of transformants than do constructs with weaker promoters such as the CaMV 35S promoter.

In bacteria and yeast, we deliver autonomously replicating vectors routinely. However, autonomous replication is not essential in yeast, because very high rates of biolistic transformation are also achieved with integrative (nonreplicating) vectors.[29] Vectors bearing plant replicons are not expected to be stable, but may reasonably be expected to increase the probability of integrative events. Likewise, vectors bearing transposable elements might greatly increase the efficiency of integration,[52] which is a limiting factor in biolistic plant transformation.

Vector size does not appear to be a limiting factor. *Escherichia coli*

[48] R. A. Jefferson, T. A. Kavanagh, and M. W. Bevan, *EMBO J.* **6**, 3901 (1987).
[49] E. Beck, G. Ludwig, W. A. Awerswald, B. Reiss, and H. Schaller, *Gene* **19**, 324 (1982).
[50] R. S. S. Datla, J. K. Hammerlindl, L. E. Pelcher, G. Selvaraj, and W. L. Crosby, *J. Cell. Biochem. Suppl.* **14E**, 279 (1990).
[51] D. McElroy, W. Zhang, J. Cao, and R. Wu, *Plant Cell* **2**, 163 (1990).
[52] J. Laufs, U. Wirtz, M. Kammann, V. Matzeit, S. Schaefer, J. Schell, A. P. Czernilofsky, B. Baker, and B. Gronenborn, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 7752 (1990).

plasmid vectors are effective up to the normal size limits of such vectors (20–30 kb). λ phage vectors (50 kb) can be bound to tungsten particles and can yield high transformation rates.[47] Likewise, intact *cells* (e.g., *E. coli*) can be delivered as biolistic projectiles,[46] indicating that entire chromosomes or genomes might be delivered by this process.

Genes can be biolistically delivered as RNA[2] or DNA, in circular or linear[35] form, and as single-stranded[35] or double-stranded DNA.

Cell Age/Physiology

In general, the optimum targets for biolistic transformation are healthy cells that are receptive to transformation and that can withstand the stresses of the bombardment process. This generally means that "young," actively dividing cells are the best. However, there are exceptions to this rule and the optimum cell age of each species must be determined empirically.

When equal numbers of cells from early-log, mid- and late-log, and stationary cultures of *B. megaterium* strain 7A17 are transformed, cells from early-log phase are transformed most efficiently.[31] There is no difference in transformation efficiency between cells from midlog, late-log, and stationary cultures of *E. coli* JA221.[32] In the yeast *Saccharomyces cerevisiae*, cells from stationary cultures are most efficiently transformed.[29] For tobacco NT1 cell suspensions,[33] early log-phase cells, 4 days past subculturing, give the highest rates of transformation. Cells 6 days old (midlog phase) and older yield dramatically lower numbers of transformants. For 'Black Mexican Sweet' (BMS) corn cell suspension cultures, the frequency of cell subculturing can also affect transformability. BMS cell suspensions subcultured three times per week give higher numbers of transformants than cultures subcultured only once a week.

Cell Size

Many organisms having different cell sizes and some cell organelles have been successfully transformed with the biolistic process. Successful organelle transformation include chloroplasts of the green alga *Chlamydomonas reinhardtii* (10 μm),[34,35] chloroplasts of tobacco (5 μm),[38–40] and yeast mitochondria.[36] The bacteria *B. megaterium* (1.3 × 4.0 μm),[31] *E. coli* (1.1–1.5 × 2.0–6.0 μm), *E. amylovora* (0.5–1.0 × 1.0–3.0 μm), *A. tumefaciens* (0.6–1.0 × 1.5–3.0 μm), and *P. syringae* pv. *syringae* (0.7–1.2 × 1.5 μm)[32] have all been transformed using M5 tungsten particles. A variety of plant cells of various shapes and sizes (20–100 μm) have

[33] C. Paszty and P. F. Lurquin, *BioTechniques* **5**, 716 (1987).

also been transformed. Target cell size is a major consideration in selection of particle size and target distance (see Tables I and III).

Cell Density

Cell density is an important parameter for the transformation of both microbes and plant cell suspension cultures. Generally, a uniform lawn of cells one cell layer thick is optimal. This provides the greatest number of targets, without extraneous cells that can interfere with plating or selection.

In bacterial systems, cells that are grown in liquid culture are prepared for bombardment by centrifugation, resuspension, and then spreading them evenly over the surface of the bombardment medium. The optimum cell number per 10-cm petri plate for *E. coli* JA221 and *B. megaterium* 7A17 is 2×10^9 and 1×10^8, respectively. More transformants per plate were produced when 3×10^9 *E. coli* cells per plate were bombarded rather than 2×10^9, but transformed colonies were too dense to count.

For tobacco cell suspension cultures, we routinely collect 5 ml of suspension, which contains a 0.6-ml settled volume of cells, onto a 7-cm diameter filter paper. However, at this density the number of stable transformants can be difficult to count, and thus the cells are often diluted and replated after bombardment (see Cell Handling, Transfers, Selection below).

Osmoticum

The addition of an osmoticum (i.e., a supplemental agent increasing osmolarity) to the bombardment medium can dramatically increase the rates of transformation. We have found this to be true for all microbial species tested and for all plant cell suspension cultures, although the optimum concentration for each species varies. Elevated osmoticum concentrations may work by protecting the cells from leakage and bursting (lower turgor pressure) and may also improve particle penetration itself.

The optimum osmotic concentration for *B. megaterium* 7A17 is approximately 1.5 *M* (0.75 *M* sorbitol plus 0.75 *M* mannitol). The optimum for *E. coli* JA221 is approximately 0.6 *M* sorbitol. For bacteria, the optimum osmotic concentration is generally slightly below the toxic level for the species. One exception is *E. amylovora*, which can grow at 1.0 *M* sorbitol. This concentration of osmoticum, however, interferes with ampicillin selection for pUC118 transformation. In this case, a concentration of 0.05 *M* still significantly increases transformation efficiency while permitting selection for the ampicillin-resistant transformants. When selecting *E. coli* JA221 (Δ*trpES leu*$^-$ *hsdR*$^-$ *recA*$^-$) transformants on tryptophan dropout

TABLE III
BIOLISTIC PARAMETERS FOR DIFFERENT APPLICATIONS[a]

Species	Cell stage/ preparation	Cell density	Vacuum (in. Hg)	Helium overlay	Target distance (cm)[b]	Power	Particle	Notes (efficiency/shot)	Ref.
Escherichia coli	Log-stationary	2×10^9	29	+[c]	6	1,000	M5	30,000 colonies	32
Bacillus megaterium	Log	1×10^8	29	nt[d]	6	1,000	M5	~30,000 colonies	31
Yeast (*Saccharomyces cerevisiae*)	Stationary	1×10^9	29	+	12.3	1,500	M10	~10,000 colonies	29
Mildew (*Uncinula necator*)	Early sporulation	Not applicable	27	nt	2.8	900	M5	~0.1 stable	33
NT1	4 days after transfer (early log)	0.4 ml scv[e]	28	No	12.3	1,000	M10 or 1-μm gold	~800 colonies	52
NT1 chloroplasts	4 days after transfer	300 mg	28	nt	9.6[f]	900	M10	~800 beu[g]	39
Wheat embryos (half shoot apical region)	4- to 5-day-old seedlings	10 explants/ plate	28	No	2.8	1,300	M10	~160 beu	—
Bean embryos (apical dome)	6–12 hr imbibed	10 explants/ plate	28	No	6.0	1,300	M10	~120 beu	—
Peach embryonic axis	2 days imbibed	10 axes/plate	28	No	6.4	1,300	M10	~380 beu	—
Peach cotyledon	10 days imbibed	5 explants/ plate	28	No	9.6	900	M10	~720 beu	—
Animal cell cultures (e.g., myotubes)	Confluent	confluent—60-mm plate	15	nt	12.3	800	1-μm gold	5–50 ng[h]	41,42
Intact mouse skin	Depilatory exposure	1 cm[2]	15	No	0.2	1,200	2- to 5-μm gold	1–60 ng	44
Intact mouse liver	Surgical exposure	1 cm[2]	0	No	0.2	1,200	2- to 5-μm gold	0.2–0.4 ng	44

[a] These parameters have proved successful, but have not necessarily been optimized.
[b] Gap distance is 1.0 cm except where noted. Flying disk flight distance is 1 cm.
[c] +, Not tested.
[d] nt, Not tested.
[e] scv, Settled cell volume.
[f] With gap distance at 0.5 cm.
[g] beu, Blue expression units.
[h] Luciferase per bombardment, transient expression.

medium that had been transformed with plasmid pKRS101 (Ap^r, *trpE*), the optimum osmoticum was 0.6 *M* sorbitol. However, the cells grow slowly at 0.6 *M* sorbitol, which is not a problem with tryptophan selection, but can interfere with ampicillin selection because of formation of satellite colonies around the transformed colony. A lower concentration (0.2–0.4 *M*) of osmoticum still produces more transformants per plate than no osmoticum but also reduces the incubation time and formation of satellites as compared to 0.6 *M*.

In tobacco cell suspension cultures, 2- to 10-fold higher rates of both transient and stable transformants are obtained when at least 300–900 mOsm/kg H_2O osmoticum is included in the bombardment medium.[55] In most of our experiments osmoticum has consisted of equal molarities of mannitol and sorbitol. Because commercial sources of mannitol may be contaminated with abscisic acid[54] we have also tested the use of raffinose as an osmoticum. Thus far, we have not seen a substantial benefit of raffinose over mannitol/sorbitol. Additionally, raffinose is more expensive and is difficult to keep in solution at high concentrations.

Another benefit to using high osmotic conditions in plant cell suspension cultures is the reduction of background cell growth. If the starting osmoticum is inhibitory but not lethal, all cell growth is initially inhibited and then as the osmoticum concentration is lowered and the cells are placed on kanamycin medium, only the kanamycin-resistant colonies resume growth. For NT1 tobacco cell nuclear transformation a concentration of 0.2–0.4 *M* (i.e., 400–700 mOsm/kg H_2O) supplemental osmoticum appears to be optimal.

For transient gene expression in tobacco chloroplasts a concentration of 0.55 *M* mannitol plus 0.55 *M* sorbitol is optimal.[39]

Tungsten Toxicity

In some cases tungsten microprojectiles are toxic to the target cells. Tungsten particles added to the medium of tobacco cell suspensions reduce cell growth, even at concentrations 10–20 times lower than that delivered to a bombarded plate. At higher concentrations, tungsten can cause extensive cell death.[19] Tungsten also dramatically acidifies the culture medium. This is not the cause for toxicity in tobacco cell suspensions, but medium acidification could be a significant problem in pH-sensitive cells. Tungsten does not appear to be toxic to all cell types (e.g., *Bacillus*).

When tungsten toxicity is believed to be a problem, the best solution would be to use gold or another inert particle type, if the appropriate

[54] J. A. Russell, M. K. Roy, and J. C. Sanford, *In Vitro Cell. Dev. Biol.* **28P**, 97 (1992).
[55] H. Belefant and F. Fong, *Plant Physiol.* **91**, 1467 (1989).

size for a particular application is available. In tobacco cell suspension cultures, bombardment with 1-μm gold instead of M10 tungsten particles increases the recovery of stable transformants.[19] Where the use of gold is not possible, then reduced concentrations of tungsten in the DNA reaction mixture, or reduced loads on the macroprojectile, may be tried. Also, the tungsten concentration in the cell environment may be reduced by washing the cells/tissues soon after bombardment. For pH-sensitive cells, the bombardment medium can be buffered. Tungsten acidification of the medium of tobacco cell suspension cultures is effectively buffered by 10 m*M* 2-(*N*-morpholino)ethane sulfonic acid (MES).[19]

Cell Handling, Transfers, Selection

Bacteria. We have developed a transfer system for bacteria that allows exposure to a high level of osmoticum during bombardment and then facilitates a gradual decrease in osmoticum and imparts selection for antibiotic resistance.[32] The transfer device is a thin agar medium layer (7 ml), which is pipetted onto a piece of supporting filter paper (8-cm diameter) with an extending tab for handling (paper + agar = "pagar"). Cells are spread onto the pagar, dried, bombarded, and then the pagar is transferred on top of a selective medium (21 ml). The selective medium includes enough antibiotic so that the final concentration following diffusion into the total 28-ml volume is correct. The pagar medium should contain the desired concentration of osmoticum. The osmoticum concentration for bombardment may be high enough to slow or actually prevent growth initially, but when the pagar is layered over the selective medium diffusion gradually and gently reduces the osmotic concentration while the antibiotic is diffusing upward.

In all microbial systems tested, resuspended cells are slowly dried onto the surface of the medium shortly before bombardment. In cases in which bacteria are bombarded while the surface of the medium is still moist, transformation efficiency is reduced. Also, bombarding moist plates results in splattering of cells and medium during bombardment, and may contaminate the surfaces of the gun for other users.

Plant Cell Suspension Cultures. To prepare plant cell suspension cultures for bombardment, the cells are collected onto 7-cm filter papers (#1; Whatman, Clifton, NJ) using a Büchner funnel. The filter papers containing the cells are then placed over pagar supports in 100 × 15 mm petri plates. The pagar supports consist of a filter paper with attached tabs (for handling), covered with 10 ml of growth medium containing the desired osmoticum, and solidified with 0.25% (w/v) Gelrite (Kelco, San Diego, CA). The cells are allowed to equilibrate with the osmoticum for at least

1 hr before bombardment. During bombardment, the vacuum should not be pulled higher than 28 in. Hg (about 710 mmHg), or the pagar supports will sometimes flip out of the plates due to sudden degassing of the Gelrite. The bombarded plates are then placed in plastic boxes and are incubated in a culture room at 24° and with indirect light.

On the day following bombardment, the osmoticum in the culture medium is reduced in two gentle steps. First, the tabs are used to remove from the old petri plate the pagar supports and cells, all of which is transferred to the new petri plates containing 10 ml of Gelrite-solidified growth medium without osmoticum. Eight to 10 hr later, the pagar support and cells are transferred to new petri plates containing 20 ml of Gelrite-solidified growth medium without osmoticum.

Two days after bombardment, either transient GUS assays are performed, or the cells are transferred to selective medium. For transient GUS assays, the filter papers containing the cells are transferred to new petri plates and 1 ml of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid, cyclohexyl ammonium salt (X-Gluc) staining solution[12] is pipetted under the filter paper so as not to disturb the cells. The cells are then incubated at 37° for 24 hr, and the number of GUS-expressing blue cells are counted.

To select for kanamycin-resistant cells, the filter papers containing the cells are transferred to 100 × 15 mm petri plates containing 20 ml of NT1 growth medium with 350 mg/liter kanamycin and 0.25% (w/v) Gelrite. Kanamycin-resistant colonies begin to appear in 4 weeks.

One of the critical factors for colony growth in tobacco NT1 cells is the gaseous environment. Wrapping the plates with Parafilm (American National Can, Greenwich, CT) delays the appearance of colonies and reduces the number of colonies recovered.[33] This is likely due to ethylene accumulation in the plates. As an alternative, wrapping the plates with venting tape (Scotch Brand #394; 3M Corporation, St. Paul, MN) gives better gas exchange yet still helps reduce entry of contaminants. With venting tape, however, desiccation of the plates occurs more rapidly. Thus, the cells must be transferred to fresh medium at least every 2 weeks.

With our optimized NT1 protocol, typically 500 to 1500 kanamycin-resistant colonies can be obtained from 1 bombarded plate. However, it is impossible to count all of these colonies if the cells are left on the original filter paper disks. Thus, the cells must be replated to a lower density. The method we use is to dilute the cells 2 days postbombardment at the time of transfer to kanamycin medium. First, the filter paper containing the cells is cut into four equal parts. Each filter paper section is then placed in a 150 × 15 mm petri dish containing 40 ml of NT1 medium with 350 mg/liter kanamycin and 0.25% (w/v) Gelrite. Subsequently, 3 ml of liquid medium is pipetted onto each plate and the plates are swirled to spread

the cells uniformly. Because it is difficult to subsequently transfer the cells until colonies appear, the plates are wrapped with Parafilm to reduce desiccation.

Experimental Design Parameters

When optimizing the biolistic process, each cell system requires special considerations. However, there are some features inherent in the process that create variation for all experiments. For example, there can be major variation between different tungsten–DNA coating events as measured by particle agglomeration and transformation efficiency. To minimize this problem, our experimental designs typically block DNA-precipitation aliquots (microcentrifuge tubes) across treatments, so that a "good" or "bad" precipitation is not confounded with a treatment effect. In addition, we see significant fluctuations in agglomeration and transformation efficiency between days and over months.

When applying the biolistic process to any species or cell type for the first time, certain basic parameters must be optimized empirically in an efficient and rational manner. In nonmicrobial systems, these parameters are best studied using a rapid series of transient gene expression experiments. In microbial systems, stable transformation experiments can be used for preliminary testing, because these can be scored very soon after bombardment. Because certain parameters naturally interact, it is logical to test such parameters using a fractional factorial design.[56] We use this design to optimize the physical parameters (power load, gap distance and target distance) of the helium gun for a new application. When we test 3 values of each of 3 parameters the fractional factorial design reduces treatment size from 27 to 13. The size of such factorial experiments is limited by how many samples can be bombarded in a single day, because we do not consider contrasts between different days to be valid. There are significant variations from shot to shot, with some shots being "failures," hence at least 3 replicates are needed per treatment (preferably 5 to 10). Using a factorial design, 13 treatments (with 5 replicates each) would require only 65 bombardments, which is a reasonable-sized experiment for a single day. The data from the fractional factorial-type experiments can be analyzed using the response surface analysis procedure of SAS (Statistical Analysis System; SAS Institute, Inc., Cary, NC). This analysis gives the combination of the three parameters that would give either a

[56] O. Kempthorne, "The Design and Analysis of Experiments." Krieger, Malabar, Florida, 1983.

maximum or minimum response (number of transformants). Based on the results of the first experiment, a second fractional factorial experiment would follow, refining the optimum values for each parameter.

The optimum conditions for the biolistic transformation of plants depends on the type of tissue to be bombarded. With intact tissues such as leaves, meristems, and cotyledons, particle penetration is often the most limiting factor and higher particle velocity may be required either by using higher power load, shorter gap distance, or shorter target distance. With cell cultures, however, cell injury is generally more limiting than particle penetration and more gentle treatments are needed. Furthermore, optimal bombardment conditions depend on whether the purpose of the experiment is for transient gene expression only (e.g., for testing promoter strength) or whether stable transformants are desired. The highest transient transformation rates are generally obtained with more violent treatments, which give better particle penetration. However, these treatments may injure cells in such a way that while they can still express the gene, they may have impaired cell division or growth. Therefore, optimal treatments for stable transformation will tend to be gentler than is optimal for transient expression studies.

A general scheme for optimizing biolistic stable plant transformation is suggested below. With cell cultures, generally we screen for transient plant tranformation in our initial experiments to optimize biological parameters and then we screen stable transformation in later experiments. First, a plasmid with a strong promoter and a marker or reporter gene must be identified. We routinely use the GUS gene for transient assay experiments and the NPTII gene for selection of stable transformants. A plasmid such as pUC118 without GUS or NPTII is used as a negative control. In all experimental designs, each microcentrifuge tube used for DNA-coating particles (precipitation event) should be treated as a block, and ideally the cells from different culture flasks should be randomly distributed or blocked among treatments as well.

The size of an experiment that can be performed in 1 day depends on the time required for cell preparation. For example, with cell suspension cultures, 2 experienced people can bombard 100 plates in 1 day. A more comfortable experiment size is 60 plates/day. However, when cell preparation time is lengthy, such as with embryo dissection, the maximum experiment size may be only 20 plates. For the actual bombardment step, typically 15 to 30 plates can be done per hour (depending on the operator and the complexity of the experiment).

In plant systems the state of the tissue and the osmoticum concentration in the bombardment medium are the two most important biological

parameters to begin to optimize. Helium gun parameters that are already known to be optimum for a similar target should be used for initial experiments (see Table III).

Experiment 1

Purpose: To optimize cell type or cell stage for bombardment.

1. Prepare five to seven plates of each cell type or cell stage on appropriate medium.
2. Set helium gun parameters at 1000 psi, gap distance at 1.0 cm, target distance at 12.3 cm, and 1 cm flying disk flight distance.
3. Coat M10 particles with pB1505 plasmid (dicots) or pACGUS (monocots).
4. Bombard the plates and incubate under standard growth conditions for 2 days.
5. Stain the tissues by covering them with x-Gluc solution. The x-Gluc solution consists of 0.5 mg/ml x-Gluc dissolved in dimethyl sulfoxide (DMSO), 10 m*M* ethylenediaminetetraacetic acid, disodium salt (EDTA), 100 m*M* sodium phosphate, 0.5 m*M* potassium ferrocyanide, and 0.1% (v/v) Triton X-100.[12] Incubate at 37° for 24 hr and count the number of blue expression units (blue-stained cell clusters) per bombardment.

Experiment 2

Purpose: To determine if osmoticum in the bombardment medium increases transient transformation rates. (*Note:* This will be further optimized in experiment 5).

1. Prepare bombardment medium with equal ratios of mannitol and sorbitol at a combined concentration of 0, 0.25, 0.5, 0.75, and 1.0 *M*.
2. Distribute the type of cells or tissues selected from experiment 1 onto the prepared plates. Use six plates (one unbombarded control) per treatment. Incubate for at least 1 hr.
3. Bombard and stain using the conditions described in experiment 1.

Experiment 3

Purpose: To determine optimal helium pressure and target distance for transient and stable gene transformation. (*Note:* For tissues with a lengthy preparation time, such as meristems, divide into two experiments, transient and stable).

1. Using the best cells or tissues and optimum osmotic treatments (determined in experiments 1 and 2), prepare 33 plates to be used for transient assays and 33 plates to be used for stable selection. There will be five replicate plates per treatment plus three negative controls for both transient and stable assays.
2. Coat M10 particles with plasmid DNA containing the GUS and/or NPTII genes.
3. Bombard the plates using the gun set at three helium pressures (1000, 1300, and 1600 psi) and two target distances (5 and 12.3 cm). Incubate the plates for 2 days.
4. Stain the cells for transient assays with x-Gluc, and transfer cells for stable selection to medium with kanamycin. Determine the best treatments based on the number of blue expression units or kanamycin-resistant colonies/plants.

Experiment 4

Purpose: To determine the optimal combination of flying disk flight distance and helium pressure for transient and/or stable gene transfer. (Again, this experiment may be divided into two parts.)

1. Using the best tissue and osmoticum concentration (determined in experiments 1 and 2), prepare 48 plates to be used for transient assays and 48 plates to be used for stable selection. There will be five replicate plates per treatment plus three negative controls for both transient and stable assays.
2. Coat M10 particles with plasmid DNA containing the GUS and/or NPTII genes.
3. Bombard the plates using three pressures (ranging from the best pressure determined in experiment 3, up to 2000 psi), three flying disk flight distances (0, 1, and 2 cm) and the best target distance determined in experiment 3. Incubate the plates for 2 days, stain with x-Gluc or transfer to kanamycin medium, and count the number of blue expression units or kanamycin-resistant colonies/plants obtained.

Experiment 5

Purpose: To optimize the concentration of osmoticum in the bombardment medium for stable transformation.

Experimental design will be the same as in experiment 2 but stable transformation will be evaluated.

Experiment 6

Purpose: To determine the best particle type (tungsten or gold) for stable transformation.

When attempting to biolistically transform a *bacterium*, osmoticum concentration is the most important biological parameter. Selecting the wrong osmoticum concentration for the initial experiments can mean the difference between 0 and 2000 transformants per plate. Begin by using physical gun parameters (power load, 1000 psi; gap distance, 1.0 cm; target distance, 6.0 cm) and biological parameters (growth phase, logarithmic; cell density, 2×10^9 cfu/plate) that are already optimized for *E. coli* JA221.[32] In the first experiment determine the range of osmoticum that produces transformants. When we used this approach to transform *E. amylovora*, *E. stewartii*, *P. syringae* pv. *syringae*, and *A. tumefaciens* we were able to transform cells in our initial experiment and determine a range of osmoticum for successful transformation.[32] Biolistic transformation of bacteria differs from plant transformation in that M5 tungsten particles are used as well as a helium flush of the vacuum chamber.

Prior to the first bombardment experiment, a method for selecting transformants must be chosen. Either direct selection (auxotrophic marker or antibiotic marker) or indirect selection (agar overlay containing antibiotic for antibiotic marker, or pagar for antibiotic markers) can be used. Also, an upper limit of osmotic concentration can be determined that allows growth of the recipient bacterium, narrowing the range of concentrations to be tested.

Bacterial Experiment 1

Purpose: To determine the approximate range of osmoticum necessary for transformation of the bacterium.

1. Prepare selective medium with 0 *M* osmoticum, three treatments of sorbitol, and three treatments of mannitol at concentrations between 0 *M* and a concentration close to the concentration that prevents growth. Use 3 plates and 1 control per treatment, a total of 28 plates.
2. Spread 1×10^9 cells/plate from a logarithmic growth culture and allow the plates to dry slowly before bombardment.
3. Control plates are prepared by mixing the DNA-coated tungsten with bacterial cells, which are then spread on the plate surface. The control plates are exposed to vacuum only and no helium blast.
4. Set the helium gun parameters: 1000 psi; gap distance, 1.0 cm; target distance, 6.0 cm.
5. Coat M5 tungsten particles with plasmid DNA.
6. Bombard plates, incubate at appropriate temperature, count putative transformants, and determine whether sorbitol or mannitol produces more transformants per plate and the approximate optimum concentration range.

7. Transformation can be confirmed by plasmid isolation, restriction digest, and visualization by agarose gel electrophoresis.

Bacterial Experiment 2

Purpose: Optimize biolistic parameters.

Prepare a fractional factorial design experiment to determine the optimum helium gun parameters. Test helium pressure (1000, 1300, and 1600 psi), target distance (6.0, 9.2, and 12.3 cm), and gap distance (low, middle, and high). Use medium that contains the osmoticum concentration determined in experiment 6a to give the greatest number of transformants per plate. Repeat using parameters suggested by the results of experiment 6b.

Using the optimum parameters determined in experiments 6a and 6b, optimize the biological parameters in the following experiments.

Bacterial Experiment 3

Purpose: Optimize the osmoticum concentration.

Bacterial Experiment 4

Purpose: Optimize the culture growth phase (early, middle and late logarithmic, and stationary).

Bacterial Experiment 5

Purpose: Optimize the cell density per plate.

Summary

The biolistic process is still rapidly evolving. We do not anticipate further major improvements in biolistic apparatus. There will probably still be further major improvements in particles, DNA coating, and vectors, as well as significant further advances in understanding of biological determinants of cell penetration and survival. The technology has currently reached the point at which it can be readily and reliably used for a wide range of applications. Given the information presented in this chapter, new applications can be optimized fairly readily.

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Development of the particle inflow gun for DNA delivery to plant cells *

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Summary. A simple and inexpensive particle bombardment device was constructed for delivery of DNA to plant cells. The Particle Inflow Gun (PIG) is based on acceleration of DNA-coated tungsten particles using pressurized helium in combination with a partial vacuum. The particles are accelerated directly in a helium stream rather than being supported by a macrocarrier. Bombardment parameters were partially optimized using transient expression assays of a ß-glucuronidase gene in maize embryogenic suspension culture and cowpea leaf tissues. High levels of transient expression of the ß-glucuronidase gene were obtained following bombardment of embryogenic suspension cultures of corn and soybean, and leaf tissue of cowpea. Stable transformation of embryogenic tissue of soybean has also been obtained using this bombardment apparatus.

Abbreviations: 2,4-D = 2,4-dichlorophenoxyacetic acid, PCV = packed cell volume, GUS = ß-glucuronidase, NOS = nopaline synthase.

Introduction

Particle bombardment offers a rapid method for delivery of DNA to plant cells for both transient gene expression (Klein *et al*, 1987) and stable transformation studies (Klein *et al.*, 1988). The main benefit of this method is that intact plant tissues can serve as the target. Most of the reports on particle bombardment utilize the same basic concept for particle acceleration and delivery: a force provided by either an explosion or expansion of compressed gas propels a macrocarrier holding particles towards an immobile object which retains the macrocarrier but permits the particles to pass. This force can be generated by a 22 caliber power load (Sanford *et al.*, 1987), a high voltage electric discharge (McCabe *et al.*, 1988), and a release of either compressed air (Oard *et al.*, 1990) or helium (Johnston *et al.*, 1991). The immobile object can be a plate with a small orifice or a screen. Recently, devices that accelerate particles directly in a stream of helium (Takeuchi *et al.*, 1992) or carbon dioxide or nitrogen (Sautter *et al.*, 1991) have been described. Although the flowing helium device (Takeuchi *et al.*, 1992) is very simple and inexpensive, severe tissue damage can result from the manual release of the particles without the benefit of a vacuum. Micro-targeting (Sautter *et al.*, 1991) has much potential but the components of this device may be difficult to obtain.

The major factor limiting particle gun technology is accessibility of devices due to high cost and complexity. The development of an inexpensive and efficient device that is simple to build and operate would aid in the distribution and utilization of this technology.

Materials and Methods

Particle Inflow Gun Design: The Particle Inflow Gun (PIG) was constructed using equipment and supplies that were readily avsilable from equipment supply companies. The vacuum chamber (Fig. 1a) was welded together from 6.4 mm steel plate and measured 16.5 x 16.5 x 30.5 cm. The vacuum chamber was painted to prevent oxidation of the metal. The front of the vacuum chamber was left open and ground smooth to provide a good seal with the door. The door was constructed from 2.5 cm thick plexiglass and a 6.4 mm thick neoprene rubber gasket was glued to and recessed in the door. The plexiglass door was attached to the steel box with two hinges. Two collars were welded into holes drilled in the top and side of the box. All of the fittings used in construction of the PIG were 1/4 inch (6.4 mm) I.D. National Iron Pipe. The vacuum/gauge/vent assembly, which consisted of two high pressure needle valves (Trerice; Detroit, MI, #735-2) and a vacuum gauge (Marshall town, Hastings, NE, #G14489), was connected to the collar in the side of the box (Fig 1a) using a cross fitting. The needle valves were rated to tolerate 140 PSI while the vacuum gauge displayed the vacuum settings down to 30 in. Hg. A 2-way solenoid (ASCO, Florham Park, NJ, Red Hat II, #JKF8262G22, with type I splice box) was connected to the collar on the top of the chamber (Fig 1b). The solenoid was controlled by a timer relay (National Controls Corp., #Q2F00001321, timer duration of 50 msec to 2 sec) set for the minimum timer duration of 50 msec. A copper line from a helium tank set at 40-80 PSI was connected to the other opening of the solenoid.

On the inside top of the vacuum chamber, a stainless steel, male Leur-lok needle adaptor (Clay Adams, Parsippany, NJ, #7553) was connected to the collar using a compression fitting (Fig 1b). A 13 mm stainless steel (Fisher Scientific, Pittsburgh, PA, #09-753-10A) or plastic (Gelman, Ann Arbor, MI, #4317) syringe filter unit could be readily attached to and removed from the device using the Leur-lok needle adaptor. A 9.5 mm plexiglass insert was designed to fit just inside of the vacuum chamber and grooves for a plexiglass shelf were cut into the left and right walls of the insert at every 1.5 cm.

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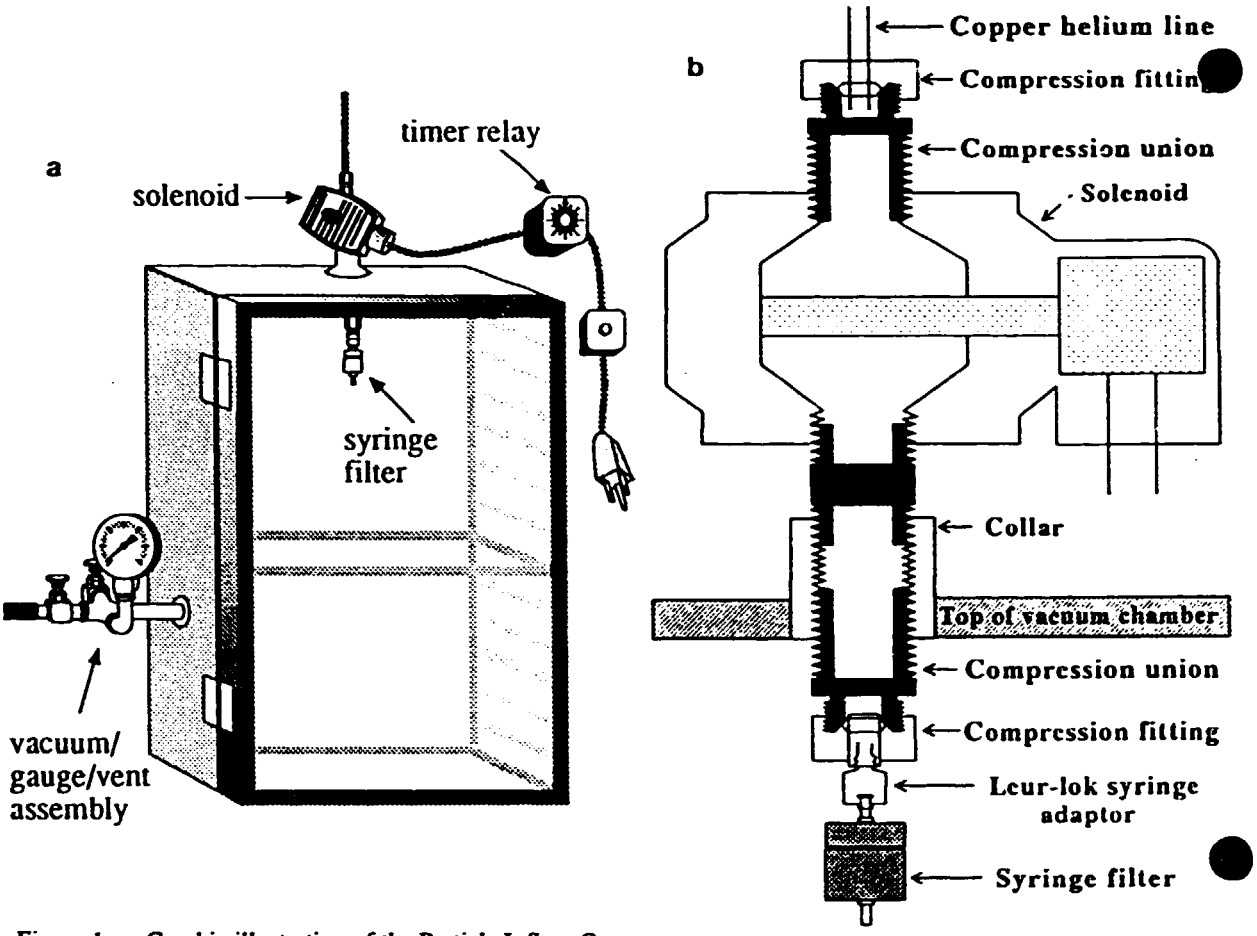

Figure 1. a. Graphic illustration of the Particle Inflow Gun.
b. Schematic showing connections from the helium line through the syringe filter. Prior to bombardment, 2 μl of DNA-coated particles was placed on the screen in the syringe filter.

Plant Tissue Preparation: Embryogenic suspension culture tissue of soybean (*Glycine max* Merrill cv "Fayette") was prepared as previously described (Finer and McMullen, 1991). One gram of tissue was placed in the center of a 3.5 cm Petri dish and the excess medium was removed. The tissue was placed uncovered in a hood for 15 min to facilitate partial drying.

Type II embryogenic callus cultures of corn (Zea mays A188 x B73) were initiated and maintained on $AgNO_3$-containing medium as described previously (Vain *et al*, 1989). Embryogenic suspension cultures were initiated from type II embryogenic callus in a medium containing MS salts (Murashige and Skoog, 1963), B5 vitamins (Gamborg *et al.*, 1968), 2% sucrose, and 1.5 mg/l 2,4-D (pH 5.7). The suspension cultures were maintained in 125 ml DeLong flasks by weekly subculture of 10-20 μl PCV of tissue into 30 ml of fresh medium. Corn suspension cultures were maintained in the light (30 $\mu Em^{-2}s^{-1}$; 16 hr day) at 150 rpm. Prior to bombardment, embryogenic corn cells (100-300 μl PCV) were filtered through either a 500 μm or 1 mm filter and evenly dispersed on a 7 cm filter paper disc (Whatman #4). Discs were stored on the maintenance medium solidified with agarose for short periods of time.

Leaf tissue of cowpea (*Vigna unguiculata*) was obtained from greenhouse-grown plants. Plants were grown under natural lighting from April to October and with supplemental lighting (12 hr day) from November to March.

DNA Constructions: For transient expression studies of soybean and cowpea, pUCGUS (CaMV35S promoter:GUS coding region: NOS terminator) was utilized (Finer and McMullen, 1990). For optimization of transient expression in embryogenic corn cells, pGB5 (CaMV35S promoter:*Sh-1* intron:GUS coding region:NOS terminator) was constructed. This plasmid was made by insertion of 1028 bp of the *Sh1* first intron/exon junctions (Vasil *et al.*, 1989) into the *Bam*HI site of pUCGUS after both the intron fragment (plus linker sites) and *Bam*HI-cleaved pUCGUS ends were made blunt with T4 polymerase.

The plasmid pHygr which contained a gene for resistance to the antibiotic hygromycin was constructed by first ligating the CaMV35S promoter as a HindIII-BamHI fragment from pBI121 (Jefferson *et al.*, 1987) into *Hin*dIII/*Bam*HI cleaved pUC119 to generate pCaMV. The NOS terminator was then ligated into pCaMV as a *Sst*I/*Eco*RI fragment from pBI121 to generate pCaMV-NOS. Finally, the *Bam*HI fragment from pLG90 (Gritz and Davies, 1983) that contained the coding region for the hygromycin resistance gene was ligated into the BamHI site of pCaMV-NOS. For stable transformation of soybean, pUCGUS was mixed with pHygr at 9:1 prior to DNA precipitation.

Particle Bombardment using the PIG: Tungsten particles (M10; provided by Sylvania Chemicals/Metals, Towanda, PA) were sterilized in ethanol and the DNA was precipitated according to Finer and McMullen (1991) for soybean and cowpea. For corn, 20 μl of DNA (20 μg), 10 μl of tungsten (1 mg), 25 μl of 2.5 M $CaCl_2$, and 10 μl of 100 mM spermidine were mixed and placed at 4°C. After 5 min, 45 μl of supernatant were removed and discarded.

For bombardment, 2 μl of the particle suspension was placed in the center of the screen in a disassembled syringe filter unit. The syringe filter unit was reassembled and then screwed into the needle adaptor. Plant tissue, in Petri dishes, was placed on the adjustable shelves at distances of 14, 17, 20, and 23 cm from the screen in the

syringe filter unit. The tissue was bombarded either unprotected or covered with a baffle. The baffles were made of either 1 mm or 500 μm nylon screens (Tetko, Inc., Elmsford, NY) and were placed either directly on top of the tissue or at a distance of 9 cm above the tissue. The baffle that was positioned 9 cm above the tissue was made by cutting off the bottom of a 400 ml disposable polypropylene beaker and attaching a screen to the bottom of the beaker. The beaker was then inverted and placed over the tissue prior to bombardment. A vacuum of 28-30 in Hg was applied and the particles were discharged when the helium (at 40-80 PSI) was released following activation of the solenoid by the timer relay.

Post-Bombardment Treatments: Embryogenic soybean and maize suspension cultures and cowpea leaf tissue were stained for transient GUS activity 2 days following bombardment according to Jefferson (1987). Selection for stably-transformed soybean lines, DNA extraction from those lines, and Southern hybridization analyses were performed as described previously (Finer and McMullen, 1991).

Results and Discussion

Particle Inflow Gun Development:

The PIG contained the following features that made it much easier to construct and operate than other devices and contributed to consistent results. 1) Expansion of compressed helium was used to propel the particles (Johnston *et al.*, 1991). This method is superior to other propulsion methods because helium is inert, leaves no residue, and gives reproducible acceleration. 2) A timer relay-driven solenoid (Morikawa *et al.*, 1989) was used to release the helium. The solenoid was more accurate than a syringe stopcock (Takeuchi *et al.*, 1992) and required no setup compared to a membrane rupture system (Johnston *et al.*, 1991). Moreover, the solenoid functioned at low helium pressures, which allowed good particle penetration and may have been less damaging to the target tissue. 3) The particles were directly accelerated in the helium stream (Sautter *et al.*, 1991, Takeuchi *et al.*, 1992) rather than being supported by a macrocarrier. Specially-constructed macrocarriers and stopping plates or screens were therefore not necessary. In the absence of such consumables, the cleanup time and therefore cycle time for each bombardment (2-3 min.) was reduced. 4) A vacuum chamber was used to hold the target tissue. The vacuum reduced the drag on the particles and lessened tissue damage by dispersion of the helium gas prior to impact. The vacuum also contributed to the pressure differential, which may have been responsible for the efficient particle acceleration.

Particle Distribution:

In order to measure effective particle penetration and transient expression, leaf tissue of cowpea was subjected to particle bombardment. Cowpea leaf tissue was selected because it gave more consistent transient expression than leaf tissue of other plants. The thousands of GUS-positive foci obtained with cowpea leaf tissue were not quantified due to confluence of the spots.

With no baffle, cells exhibiting transient GUS expression were mostly restricted to a 3.5 cm diameter ring that was 3-4 mm thick (Fig 2a). The only tissue that expressed GUS within this ring was veinal tissue and the expression was deep within the tissue, indicating subsurface penetration of particles and damage to the surface tissues. Most of the non-veinal tissue in the center of this ring appeared to be damaged. This central necrotic area is similar to that reported by Klein *et al.* (1988) for tobacco leaves and suspension culture cells.

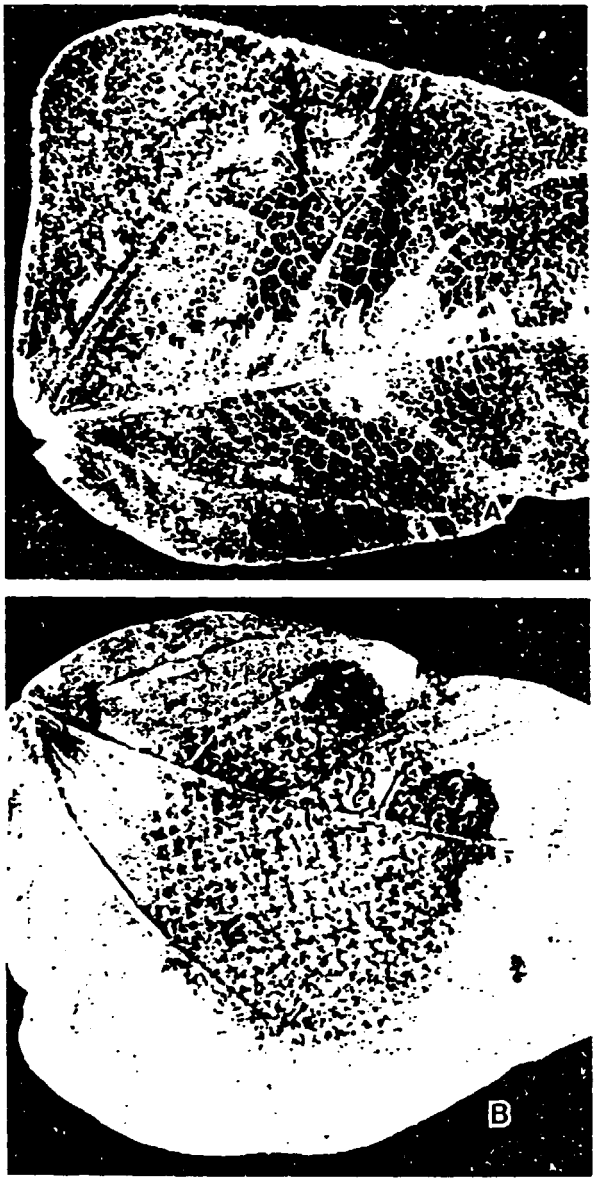

Figure 2. a. Transient expression of the GUS gene in leaf tissue of cowpea bombarded with no baffle (80 PSI, tissue 17 cm from syringe filter). b. Transient expression with 1 mm baffle placed directly on the leaf tissue. Note the differences in GUS activity staining patterns on the periphery and interior of the bombarded leaf.

Use of the 1 mm baffle directly on top of the cowpea leaf tissue during bombardment resulted in a striking alteration in distribution of GUS expression (Fig. 2b). In the centermost portion of the blast, GUS-expressing cells reflected a positive impression of the screen while on the periphery of this area, the GUS-expressing cells gave a negative impression. This distribution indicated that in the center of the bombarded area, the particles moved around the fibers of the screen and penetrated the cells that were protected or shielded

PCV (μl)	filtration (μm)	pressure (PSI)	distance (cm)	baffle (500 μm)	blue foci[1]
300	<1000	**40**[2]	17	-	781 [b3]
300	<1000	**50**	17	-	1031 [b]
300	<1000	**60**	17	-	1105 [b]
300	<1000	**70**	17	-	1258 [ab]
300	<1000	**80**	17	-	1890 [a]
100	<1000	70	17	-	480 [b]
300	<1000	70	17	-	1258 [a]
100	**<1000**	60	17	-	522 [b]
100	**<500**	60	17	-	1258 [a]
100	<500	60	**14**	-	935 [a]
100	<500	60	**17**	-	1302 [a]
100	<500	60	**20**	-	1236 [a]
100	<500	60	**23**	-	1344 [a]
100	<500	60	17	-	1516 [a]
100	<500	60	17	+	1440 [a]

Table I. Effect of factors on transient expression of the GUS gene in embryogenic suspension culture tissue of corn. [1]Each value represents the mean of from 4 - 11 bombardments. [2]Parameters in bold type were varied within experiments. [3]Values followed by different letters are significantly different at P=0.05 by one way analysis of variance.

from the blast by the screen. The leaf cells in the areas between the fibers of the screen were apparently insufficiently protected and did not survive the impact of the blast. On the periphery of the ring, the GUS-positive regions were located between the fibers while the GUS-negative regions were shielded from the particles by the screen. It was not determined if the damage in the center of the blast resulted from the particles, the solution carrying the particles, or the helium burst. Although baffles have been used to obtain stable transformation in cotton (Finer and McMullen, 1990), corn (Gordon-Kamm *et al.*, 1990), and soybean (Finer and McMullen, 1991), the specific effect of these baffles were not well-documented.

Optimization of Bombardment Parameters:

Due to the simplicity of the PIG, it was necessary to modify only a few parameters for optimization. Bombardment parameters were optimized using embryogenic maize suspension cultures and the initial conditions were as follows: 60 PSI helium, 17 cm distance from syringe filter to tissue, 300 μl PCV of cells, cells filtered through 1 mm filter, no baffle, and 2 μl of the particle preparation.

The effect of different pressures of helium is shown in Table I. Transient expression of the GUS gene increased with increasing pressure up to a maximum average of 1,890 blue foci per shot with 80 PSI. However, at the highest pressure, many cells were dislodged from the Petri dish and a pressure of 60-70 PSI was selected for future experiments.

In comparing 100 μl versus 300 μl PCV of target cells (Table I), the number of blue foci increased in proportion to the PCV. At a PCV of 500 μl, 2,698 blue loci were obtained from a single shot. This largest PCV gave the greatest confluence of cell clumps and the most surface area for targeting. Because small amounts of embryogenic tissue are generally more suitable for liquid culture (Finer and McMullen, 1990, 1991), we examined the use of smaller clumps of tissue, which could provide a large surface area for targeting while maintaining a low PCV. Filtration of the suspension culture tissue through a 500 μm instead of a 1 mm filter led to a 2.6-fold increase in transient expression of the GUS gene (Table I). In addition to providing a large target area for bombardments, the small clumps of tissue were less likely to be dislodged from the filter disc from the impact of the bombardment. Large clumps of tissue protrude further from the filter paper disc and may therefore be more vulnerable to displacement by the helium burst.

Evaluation of various distances between the syringe filter and the tissue surprisingly revealed no significant differences in bombardment efficiency (Table I). This is in contrast to earlier experiments, using larger cell clumps (<1 mm), where cell loss was greater at shorter distances (unpublished). Also surprising was the absence of a beneficial effect from use of a 500 μm baffle placed 9 cm above the tissue (Table I). It was anticipated that the baffle would give an effect similar to that seen with the cowpea leaf tissue. However, parameters such as the size and location of the baffle, as well as the pressure utilized for the corn bombardments were different from those used for the cowpea leaves.

A comparison between the stainless steel and plastic syringe filter units (Takeuchi *et al.*, 1992) gave insignificant differences in the number of blue foci obtained per bombardment (unpublished). Plastic syringe filters were preferred because of lower cost and lower reactivity of the plastic with solutions. Upon repeated

usage of the stainless steel filter units, oxidation of the metal was apparent in the funnel area below the screen. This oxidation may affect particle acceleration and helium flow. Although the plastic filters units were only rated to withstand 40 PSI (stainless steel units were rated at 100 PSI) such pressures were never present in the filter unit itself as it was simply a holding or flow-through device for the particles.

Transformation of Soybean:

An average of 11.5 stably-transformed, hygromycin-resistant clones were obtained for each bombardment of embryogenic soybean tissue. Every hygromycin-resistant soybean clone that has been obtained and analyzed to date (Finer and McMullen, 1991; and unpublished) contained the introduced hygromycin resistance gene. This indicated good efficacy in the hygromycin selection scheme. The number of clones obtained using the PIG was over 3 times that reported earlier for soybean (Finer and McMullen, 1991) using an older gunpowder version of the commercially-available particle gun (Biolistics, Model BPG). Transient expression of the GUS gene in embryogenic soybean tissue increased 2.5-fold, from an average of 709 blue foci with the gunpowder version to 1,812 with the PIG.

Southern hybridization analysis of a hygromycin-resistant, GUS-positive clone that was obtained using the PIG confirmed the presence of the intact expression unit for both the GUS and hygromycin resistance genes (Fig. 3). Based on the number of hybridization bands obtained with *Hin*dIII-digested DNA and the intensity of the hybridization signals, we estimate the presence of 3-4 copies of the GUS gene and only one copy of the hygromycin resistance gene. A higher copy number for the GUS gene was expected because pHygr and pUCGUS plasmids were mixed at 1:9 prior to DNA precipitation for bombardment and more of the pUCGUS plasmid was present for recombination and integration. Digestion of genomic DNA with *Bgl*II-restricted DNA (plasmids do not contain *Bgl*II restriction sites) revealed a high molecular weight band, indicating integration of the introduced DNAs into plant genomic DNA.

Conclusion

We report here on the development and partial optimization of the Particle Inflow Gun. This device was simple to operate, inexpensive and performed comparably to commercial devices. After preliminary optimization of this device for transient GUS expression, the factors that had the greatest influence on transient expression of the GUS gene in embryogenic corn tissue were the cell preparation methods. Stable transformation of embryogenic soybean has been obtained using this device and efforts are underway to generate transgenic corn plants.

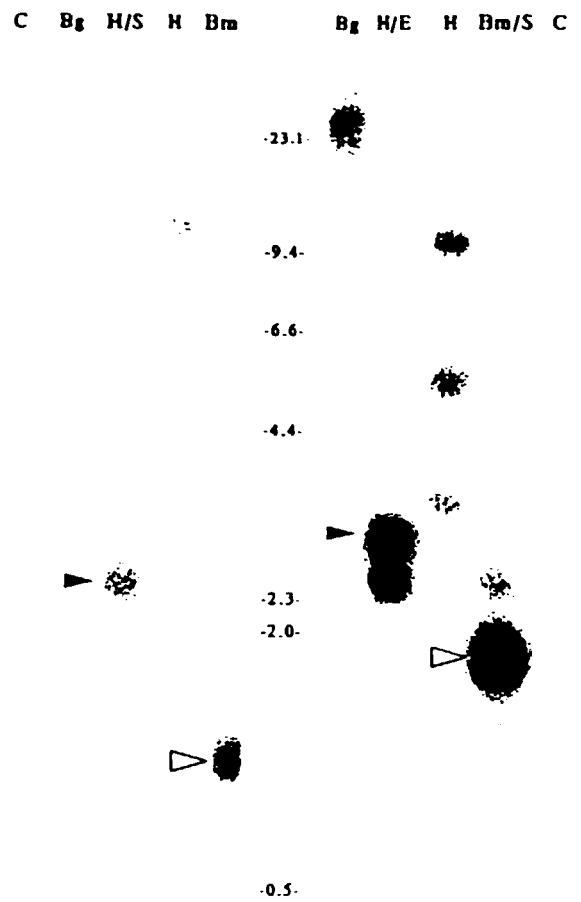

Figure 3. Southern hybridization analysis of a transformed soybean clone. DNA from nontransformed soybean cultures (C) was digested with *Hin*dIII. DNA from transformed soybean (all other lanes) was digested with various combinations of *Hin*dIII (H), *Bgl*II (Bg), *Sst*I (S), *Bam*HI (Bm), and *Eco*RI (E). *Hin*dIII digests pHygr (5.2 kb) and pUCGUS (6.1 kb) once and there are no restriction sites for *Bgl*II. *Hin*dIII/*Eco*RI cleaves the intact 2.9 kb expression unit for the GUS gene (right black arrow) and *Hin*dIII/*Sst*I cleaves the intact 2.2 kb expression unit for the hygromycin resistance gene (left black arrow). Digestion with *Bam*HI/*Sst*I and *Bam*HI releases the intact coding unit for the GUS gene (1.8 kb, right white arrow) and the hygromycin resistance gene (1.0 kb, left white arrow) respectively. The DNA on the right side of the figure was probed with the coding unit for the GUS gene and the DNA on the left side of the figure was probed with the coding unit for the hygromycin resistance gene.

Acknowledgments: The authors wish to thank Curt Hannah (University of Florida) for supplying the clone for the *Sh1* intron, and Kevin Simcox and Roy Gingery for their critical review of this manuscript. We also thank Jeff Imhoff and Jake Yoder for helpful suggestions on design and construction of the PIG and Lauren Karr for the graphic illustrations. JJF wishes to gratefully acknowledge Noel Keen (University of California at Riverside) for his openness in allowing early exposure to the flowing helium device. Financial support for this project from Limagrain Genetics and Nickerson Seeds is greatly appreciated. Salaries and additional research support were provided by State and Federal funds appropriated to OSU/OARDC and USDA-ARS. Mention of trademark or proprietary products does not constitute a guarantee or warranty of the product by OSU/OARDC or USDA, and also does not imply approval to the exclusion of other products that may also be suitable. Journal Article No. 34-92

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POLYMERASE CHAIN REACTION

INTRODUCTION

The polymerase chain reaction (PCR) method of DNA amplification has clearly revolutionized, and replaced, many gene cloning and analysis procedures. PCR allows the rapid amplification of target DNA sequences by using a thermostable DNA polymerase and a set of DNA convergent primers (1). Up to a 10^6 fold amplification of target sequences can be obtained in a single PCR run. The purpose of this lecture is to describe some uses of PCR in gene isolation, and thus the primary focus will be on using PCR for amplifying mRNA molecules.

BASIC PCR

Several recent reviews on PCR techniques are available to provide background information (2-4). Also, a collection of various PCR protocols has recently been published (5). Starting with a single-strand DNA molecule as a template, the molecule is: first denatured by a brief (40 to 60 seconds) exposure to 94° C temperature, then cooled to a lower temperature (40° to 60° C) to allow hybridization of a oligonucleotide primer (primer # 1), and then the ss-DNA: Primer #1 hybrid is incubated at 71° C to allow *Taq* Polymerase to synthesize a DNA strand complementary to the ss-DNA. The resulting double-strand (ds) DNA molecule is then denatured, and a second oligonucleotide primer (primer # 2) primes *Taq* Polymerase mediated synthesis of new copy of the original ss-DNA molecule. This cycle of denaturation, primer hybridization, and DNA synthesis is repeated 25 to 40 times in a single PCR run.

PCR FOR GENE ISOLATION

A. Amplification of mRNA populations.

PCR amplification of cDNA molecules is performed by a modification of the RACE procedure of Frohman et al. (6). Figure 1 presents a schematic of this technique. First-strand cDNA is made from poly A+ RNA using reverse transcriptase and an oligo dT primer. After mRNA hydrolysis the 3' ends of the cDNA molecules are "tailed" with dA residues by using terminal transferase and dATP.

Terminal transferase reaction:

	cDNA (20 ng)
	d.H2O
6 ul	5X terminal transferase buffer
1 ul	5 mM dATP
1 ul	terminal transferase (500 units/39 µl)
30 ul	total volume

The reaction is incubated at 37°C for 8' after which the enzyme is heat killed at 65°C for 15'. 70 ul of d.H2O are added to make the concentration of tailed cDNA 0.2 ng/ul. This tailed cDNA is then amplified by PCR using an oligo-(dT) primer that also includes a Hind III restriction site:

PCR amplification reactions:

Primer: '5-CCCGGGAAGCTTAAGCTTTTTTTTTTTTTT-3'

8 ul	tailed cDNA (1.6 ng)
5 ul	10X PCR Buffer
5 ul	DMSO
4 ul	dNTP's @ 25 mM each
1 ul	primer @ 1.65 µg/µl
27 ul	d.H2O
50 ul	

This mixture is incubated at 95°C for 5', put it in a 37°C water bath, and 0.5 ul *Taq* polymerase (5 ul from Perkin-Elmer) is added. After layering 35 ul mineral oil on top of the reaction mixture, the tailed cDNA molecules are subjected to PCR amplification as follows: 1 cycle of 60°C x 40' (second strand synthesis), followed by 35 cycles of 95°C x 1' (denaturing), 47°C x 2' (annealing), 65°C x 0.5' (initial elongation at this temperature because the primer makes many weak A:T bonds with the cDNA), and 71°C x 3' (more elongation). After 35 cycles one 15' incubation at 71°C is included to finish off any incompletely polymerized strands. The aqueous phase of the reaction mixture is put through a Sephadex G50 column to clean it up prior to using this material in other reactions.

At this point the PCR amplified ds-cDNA can be used as substrate for slot blot analysis, or can be radioactively labelled by random-priming or nick-translation to make hybridization probes. Such uses are invaluable when only small amounts of mRNA are available for analysis. However, if the goal of cDNA amplification is to make a cDNA library from the amplified material, then further manipulation of the samples is required before cloning into the vector of choice.

B. Amplification of mRNA populations for cDNA cloning.

Many of the amplified ds-cDNA molecules that result from the above procedure have unusual structures due to limitations in the amounts of nucleotides and primers, and due to the complementary sequences at the ends of each molecule. While the amplified DNA can be used for making probes, etc., attempts to efficiently clone these molecules have failed, even after digestion with HindIII to produce protruding single-strand end sequences. Successful cloning of ds-cDNA amplified in this manner requires two PCR cycles following the addition of fresh primer and nucleotides. The resulting ds-cDNA molecules are then purified by Sephadex G-50, digested with HindIII to create "sticky" ends, and ligated into a vector cut with HindIII.

C. PCR amplification of specific genes from mRNA.

The RACE procedure for the amplification of cDNA was originally designed for amplifying specific gene sequences (see ref. 6), and has proven to be very useful in obtaining homologous genes from different plant species. This method requires that some DNA sequence information is known from a region of the gene that is likely to be conserved between species. As shown in Figure 2, the 3' RACE procedure allows the amplification of the 3' end of a gene based on one internal sequence primer, and one poly (dT) primer which hybridizes to the poly (A) tail of the mRNA. Depending upon the quality of the internal sequence information, it may be necessary to design a degenerate internal primer, or to design two internal primers such that second primer is "nested" within the region between the first internal primer and the poly (A) tail. Creating "nested" primers can greatly increase the specificity of the sequence isolated by demanding that two independent PCR runs amplify the same target gene. While PCR amplification conditions will need to be determined for each set of primers used, it should be noted here that due to the high (dT) content of the primer which hybridizes to the poly (A) tail, the internal primers need to have a dA:dT composition similar to that to the (dT) primer. Cloning and confirmation of the desired product, as will be discussed, requires several independent PCR runs in order to avoid PCR artifacts due to the relatively low replication fidelity of the PCR technique.

D. PCR as a technique to measure gene expression in plants.

The 3' RACE procedure described above can be used as a very sensitive measure for the occurrence of specific gene transcripts. By using a gene-specific internal primer and a poly (dT) primer it is possible to amplify sequences from a specific target mRNA in a solution of total nucleic acids. The preparation of total nucleic acids can be from a very small amount of tissue, even from a single cell.

By using the 3'RACE method one can obtain a rough, qualitative measure of the expression of a particular gene. Results from this procedure must be carefully interpreted, however, because the PCR process itself can introduce large differences in the degree of amplification. If one requires a more accurate determination of gene expression patterns, then the technique of "competitive PCR" (7) should be used. As shown in Figure 3, competitive PCR is based on the co-amplification, in the same reaction tube, of the endogenous target cDNA (made from the mRNA population to be measured) and an altered template DNA that uses the same internal and poly (dT) primers as the endogenous cDNA (see Figure 4). The altered template may be created from a cloned version of the gene to assayed by creating a deletion or insertion, or a change in a restriction enzyme site (7). After the PCR run the amplified endogenous sized molecules are separated from the competitive template molecules by gel electrophoresis, and the ratio of the two is compared. When the ratio is 1:1, the starting concentration of the endogenous cDNA can be determined given that the input concentration of the competitive template is known. This quantitative method is able to easily detect 2 to 5 fold changes in the level of transcripts, and is very useful for measuring changes in gene expression in tissue samples which are difficult to obtain.

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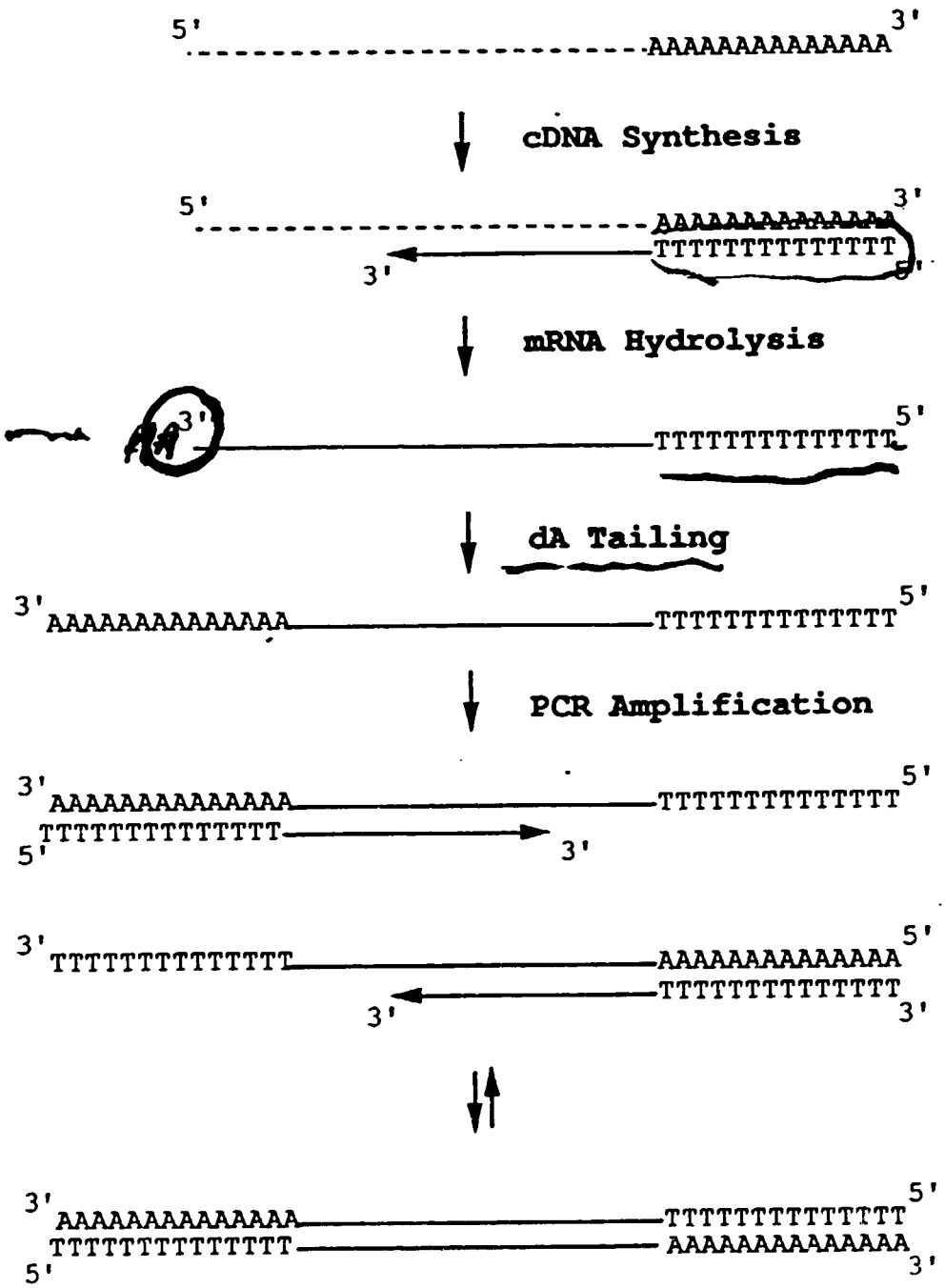


Figure 1. PCR amplification of a population of cDNA molecules.

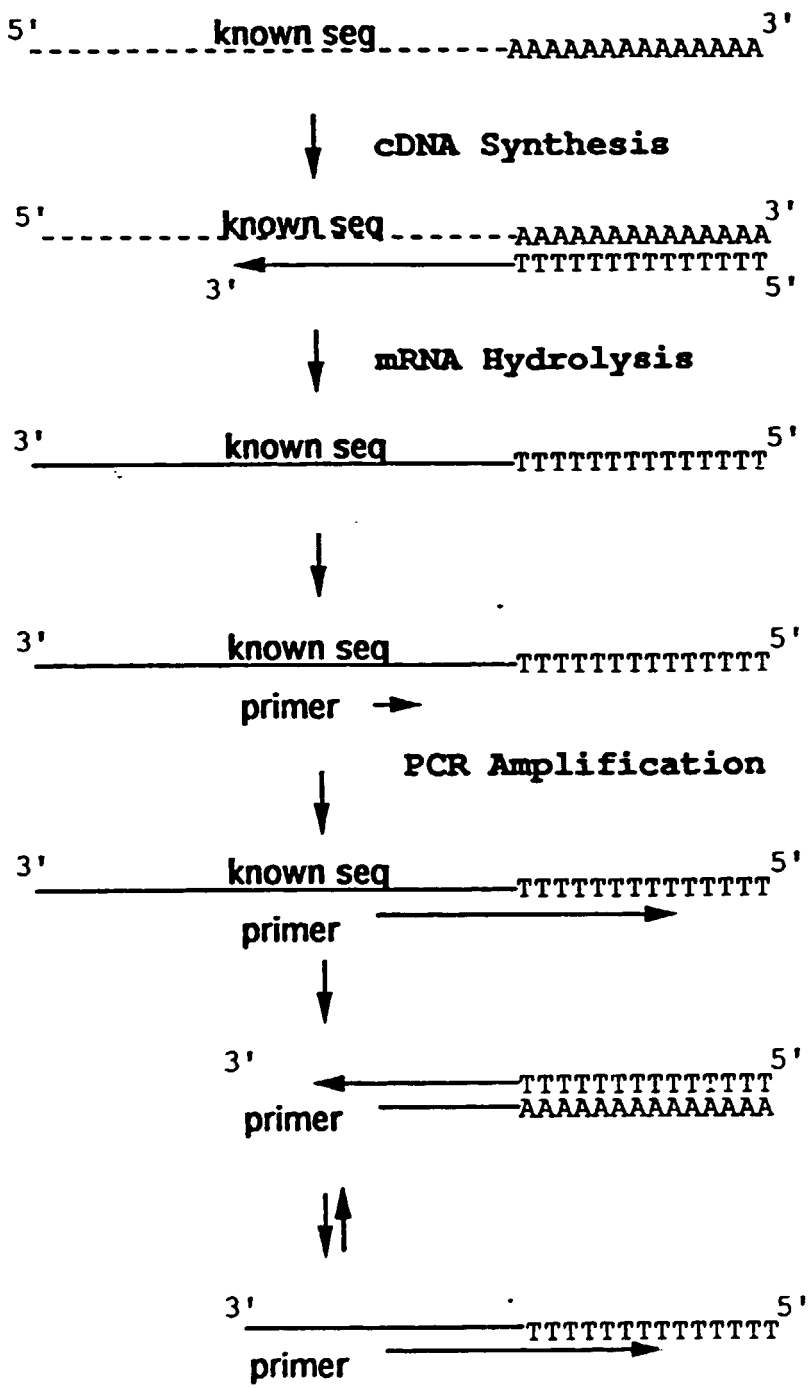


Figure 2. PCR amplification of a specific gene based on sequence information and gene expression as mRNA.

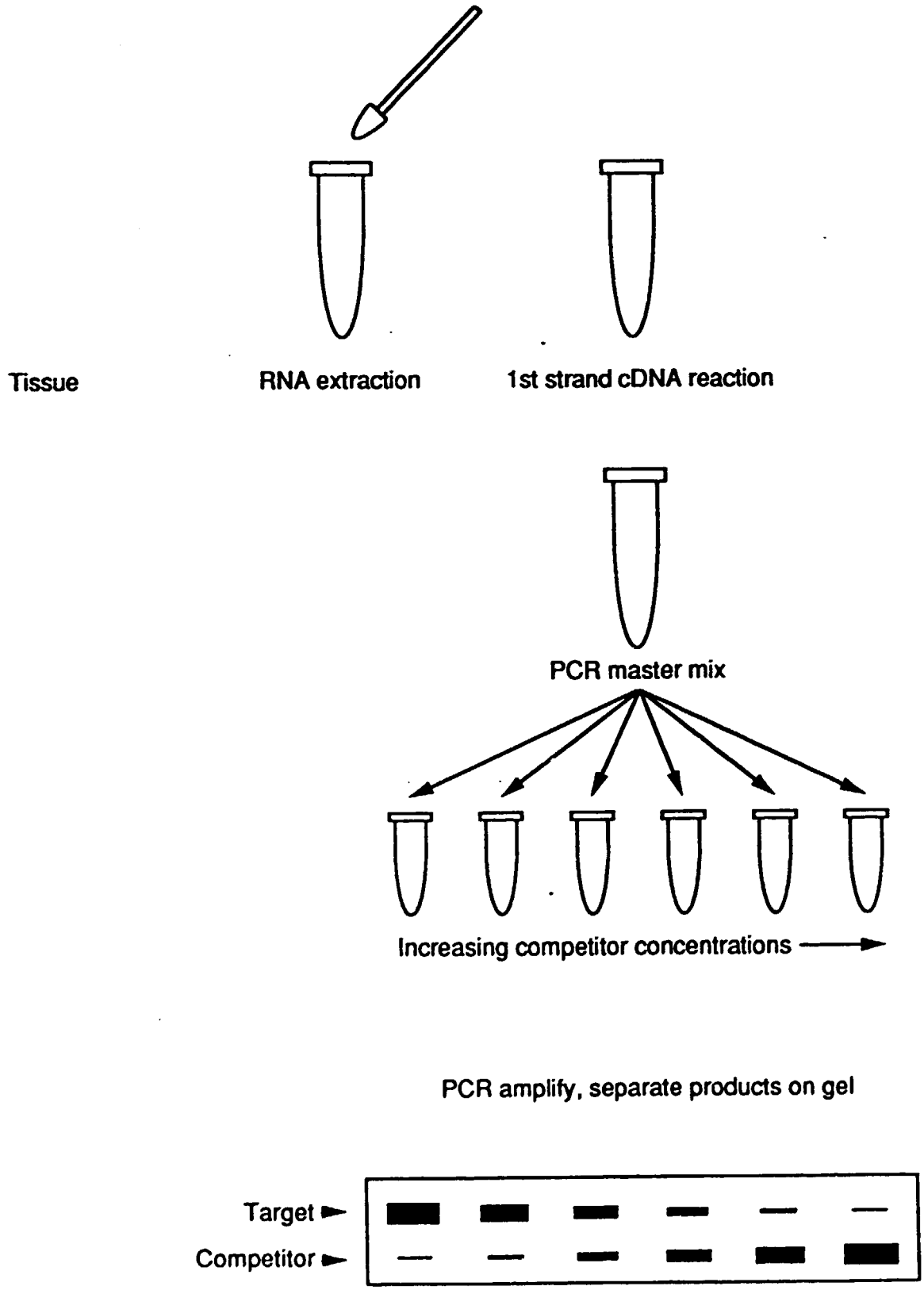
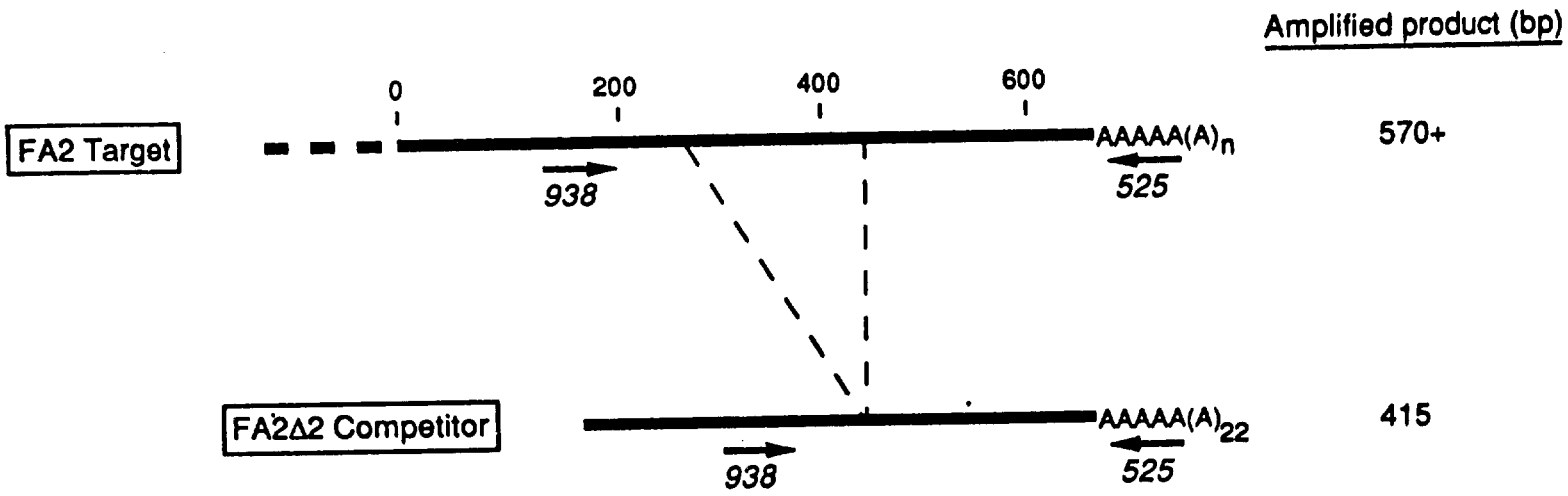


Figure 3. Competitive PCR to quantitate mRNA levels.



Primer 525: poly(dT)

Primer 938: FA2 specific

Figure 4. Competitive PCR template for the tobacco FA2 gene.

CONSTRUCTION OF SUBTRACTED cDNA LIBRARIES

INTRODUCTION

The ability to isolate clones of genes will remain an important technique for molecular plant breeding. In some plant species, especially those with well characterized transposable element systems, it is possible to isolate genes of interest based on mutant phenotypes associated with a "tagged" mutant allele. However, many plant species and plant processes are not amenable to a "tagging" strategy for gene cloning, and thus other methods must be employed. Perhaps the most significant (and challenging) approach for gene isolation is cDNA cloning. cDNA cloning permits the isolation of genes based simply on the transcription of the gene into mRNA molecules. Methods for cDNA cloning have improved greatly in the past few years, and it is the purpose of this lecture to discuss how these improvements can assist plant biologists in the isolation of genes based on their pattern of transcriptional expression.

THE PROBLEM: mRNA sequence complexity

Isolating plant genes based on the expression patterns is possible if one can produce a representative clone library using mRNA as the starting material. This mRNA is then converted into complementary DNA (cDNA) using the enzyme reverse transcriptase (RTase). A major problem occurs at the outset of cDNA library construction: the abundance of the desired mRNA in the pool of cellular RNA may be quite low, and thus will not be detected when screening the library for this DNA sequence. Most of the mRNA in plant cell represents a relatively few number of highly transcribed genes. Thousands of plant genes produce transcripts at such low levels that they would not be found in standard cDNA libraries. In addition, the large number of different genes which are transcribed at a low level means that the sequence complexity of the low abundance mRNA is very high. In order to account for this problem most researchers now use a step in which the cDNA pool to be used for library construction is first hybridized in solution with a second mRNA population, and then the DNA:RNA hybrid molecules are removed prior to making the library. The resulting library is described as a "subtracted" cDNA library, and should be enriched for lower abundance mRNA sequences. Often the subtracting mRNA population is from a different but related tissue, and thus the library is considered a TISSUE A minus TISSUE B (A - B) subtracted cDNA library.

TECHNIQUES AND CONSIDERATIONS

A. Outline of cDNA synthesis and cloning.

The basics of cDNA cloning can be found in references 1-4. Some key points will be briefly summarized here and in Figure 1. After preparing intact total RNA from the tissue of choice, the poly (A)+ mRNA should be enriched by purification with oligo (dT) cellulose. Even with several passages over an oligo (dT) cellulose column there will remain a significant amount of ribosomal and poly (A)- RNA. cDNA synthesis by RTase can be "primed" either by an oligo (dT) containing primer, or by random hexamer primers (5). After first-strand cDNA synthesis second-strand synthesis is completed by RNase H. The resulting double-strand (ds) DNA molecules are "repaired" with T4 DNA polymerase I, and after the addition of linkers, can be cloned into an appropriate bacteriophage or plasmid vector.

B. Improvements.

In the past few years several improvements have been made in the construction of cDNA libraries. Perhaps the most important are: 1) the ability to "directionally clone" cDNA molecules such that the orientation of the 5' and 3' ends of the mRNA sequence are known, and 2) the development of methods for making subtracted cDNA libraries. Producing a "directional" cDNA library requires only a modification in the primer used for first-strand synthesis by RTase, and the use of a specially designed (and now commonly available) vector. Figure 2 diagrams the use of a directional cloning vector for creating a subtracted cDNA library. Thus, every cDNA library made should be done so using a directional cloning procedure. The merit of producing a subtracted cDNA library requires a bit more consideration, but it is well worth the effort if sufficient starting material is available.

Two basic strategies exist for producing subtracted cDNA libraries, and choosing a strategy will depend on the ease with which plant material can be collected for mRNA isolation. In constructing an A-B library, if tissue is easily obtained then one would most likely drive the subtractive hybridization with mRNA derived directly from tissue A. On the other hand, if tissue is difficult to obtain then one would most likely use *in vitro* derived tissue A RNA to drive the subtractive hybridization. This latter method demands the use of cloning vectors containing promoters for T3, T7 or Sp6 RNA polymerases (as shown in Figure 3 for the vector λCAT).

C. Subtraction.

First-strand cDNA is made from 1.0 to 2.0 μg of tissue A mRNA by RTase activity in the presence of a small amount of α–32P dCTP. Radioactive labelling of the cDNA allows one to monitor the recovery of cDNA after each round of subtraction. First-strand cDNA yield is determined as follows:

incorporated cpm/total cpm X 100% = % incorporation

nmoles dNTP/μl X reaction vol. (μl) X % incorporation/100 = nmoles of dNTP incorporated

nmoles dNTP incorporated X 330 ng/nmole = **ng cDNA synthesized**

Usually between 15 and 40 % of the mRNA will be converted into first-strand cDNA. For subtractive hybridization of the first-strand cDNA one should use approximately a 50-fold excess of tissue B mRNA per hybridization cycle. The most straightforward method for detecting and removing DNA:RNA hybrid molecules from the cDNA pool is by biotin-streptavadin conjugation (6). Prior to solution hybridization the tissue B mRNA is photobiotinylated. After hybridization, single-stranded (ss) mRNA and DNA:RNA hybrid molecules are removed from solution by conjugation with streptavadin and several extractions with phenol and chloroform. The amount of unsubtracted ss-cDNA can be estimated by the % of cpm remaining after extraction. Multiple rounds of subtraction can be carried out, although it is likely that for most purposes two round of subtraction should be sufficient.

In situations where Tissue A is difficult to obtain it is still possible to make subtracted cDNA libraries by using *in vitro* synthesized cRNA molecules. Tissue A mRNA is first converted into ds-cDNA and directionally cloned into a vector that has Sp6 and T7 RNA polymerase promoters flanking the site of cDNA insertion (7; see Figure 2). Then, tissue A "sense-strand" cRNA is made *in vitro* using one of these promoters. This cRNA is then used templates for RTase directed cDNA synthesis. The first-strand cDNA produced in this manner can be subjected to solution hybridization with tissue B mRNA as described above. Thus by starting with only a small amount of tissue A mRNA one can produce enough first-strand cDNA to use in subtractive hybridization reactions. The real power of this method is that subtracted cDNA libraries can now be made from very small amounts of tissue, and the more specific the starting material the better the quality of the cDNA library. In addition, most of these new cDNA library vectors have associated benefits in terms of ease of obtaining cDNA inserts from the recombinant vectors. Methods for isolating cDNA inserts from such a cloning vector are described in Appendix I and Appendix II.

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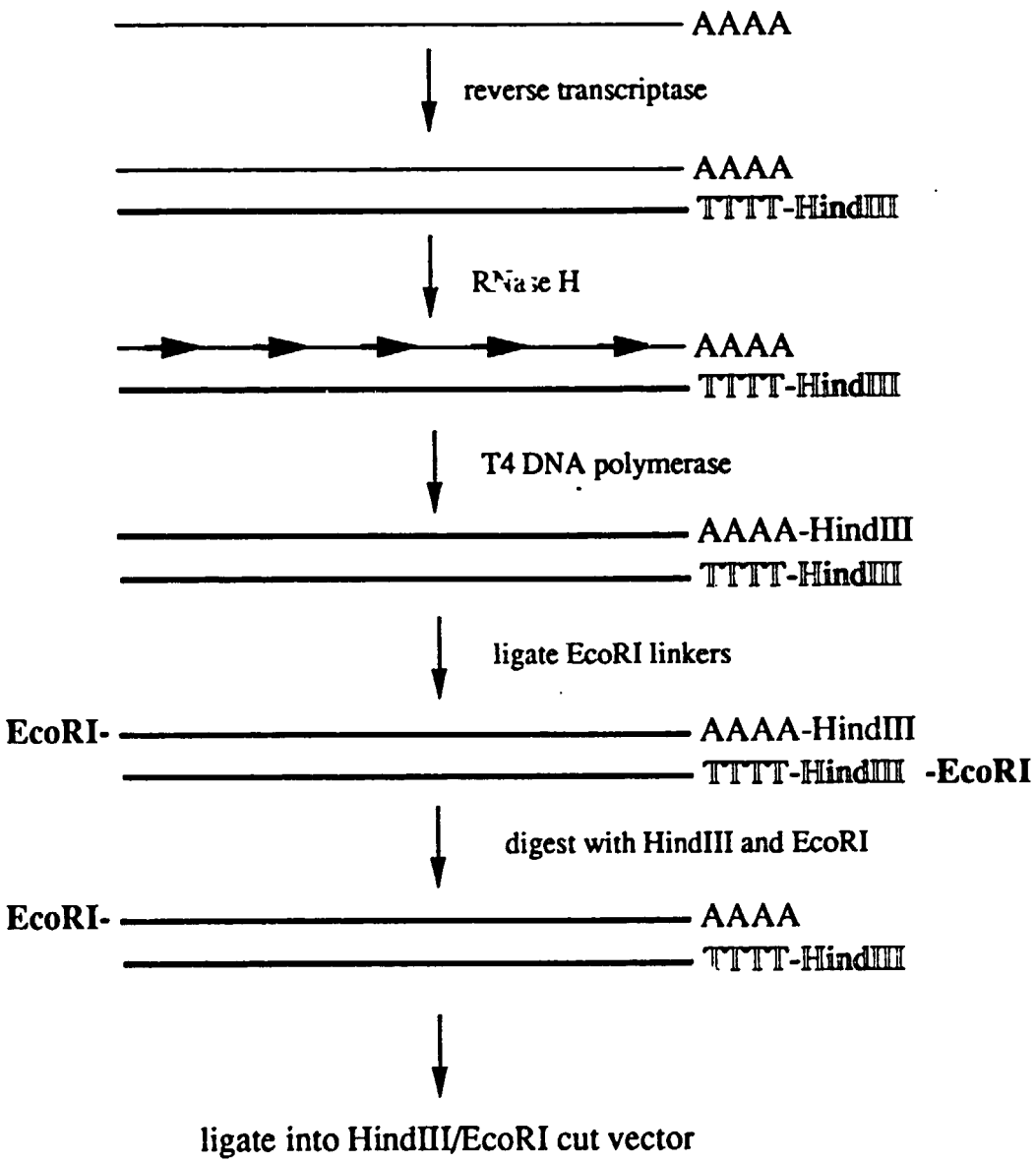


Figure 1. cDNA synthesis for directional cloning into λCAT.

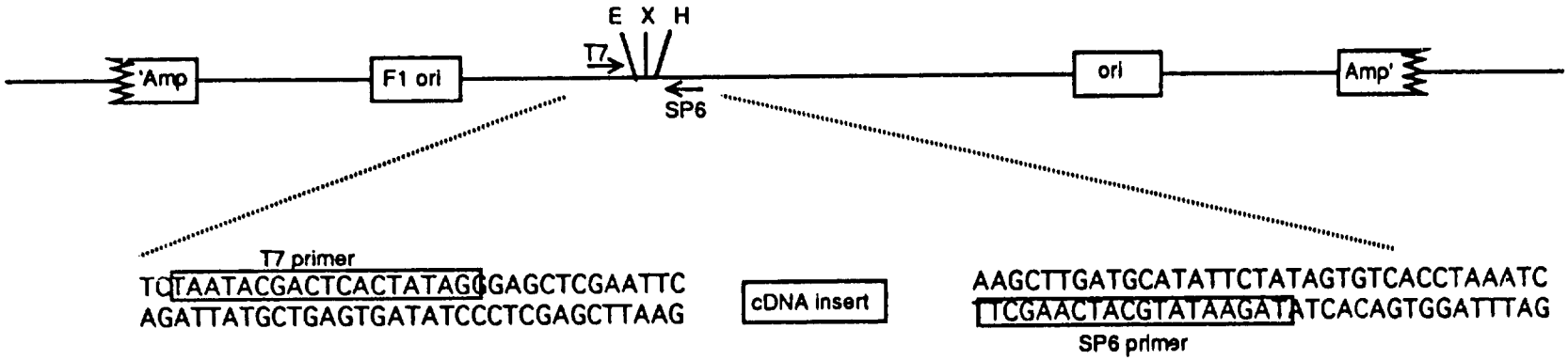
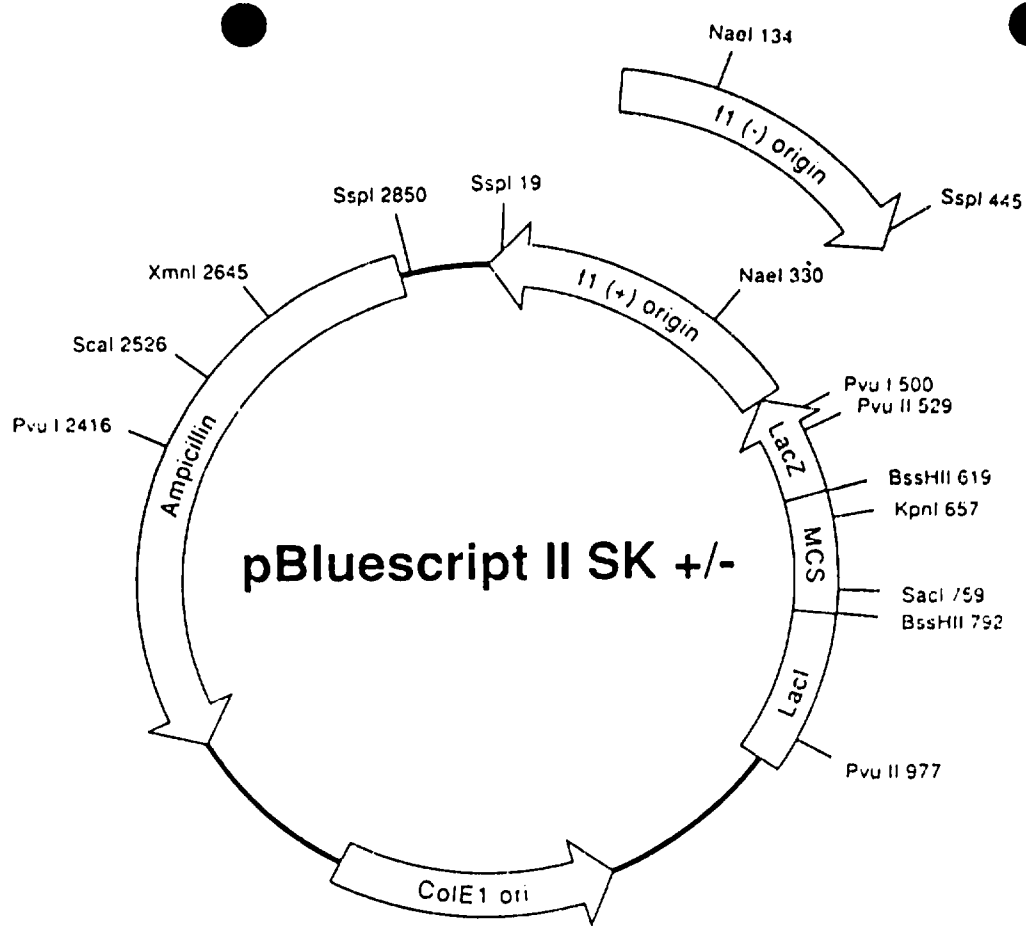


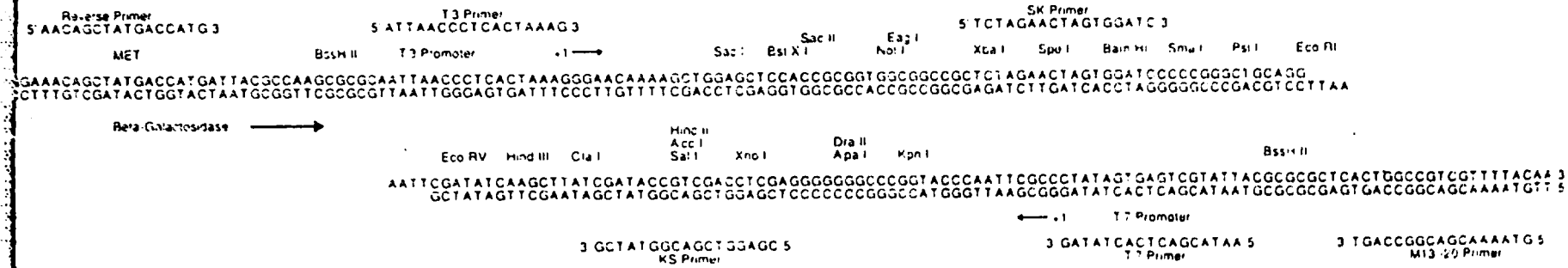
Figure 2. Directional cloning vector lambda CAT.

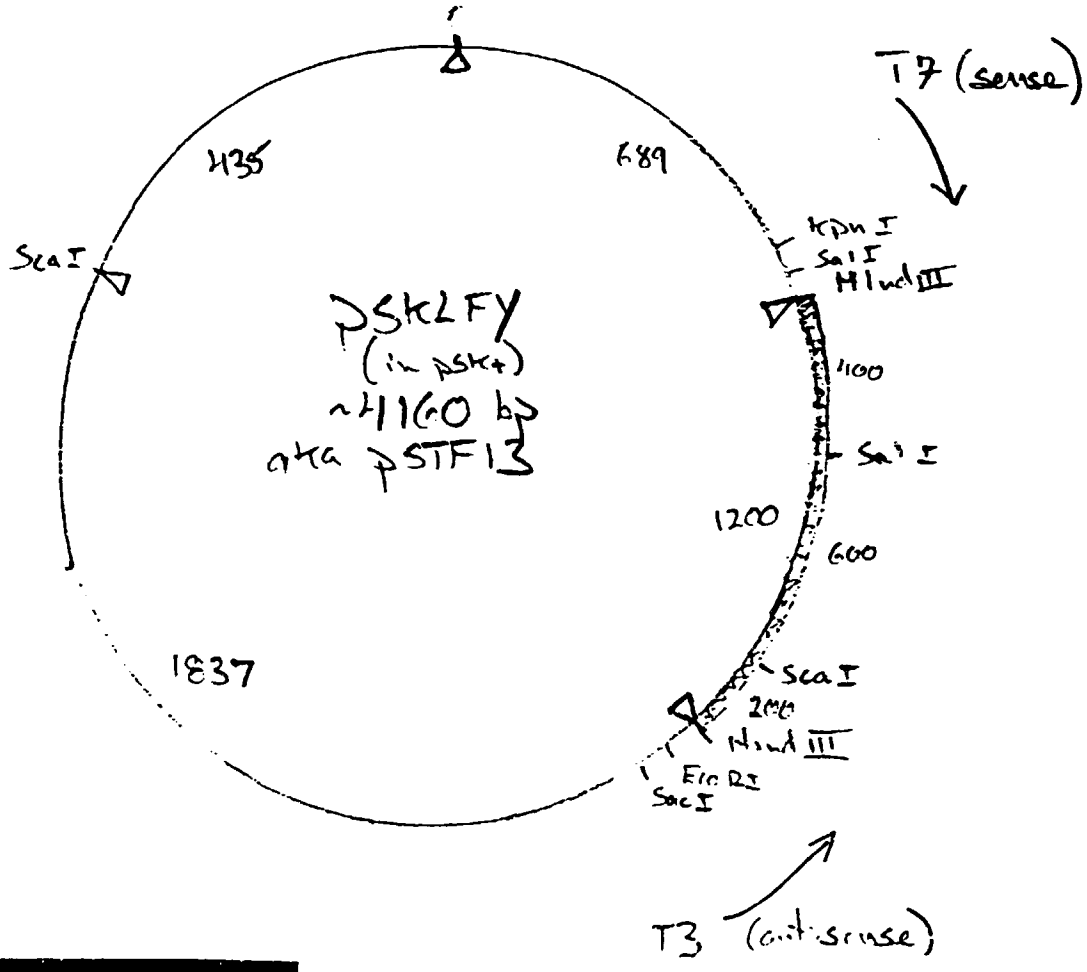
E = EcoRI; X = XbaI; H = HindIII

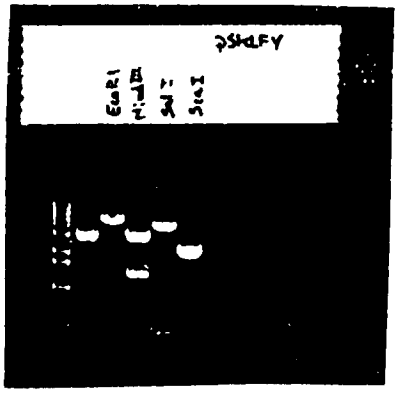
pBluescript® II SK

The pBluescript® II SK (+/-) plasmid is a 2961 basepair phagemid derived from pUC19. The vector posesses an f1 phage origin, a colE1 origin, and T3 and T7 promotors flanking a novel polylinker containing 21 unique restriction sites. These sites are clustered with 3' overhangs flanking 5' overhangs to facilitate generation of nested deletions with exonuclease III. The pBluescript II SK (+/-) plasmid contains a lacZ promotor for blue/white color selection or fusion protein induction with IPTG. The SK designation indicates the polylinker is oriented such that β-galactosidase transcription proceeds through the Sac I site first and the Kpn I site last. The pBluescript II SK (+/-) plasmid carries an f1 origin of replication, allowing single strand DNA rescue, via helper phage infection, for site-specific mutagenesis or single strand sequencing. The rescued strand of SK (+) will contain the coding strand of the β-galactosidase gene (top strand of the polylinker), the rescued strand of SK (-) will contain the non-coding strand (bottom strand of the polylinker).






T7 (sense)
435
689
ScaI
KpnI
SalI
HindIII
pSKLFY
(in pSK+)
~4160 bp
aka pSTF13
1100
SalI
1200
600
1837
ScaI
2mg
HindIII
EcoRI
SacI
T3 (anti-sense)


pSKLFY
EcoRI
HindIII
SalI
SacI

F2 Plant	Genotype
3	tlf/tfl
10	tfl/+
11	tfl/+
12	tfl/+
17	tfl/+
19	tfl/+
26	tfl/tfl
28	+/+
31	tfl/+
[illegible]	tfl/tfl
50	tfl/+
57	tfl/+
60	tfl/tfl
65	tfl/+

2.2. DNA extraction (modified from Murray and Thompson, 1980)

Grind lyophilized tissue using a mortar and pestle or coffee grinder. If necessary, the addition of dry ice pellets can aid grinding.

Transfer 200-500 mg lyophilized and ground tissue to a 16 ml polypropylene tube and add 6-9 ml of fresh CTAB extraction buffer that has been prewarmed to 60°C.

If dry ice was used for grinding, be sure it has sublimed completely prior to the addition of extraction buffer.

For fresh tissue extraction, add an equal amount (w/w) of a 2X stock of extraction buffer to frozen and ground tissue.

CTAB extraction buffer: 100 mM Tris-HCl (pH 7.5), 1% CTAB (Cetyl or Mixed alkyl-trimethyl-ammonium bromide), 0.7M NaCl, 10 mM EDTA, 1% β-mercapto-ethanol (BME).

Mix the suspension well by rapping the tube upside down on a hard counter to suspend the powder. There is no need to be gentle at this stage. Incubate the mixture at 60°C for 30-90 min with occasional inversion.

Place the tubes at room temperature for 5 min to cool, then add 4.5 ml chloroform:octanol (or isoamyl alcohol) (24:1 v/v). Mix the tubes by gentle inversion for 5-10 min and then spin the tubes for 10 min in a table-top centrifuge at high-speed. Pour or pipet off the upper phase to new tubes and add 1/10th volume of 10% CTAB in 0.7 M NaCl (prewarmed to reduce viscosity).

Repeat the chloroform extraction. Pipet off the aqueous phase to new tubes and add an equal volume of precipitation buffer: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% CTAB. Mix by inversion and let the tubes sit at room temperature for 30 min.

Spin the tubes in a table-top centrifuge at moderate to high speed. Decant off the supernatant. If a pellet does not form (especially true with fresh tissue extraction), add more precipitation buffer to lower the salt concentration further and respin the tubes. The critical point here is the reduction of the salt concentration which results in the co-precipitation of the DNA with the CTAB.

Resuspend the pellet in 450 μl of 1 M NaCl. Heat the tubes to 56°C if needed to dissolve the pellet. Transfer the entire volume to microfuge tubes and add 900 μl of EtOH. Mix the tubes by inversion to reprecipitate the DNA. Spin the tubes for 5 min at room temperature and decant off the supernatant. Wash the pellets twice with 1 ml each of 70% EtOH.

Redissolve the pellet in 100-400 μl TE and add 1-4 μl of 10 mg/ml RNAse A and incubate at 37°C for 15 min.

Extract with phenol and then, if necessary, with chloroform. Add 1/2 volume 7.5 M NH_4OAc and 3 volumes of EtOH to reprecipitate the DNA. Spin for 5 min. Resuspend the pellet in 100-400 μl TE. Yield should be 20-40 μg of DNA per 50 ml flask (200-300 mg lyophilized tissue).

Arabidopsis Mini Prep

1 thumb-size leaf = 25 mg. -- need ~30 leaves/prep.

0. water bath to 70°C, pre-cool mortar and pestle
1. weight 0.75-1.0g leaf tissue, freeze in liquid nitrogen, grind to fine powder in mortar and pestle
2. transfer powder into 30 ml plastic centrifuge tube containing 15 mls EB
3. add 1 ml 20% SDS, mix well by vigorous shaking, incubate 65-70°C, 10 minutes
4. add 5 ml (5M) Potassium Acetate, shake tubes vigorously, incubate on ice, 20 minutes
5. spin 13,500 rpm w/ JA17 rotor, 20 minutes, pour supernatant through a miracloth filter (placed in a funnel) into a clean 30 ml tube containing 10 mls isopropanol
6. mix gently, incubate 30 minutes in freezer
7. pellet DNA, 13,000 rpm, 15 minutes, gently pour off supernatant and dry lightly by inverting 10-20 minutes on paper towels
8. dissolve pellets in 0.7 ml 50mM Tris, 10mM EDTA pH8 (draw liquid up and down over pellet until gone)
9. transfer to Eppendorf tubes, spin 10 minutes, room temperature [important to spin down carbohydrate (insoluble pellet)]
10. transfer to new Eppendorf tubes containing 75 ul 3M NaOAC. Mix solution, then add 500 ul isopropanol, mix well, spin 30 seconds in microfuge, room temperature
11. rinse with ice cold 80% ethanol. Spin tube in microfuge and use drawn out glass pipette to remove last liquid; get pellet as dry as possible. Place open tube in 65° oven for 3-5 minutes. [Do not speed vac; you will create an insoluble product!] Add 100 or so uls of pH 7.5 TE. Close tube and incubate at 65°C for at least 30 minutes

Notes on At minipreps

0. leaf material used so far has been approximately 2 weeks old
1. yield is approximately 10 ug DNA for 1 g leaf material
2. it is important not to let leaf material thaw during grinding procedure until it is in the EB, which contains nuclease inhibitors to prevent DNA digestion (the ground powder can be poured into centrifuge tubes with liquid nitrogen)
3. the ground powder can be used right away or it can be stored at -70°C until needed or stored at 4°C in EB
4. in step 4, most proteins and polysaccharides are removed as a complex with the insoluble K-dodecyl sulfate ppt
5. in step 9, the insoluble debris is ppt'd out
6. in step 10, the high MW DNA should be separated from polysaccharides
7. preps can sometimes be difficult to cut; using 20 units of EcoRI per 1 ug of DNA and cutting at least 3 hours seems to work in general

Genomic Restriction Digest

A. Take to O.D. 260/280 of your prep and use 70-100 ugms total nucleic acid per sample to cut. This gives you enough DNA in each lane that you should be able to clearly see chloroplast bands and repeats on the get.

B. Add 70 ugms total nucleic acid (in 91-94 uls total volume with nanopure H_2O). Add 15 ul of the appropriate enzyme buffer. Add 15 ul of 10 mM spermidine, and 15 ul of 0.5% (v/v) nonidet p 40 nonionic detergent. Add 10 ul of 2.0 ug/ul DNase free RNase A. Add 2-3 uls of restriction enzyme, 40-60 units total. Add ddH_2O to a total volume of 150 uls. Digest for at least 12 hours at the appropriate temperature.

C. Precipitate DNA by adding 17 uls 3M sodium acetate (pH 5.2), then add 107 uls isopropanol; spin at 4°C for 5 minutes. Rinse with ice cold 80% ethanol, and spin down and remove as much ethanol as possible with a drawn out glass pipette. Heat in 65°C oven for 4-5 minutes to dry pellet. Resuspend in 30-40 ul TE and heat at 65°C for 30+ minutes to get DNA into solution. Add loading buffer and run on gel.

Modification for Plasmid / Phage DNA

1) Digest 0.1 - 1 µg DNA c 1 unit enz / µg DNA for 1 hr.

2) Use size of comb so as to not overload and get smeared bands. Gels can be run at higher voltage.

3) same - except leave on box shorter time, (30".) for K DNA

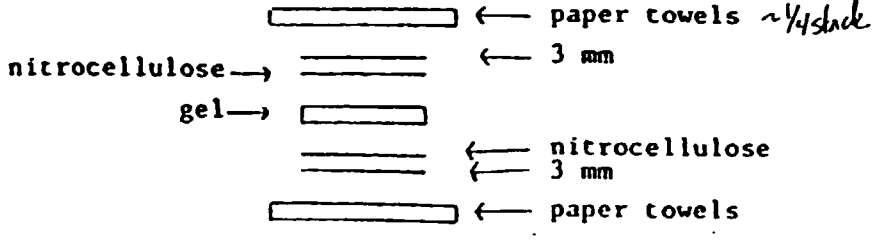
4) same

5) same

6) Use nitrocellulose. or genetran if going to probe several times Can use with set up as described, blotting for 2 hours - overnight. Can also use dry blot and transfer to 2 filters:

see Maniati's for diagram

Dry blot - used to transfer to 2 filters



label nitrocellulose top and bottom filters with ballpoint ink.

Blot 2 hr. — overnight

7) Air dry 30 - 60 min.

8) same

9) skip - not necessary

10) prehyb 2 hr - o/n

11) No need to use dextran sulfate. Denature probe in 0.5 ml hyb. buffer - add to bag. Hybridize 6 hr - o/n. Use 5 x 10^6cpm/blot - specific activity can be 1 x 10^7cpm/µg - 1 x 10^8cpm/µg.

12) Pour off hyb. buffer - store @ -20°C. Can be used again if denatured by heating at 80°C for 10 min. No need to do formamide wash.

13) Rinse blot in blot wash buffer. Pour first rinse in radioactive waste. Wash 2 x, 30 min. each at 50°C in blot wash buffer:
0.1 x SSC
0.1% SDS
(5 ml 20 x SSC, 10 ml 10% SDS/liter H_2O)

Blot filter dry with paper towels - wrap in saran wrap and expose to film with screen. I usually develop after about 5 - 6 hr. and determine if shorter or longer exposure times are necessary.

Southern Blots - Maize DNA

1) Digest 4-5 µg of genomic DNA, with 2 units enzyme/µg DNA for 1 hr. Add another 2 units enzyme/µg DNA - digest another hour.

2) Load onto hardened agarose gel (0.5 - 1.2% agarose - depending on average fragment sizes and ≈1-5µg/mℓ EtBromide in 1 x TBE). Use large wells on combs, to help prevent overloading and smeared bands. Run gel overnight at 30 - 40 V.

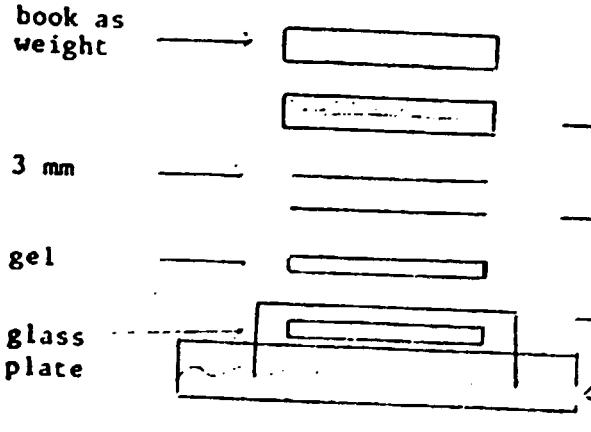
3) Cut away one corner of the gel to mark orientation. (lower left) Take picture using a ruler next to the size standards (I usually use EcoRI/Hind III or Hind III digested λ DNA). Leave on u.v. box for 1½ min. to nick DNA-(helps transfer of large fragments).

4) Denature by incubating gel at room temperature in denaturing buffer 2 times, 20 min. each on a shaker platform. Always remove buffer with aspirator and pasteur pipette.

5) Remove denaturing buffer and rinse once quickly in neutralizing buffer, then incubate 2 more times, 30 min. each in neut. buffer, shaking at room temperature.

6) Remove neutraliz and place in 10 x SSC (175.3 g NaCl, 88.2 g Na citrate per 1000 mℓ is 20x stock).* Measure gel and cut out genetran and one piece 3mm paper to fit gel. Label genetran in corner using ballpoint pen as to date & experiment. Set up plate and wick as drawn below. Place gel on wick. Rinse genetran in water, then in SSC and place on gel. Carefully remove any air bubbles by rubbing with gloved fingers. Place 3mm and paper towels on top. Use a book as a weight. Blot overnight. Note: be sure to mask around gel using exposed film or parafilm to insure that solution wicks through, not around gel.

* cut top of gel off (also wells).



: morning disassemble set.up and air dry genetran filter
lry paper towels for =1 hour (it absorbs more water than
·ocellulose).

2 hr. in 80°C vacuum oven.

dry blot in 0.1 x SSC, 0.1% SDS for 1 - 3 hr. at 60 - 65°C -
ing.

59.50 e in hybridiz buffer and prehyb @ 42°C for 6 hr. → overnight.
59.50 ℓ for blot =13 x 18 cm. I use seal-a-meal bags.
59.50
59.50 idization: Heat up 10mℓ hyb and add dextran sulfate to
59.50 0.5g/10mℓ). When in solution remove 1mℓ and add labeled
59.50 to it - denature probe by heating at 80°C for 5 - 10'.
59.50 while remove prehyb buffer and add remaining 9 mℓs to
59.50 er. Once probe is denatured add to bag. Seal and
59.50 bate @ 42°C while shaking. I usually incubate for 36 hr.,
59.50 24 hr. will do okay if in a hurry. Signal does seem
59.50 ncrease at 36 vs. 24 hr. - even though theoretically
59.50 :ion should be complete.
59.50
59.50 : 15 - 20 x 10^6 cpm/blot with specific activity at least
59.50 .0 ^{8} cpm/µg.
59.50
59.50 'e probe. Store at -20°C if want to save to use on plasmid
59.50 .. I do not use recycled probe on genomic Southerns. Wash
80.33 in 100mℓ formamide wash buffer for 30 min. at 42°C.
80.33 'e and discard in radioactive waste.
80.33
87.89
96.01 2x quickly in blot wash buffer (0.1 x SSC, 0.1% SDS) at room
88.73 , then 3x at 60 - 65°C in =500mℓ ea time blot wash buffer,
80.33 45 min. each wash.
98.73
93.91 dry and wrap in saran wrap. Do not let dry completely,
80.33 ckground will be high for next hybridization. Expose
80.33 ight - examine signal and determine if shorter or longer
88.73 ures are necessary.
wild type,

for Rehyb: Heat up =500mℓ of 0.1 x SSC, 0.1% SDS to 95-100°C.
on blot - shake at room temperature for =10 min. Repeat
times washing with at least 1 - 1.5 liters buffer. Expose
eck that signal is removed. Prehyb 1 - 5 hr. - hybridiz
scribed above. Never let genetran dry out completely.
in saran wrap.
(0.1 gm)

6 < 9

59.50
59.50

59.50 perature I use is a function of GC content. It is not
idea to wash genetran below 58°C.
59.50
59.50
59.50

RECIPES

1. **BBL plates**

Red, Yellow	BBL Plate Agar				
Agar	10 gm	20 gm	30 gm	40 gm	50 gm
NaCl	5 gm	10 gm	15 gm	20 gm	25 gm
BBL Trypticase	10 gm	20 gm	30 gm	40 gm	50 gm
H_2O	1 liter	2 liter	3 liter	4 liter	5 liter

Cool to 50° and pour.

2. **BBL Top Agar**

Agar	6.5 gm	13 gm	19.5 gm	26 gm	32.5 gm
NaCl	5 gm	10 gm	15 gm	20 gm	25 gm
BBL Trypticase	10 gm	20 gm	30 gm	40 gm	50 gm
H_2O	1 liter	2 liter	3 liter	4 liter	5 liter

Dissolve in steamer. Mix well. Continue mixing while bottling. Autoclave for 20 minutes slow exhaust.

3. **Maniatis SM Buffer**: This buffer is used for storage and dilution of bacteriophage l stocks.

Per liter:		
NaCl	5.8 gm	
$MgSO_4 \cdot 7H_2O$	2 gm	
1 M Tris· Cl (pH 7.5)	50 ml	
2% gelatin solution	5 ml	(0.01%)
H_2O to 1 liter		

Sterilize the buffer by autoclaving for 20 minutes at 15 lb/sq. in. on liquid cycle. After the solution has cooled, dispense 50-ml aliquots into sterile containers. SM may be stored indefinitely at room temperature.

A 2% gelatin solution is made by adding 2 g of gelatin to a total volume of 100 ml of H_2O and autoclaving the solution for 15 minutes at 15 lb/sq. in. on liquid cycle.

4. **LB + 4X AMP Plates** (1 liter)

Plates:	Agar	15 gm
	Tryptone	10 gm
	Yeast extract	5 gm
	NaCl	10 gm
	H_2O	1 liter

Autoclave. Cool to ~60°. Add 1.0 ml sterile ampicillin solution (100 mg/ml). (For broth, leave out 15 gm agar)

5. **LB + 4X AMP Broth** (1 liter)

Broth:	Tryptone	10 gm
	Yeast extract	5 gm
	NaCl	10 gm
	H_2O	1 liter

6. **SOB transformation buffers**

TFB (200 ml)	MES	0.4 gm
	RbCl	2.42 gm
	$MnCl_2 \cdot 4H_2O$	1.78 gm
	$CaCl_2 \cdot 2H_2O$	0.3 gm
	$[Co(NH_3)_6]\ Cl_3$	0.16 gm

Adjust pH to 6.3 with 1N KOH. Filter sterilize.

7. **STET buffer**

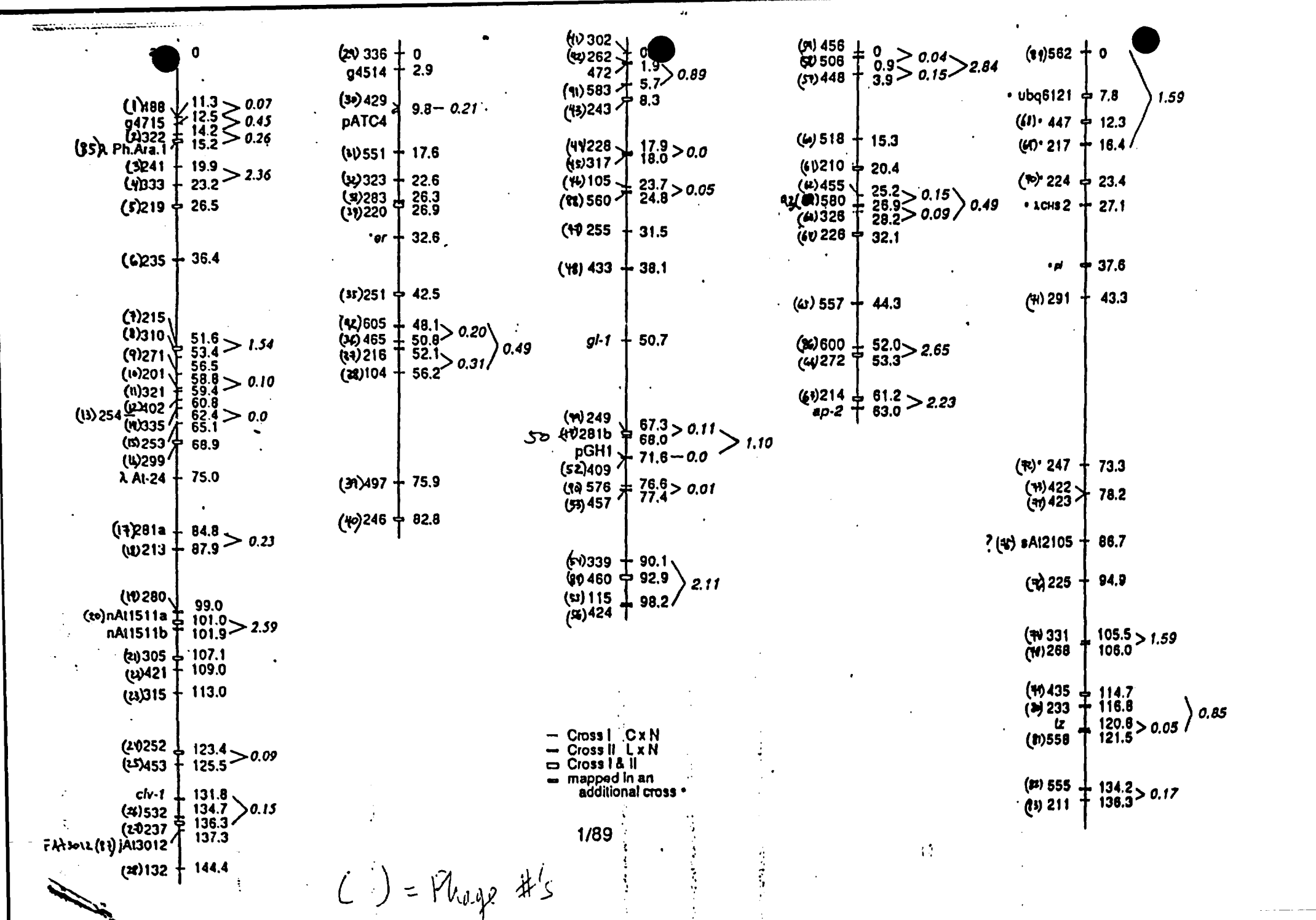
0.1 M NaCl
10 mM Tris · Cl (pH 8.0)
1 mM EDTA (pH 8.0)
5% Triton X-100

8. **50 X TAE buffer**

Buffer	Working solution	Concentrated stock solution (per liter)
Tris-acetate (TAE)	1x: 0.04 M Tris-acetate 0.001 M EDTA	50x: 242 g Tris base 57.1 ml glacial acetic acid 100 ml 0.5 M EDTA (pH 8.0)

9. **10X Ligation buffer**

0.5 M Tris · Cl (pH 7.6)
100 mM $MgCl_2$
100 mM dithiothreitol
500 µg/ml bovine serum albumin (Fraction V; Sigma) (optional)


0
(1)488 11.3 > 0.07
g4715 12.5 > 0.45
(2)322 14.2 > 0.26
(35)λ Ph.Ara.1 15.2
(3)241 19.9 > 2.36
(4)333 23.2
(5)219 26.5
(6)235 36.4
(7)215
(8)310 51.6 > 1.54
(9)271 53.4
56.5
(10)201 58.8 > 0.10
(11)321 59.4
(12)402 60.8
(13)254 62.4 > 0.0
(14)335 65.1
(15)253 68.9
(16)299
λ A1-24 75.0
(17)281a 84.8
(18)213 87.9 > 0.23
(19)280 99.0
(20)nAI1511a 101.0
nAI1511b 101.9 > 2.59
(21)305 107.1
(22)421 109.0
(23)315 113.0
(24)252 123.4 > 0.09
(25)453 125.5
clv-1 131.8
(26)532 134.7 > 0.15
(27)237 136.3
FAT3012(17) jAI3012 137.3
(28)132 144.4
(29) 336 0
g4514 2.9
(30)429 9.8 — 0.21
pATC4
(31)551 17.6
(32)323 22.6
(33)283 26.3
(34)220 26.9
•er 32.6
(35)251 42.5
(42)605 48.1 > 0.20
(36)465 50.8 > 0.49
(37)216 52.1 > 0.31
(38)104 56.2
(39)497 75.9
(40)246 82.8
(41) 302 0
(42)262 1.9
472 5.7 > 0.89
(91)583 8.3
(43)243
(44)228 17.9 > 0.0
(45)317 18.0
(46)105 23.7 > 0.05
(92)560 24.8
(47) 255 31.5
(48) 433 38.1
gl-1 50.7
(49) 249
50 (47)281b 67.3 > 0.11
68.0 > 1.10
pGH1 71.6 — 0.0
(52)409
(93) 576 76.6 > 0.01
(53) 457 77.4
(54)339 90.1
(90) 460 92.9 > 2.11
(55) 115 98.2
(56)424
(57) 456 0
(58) 506 0.9 > 0.04 > 2.84
(57)448 3.9 > 0.15
(60) 518 15.3
(61)210 20.4
(62)455 25.2 > 0.15
(59)580 26.9 > 0.49
(63)326 28.2 > 0.09
(64) 226 32.1
(65) 557 44.3
(66)600 52.0 > 2.65
(44)272 53.3
(67)214 61.2 > 2.23
sp-2 63.0
(91)562 0
• ubq6121 7.8 > 1.59
(61)• 447 12.3
(60)• 217 16.4
(70)• 224 23.4
• LCHS2 27.1
•pl 37.6
(71) 291 43.3
(72)• 247 73.3
(73)422
(37) 423 78.2
? (75) sAI2105 86.7
(76) 225 94.9
(74) 331 105.5 > 1.59
(74)268 106.0
(77)435 114.7
(78) 233 116.8 > 0.85
lz 120.6 > 0.05
(81)558 121.5
(82) 555 134.2 > 0.17
(83) 211 136.3
— Cross I C x N
— Cross II L x N
Cross I & II
mapped in an additional cross •
1/89
( ) = Phage #'s

ARABIDOPSIS THALIANA: ISOLATING GENES BASED ON MUTANT PHENOTYPES

INTRODUCTION

Arabidopsis thaliana has several features which facilitate the molecular cloning of genes identified by mutation (1). *Arabidopsis* has a small nuclear genome (80,000 kb) with little repetitive DNA, and two well-developed restriction fragment length polymorphism (RFLP) maps (2,3), which are currently being integrated into a single physical map by using several yeast artificial chromosome (YAC) libraries (4,5). Thus it is likely that a gene identified by a mutation will be within 100 to 200 kb, or one to two YAC clones, from the nearest RFLP marker, which makes it possible to clone genes by techniques such as chromosome walking. *Arabidopsis* can therefore offer an experimental system to isolate genes that may be useful for the manipulation of the growth and development of agriculturally important crop species. For example, identifying and cloning an *Arabidopsis* gene that confers resistance to a particular pathogen may allow the isolation of the homologous gene from other plants. By recombinant DNA techniques such a gene may be engineered to provide pathogen resistance in these other plant species. Thus *Arabidopsis* can provide a valuable gene pool from which to begin investigating the genetic basis of plant traits in a variety of species.

GENERATION OF MUTANT ALLELES

Mutational studies have commonly used ethylmethane sulfonate (EMS) to induce point mutations in genes, or X-rays to induce chromosomal lesions such as deletions and translocations. In addition, recent work has indicated that diepoxybutane (DEB) and diepoxyoctane (DEO) are highly mutagenic in *Arabidopsis* (6), and may be useful in creating deletion alleles of the gene of interest. These have been used to create deletion mutations in *Drosophila* (7).

Mutagenesis with EMS and DEB is done by soaking seed in dilute solutions of the chemicals (3% EMS and 10 mM DEB) for 4 hours (in the dark), and then washing the seed several times with water. X-ray mutagenesis is done to dry seed at between 7 krad and 20 krad. These seed, designated the M1 seed, are then planted to give rise to the M1 plant generation. Seed collected from the M1 plants, designated the M2 seed, are then planted and assayed for mutant phenotypes. Given that *Arabidopsis* is normally a self-fertile plant, the M2 population will contain plants that are homozygous for the induced mutations, and thus one can screen for recessive mutations in this generation. Mutations of interest should be backcrossed to wild type about 5 times to create a nearly isogenic mutant line.

CLONING STRATEGIES

Two approaches are available for cloning genes identified by the above mutagenesis techniques: (A) chromosome walking and (B) deletion cloning. In addition some laboratories have developed methods of gene-tagging in order to clone genes. The gene-tagging approaches make use of either the T-DNA of *Agrobacterium tumefacians*, or the Ac/Ds transposable element system of maize. As both of these methods are not widely available to most laboratories I will not discuss them in this paper.

A. Chromosome walking.

This method is perhaps best described by example, and thus I will outline the walking approach we are taking to clone a gene (*TFL*) which we mapped relative to nearby RFLP markers. Given our knowledge of the location of the *TFL* locus, chromosome walking, while a difficult task, is our primary approach for cloning the *TFL* gene. The *tfl* mutation maps on *Arabidopsis* chromosome 5 between RFLP markers #447 and #3715, about 1.0 cM (~ 140 kilobases) from #447 (Figure 1). The first aim of the walk is to "link" the region between RFLP markers #447 and #3715 with overlapping YAC clones. DNA from this region is then used to refine the map position of the *TFL* locus relative to new RFLP markers discovered in the YAC DNA inserts. YACs that hybridize to RFLP markers #447 and #3715, or to nearer RFLP markers, serve as the starting points for the chromosome walk. The walk begins by isolating both end (junction) sequences of the *Arabidopsis* insert DNA in the YAC using either inverse PCR (8) or direct "end cloning" procedures. We then identify RFLPs in these sequences in order to map each end of the YACs relative to the *tfl* mutation by standard RFLP mapping. (If no RFLPs are found in the junction sequences, then RFLPs are sought in more "internal" sequences). Given that the average size of the *Arabidopsis* DNA inserts in the YAC clones is 150 kb (about 1 cM), the RFLP mapping of end sequences relative to *tfl* is crucial for establishing the direction of the chromosome walk, and whether the *TFL* locus is likely to be contained within the YAC being analyzed. Mapping both ends of the DNA insert also helps to prevent using a YAC clone that contains a discontinuous insert due to cloning artifacts.

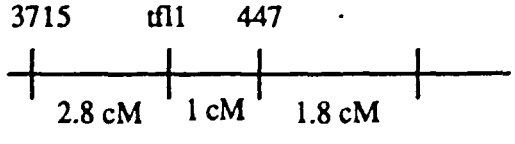


Figure 1. Map position of *TFL* relative to surrounding RFLPs.

To identify the *TFL* locus we will transform homozygous *tfl/tfl* cells with overlapping fragments of the YAC inserts (or cosmid clones) in order to complement the *tfl* mutation. Transformations will be performed using a modification of the *Agrobacterium* mediated root transformation procedure (9). The DNA fragment that complements the *tfl* mutation will be tested for complementation with other *tfl* alleles. Testing the cloned DNA with several different alleles will confirm the isolation of the *TFL* locus. Complementation analysis using a set of deletions of the *TFL*-containing DNA fragment will be used to define the region of the DNA insert containing the functional *TFL* locus.

B. Genomic subtraction as a method of gene cloning.

Deletion alleles can be used with the recently described technique of genomic subtraction cloning, or deletion cloning (10,11). This technique allows the cloning of DNA sequences by polymerase chain reaction (PCR) amplification of the sequence that is present in the wild type parent, and missing in the mutant. The small genome size, and low amount of repetitive DNA in *Arabidopsis*, make this a feasible approach for cloning genes identified by deletion alleles where the deletion is approximately 1 kb or larger in size. While this is a relatively new technique, it has been successfully used to clone chromosomal DNA based solely on subtractive hybridization and PCR amplification using DNA from isogenic wild type and deletion strains (10).

An example of using subtractive hybridization to clone a gene for which a suitable deletion exists would be as follows. Two subtractive hybridizations are carried out in a series of parallel reactions: a) wild type DNA subtracted with deletion strain DNA (wt-Δ), and b) deletion strain DNA subtracted with deletion strain DNA (Δ-Δ). Sequences that are not removed by subtractive hybridization of wild type DNA with deletion strain DNA are cloned into a lambda vector to create a wt-Δ subtracted genomic library. This library is then differentially screened with labeled DNA from the wt-Δ and theΔ-Δ subtractions in order to identify clones containing unsubtracted wild type DNA sequences. Cosmid clones that hybridize to the wt-Δ sequences will then be isolated in order to obtained larger wild type DNA fragments from the region surrounding the deletions. To confirm that the positive wt-Δ clones contain desired wt DNA sequences, one can carry out RFLP mapping (using RFLPs identified in cosmid clones), and hybridization analysis with existing YAC clones from the region near the desired locus. Potential clones obtained by this method are then tested for complementation of mutant alleles by stable plant transformation as described above.

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Leafy Seq Length: 1267 March 24, 1992 16:32 Type: N Check: 9935

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   1  AAGCTTACGG TATACGTTAT TACACTGCTG CTAAAATGGG GGACGTAGGT
      HindIII
  51  TTTACGGTGA AACTCTATT GGACATGAAA GATGAGGAAC TTGATGATAT
 101  GATGAATAGC CTTTCACAGA TTTTCAGATG GGAACTCTC GTCGGAGAAA
 151  GGTACGGTAT CAAAGCTGCA ATCAGGGCGG AACGGCGGAG GCTTGAGGAG
      RsaI
 201  GAAGAACTAC GGCGGCGCAG CCACCTTCTG TCTGATGGTG GAACTAATGC
 251  CCTTGACGCT CTCTCACAAG AAGGGTTGTC TGAGGAACCA GTGCAGCAGC
 301  AAGAGAGAGA AGCAGTTGGA AGCGGCGGAG GGGGAACGAC ATGGGAAGTG
 351  GTGGCGGCAG TTGGCGGTGG AAGAATGAAA CAAAGAAGGA GGAAGAAGGT
 401  GGTGTCGACG GGCAGGGAGA GAAGGGGAAG ACCGTCGGCG GAGGAGGATG
      SalI/AccI
 451  AAGAAACGGA GGAAGGTCAA GAAGATGAGT GGAATATTAA CGACGCCGGG
 501  GGAGGAATAA GCGAGAGGCA AAGGGAGCAT CCTTTTATCG TGACGGAGCC
 551  AGGTGAGGTG GCGCGTGGGA AAAAGAACGG CTTGGATTAC TTGTTCCACC
 601  TCTACGAGCA ATGCCGGGAT TTCTTGATTC AAGTTCAGAA TATTGCCAAG
 651  GAACGTGGTG AAAAATGTCC CACTAAGGTA ACAAATCAGG TGTTCAGGTA
                                                  RsaI
 701  CGCGAAGAAG GCAGGGGCAA GCTACATAAA TAAGCCAAAA ATGCGACACT
 751  ACGTGCATTG CTACGCACTT CATTGCCTTG ATGAGGAGGC CTCCAATGCG
      BsaAI                    SluI
 801  CTAAGAAGAG CTTTCAAGGA GCGAGGAGAG AATGTTGGGG CATGGAGACA
 851  AGCATGTTAC AAGCCCCTGG TAGCCATAGC TGCTCGACAA GGCTGGGATA
                                                  RI
 901  TCGACACCAT CTTTAATGCA CATCCTCGAC TCGCCATTTG GTATGTCCCC
 951  ACCAGGCTCC GCCAGCTTTG CCATTCTGAA CGAAGCAACG CTGCTGCTGC
1001  TGCTTCTAGC TCGCTTTCTG GTGGTGTTGG TGATCACCTG ACCGCATTTC
      RsaI                    BsaAI  BspMI
1051  AAGTTGAGAG TAGAGTACTC AGTTTAATCT TTTCTGACTT TGCTTGCCCC
                 RsaI/ScaI
1101  TATATCTAGC TCCTACTTTG TTCTAGTTGT GATGTAATCT TTTCGGTTTA
1151  GAGACTAAAA TATCTGCATA ACATGGGTGT CCCTCAATTC AGTTCTGCAA
1201  ATTCTGTAAC TGCTTTAGTT TAGAACTTTT GACATATACT AATGGTTTGG
1251  TAAAAAAAAA AAAAAAA
```

CTAB plasmid miniprep

1. Harvest cells by centrifuging in 1.5 ml microfuge tube for 3 min (one per culture should be adequate).

2. Remove supernatant and resuspend cells in 200 µl of STET buffer. Add 20 µl of lysozyme solution (10 mg/ml) and let sit at room temperature for 5 min.

3. Place tube in boiling water bath for 45 seconds.

4. Centrifuge for 10 min. Remove pellet of cell debris with toothpick.

5. Add 8 µl of 5% CTAB (warmed to 37°), centrifuge for 5 min.

6. Remove as much of the supernatant as possible. Resuspend pellet in 200 µl of 1.2 N NaCl with vigorous pipetting. Add 600 µl ethanol. Centrifuge for 5 min.

7. Remove supernatant, rinse with 70% ethanol, air dry pellet. Resuspend pellet in 30 µl TE, add 10 µg of heat treated RNAse A, and incubate at 37° for 15 min. DNA is now ready for restriction, transformation, or sequencing. For restriction use 2-4 µl of DNA in a minimum of 20 µl per reaction.

STET buffer (100 ml):

Sucrose	8g
Triton X-100	0.5 ml
EDTA (0.5 M)	10 ml
Tris (1M, pH 8)	1.0 ml
Water	to 100 ml
	Autoclave

CTAB: Hexadecyltrimethyl ammonium bromide. Make 5% aqueous solution. Store at -20° C. Warm at 37° prior to use.

Competent Cell Prep and Bacterial Transformations

Competent cells

1. Grow an overnight culture of cells. Dilute 1/100 of the overnight into 50 ml of desired medium (SOB has been recommended), and grow until OD_{550} is .45–.55.

2. Chill flask on ice. Centrifuge cells in two chilled, sterile 50 ml Oakridge tubes in JS13.1 rotor at 6,000 rpm (~6,000xg) for 5 min at 4°. Pour off supernatants and resuspend each pellet in 8 ml of ice-cold TFB. Leave on ice for 15 min.

3. Centrifuge tubes as above. Resuspend one pellet in 2 ml TFB, transfer to the other tube, and resuspend that pellet. Add 70 µl of cold N,N-dimethylformamide and leave on ice for 5 min.

4. Add 70 µl of 0.7 M ß-mercaptoethanol (3.6 µl ß-me + 71.4 µl water). Leave on ice for 10 min.

5. Add 70 µl of N,N-dimethylformamide, and leave on ice for 5 min before transforming cells.

Transformation

1. In chilled glass tubes mix 100–200 µl of competent cells with desired amount of DNA or ligation mix for the transformation. Leave on ice 30–45 min.

2. Transfer tubes to 42° water bath for 90 seconds. Add 800 µl of growth medium (LB, SOB, etc.) and shake tubes at 37° for 20 min.

3. Plate 100 µl of cells on selective medium. Centrifuge remaining 900 µl of cells in microfuge for 2 min, resuspend in about 80 µl of growth medium, and plate on selective medium.

Recipes

SOB (1 liter):

Tryptone	20 g
Yeast Extract	5 g
NaCl	0.585 g
KCl	0.186 g
$MgCl_2.6H_2O$	2 g
$MgSO_4.7H_2O$	2.5 g

Adjust pH to 6.9

TFB (200 ml):

MES	0.4 g
RbCl	2.42 g
$MnCl_2.4H_2O$	1.78 g
$CaCl_2.2H_2O$	0.3 g
$[Co(NH_3)_6]Cl_3$	0.16 g

Adjust pH to 6.3 with 1 N KOH. Filter sterilize

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TECHNICAL ADVANCE

A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers

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Summary

A set of mapping markers have been designed for *Arabidopsis thaliana* that correspond to DNA fragments amplifed by the polymerase chain reaction (PCR). The ecotype of origin of these amplified fragments can be determined by cleavage with a restriction endonuclease. Specifically, 18 sets of PCR primers were synthesized, each of which amplifies a single mapped DNA sequence from the Columbia and Landsberg *erecta* ecotypes. Also identifed was at least one restriction endonuclease for each of these PCR products that generates ecotype-specific digestion patterns. Using these co-dominant cleaved amplified polymorphic sequences (CAPS), an *Arabidopsis* gene can be unambiguously mapped to one of the 10 *Arabidopsis* chromosome arms in a single cross using a limited number of F_2 progeny.

Introduction

Genetic maps consisting primarily of restriction fragment length polymorphic (RFLP) markers are being constructed for a variety of plants including maize (Burr and Burr, 1991; Helentjaris, 1987), barley (Tragoonrung *et al.*, 1992), wheat (Kam-Morgan and Gill, 1989), rice (McCouch *et al.*, 1988), tomato (Bernatsky and Tanksley, 1986), lettuce (Landry *et al.*, 1987), *Brassica oleraceae* (Slocum *et al.*, 1990) and *Arabidopsis thaliana* (Chang *et al.*, 1988; Nam *et al.*, 1989). RFLP maps are well-suited to mapping newly cloned DNA sequences. However, most plant genes are first identified by mutation. Mapping such a mutation on to a pre-existing RFLP map is a lengthy procedure requiring the isolation of DNA from individual F_2 plants or F_3 families, and performing DNA blot analysis using each of the RFLP markers as a hybridization probe.

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Recently, techniques based on the polymerase chain reaction (PCR) (Mullis and Faloona, 1987) have been used in addition to or in place of traditional RFLP markers in genetic analysis (Cox and Lehrach, 1991). In contrast to traditional RFLP markers, PCR-generated markers can be scored using a small sample of DNA without the use of radioactivity and without the time-consuming DNA blotting procedure. One widely used PCR-based approach involves the use of single short PCR primers of arbitrary sequence (called RAPD primers for random amplified polymorphic DNA; Reiter *et al.*, 1992; Williams *et al.*, 1990). A major advantage of RAPDs is that they provide large numbers of markers. On the other hand, because the amplification of a specific sequence or sequences using a RAPD primer is frequently sensitive to PCR conditions, including template concentration, it can be difficult to correlate results obtained by different research groups (Devos and Gale, 1992). A second limitation of the RAPD method is that it usually cannot distinguish heterozygotes from one of the two homozygous genotypes (Williams *et al.*, 1990). Finally, RAPD primers frequently amplify more than one sequence, which can complicate the analysis (Riedy *et al.*, 1992). In this report we describe a simple PCR-based strategy to map a gene to one of the 10 *Arabidopsis* chromosome arms which is not subject to the limitations of the RAPD method.

Results

Polymorphisms in amplified sequences revealed by restriction endonuclease digestion

As illustrated in Figure 1(a), the PCR-based strategy that we used to map *Arabidopsis* genes is based on a method used previously to detect RFLPs in cereals (Tragoonrung *et al.*, 1992; Weining and Langridge, 1991; Williams *et al.*, 1991). The method utilizes amplified DNA fragments that are digested with a restriction endonuclease to display an RFLP. To facilitate the development of a set of PCR markers that could be used to map a mutation to one of the 10 *Arabidopsis* chromosome arms, we first identified a set of *Arabidopsis* genes that had already been mapped and sequenced and then used these mapped sequences to design PCR primers.

As of 4 March, 1992, 26 of the 254 *Arabidopsis* genomic or cDNA sequences in GenBank had assigned map positions in AAtDB (an *Arabidopsis thaliana* database,

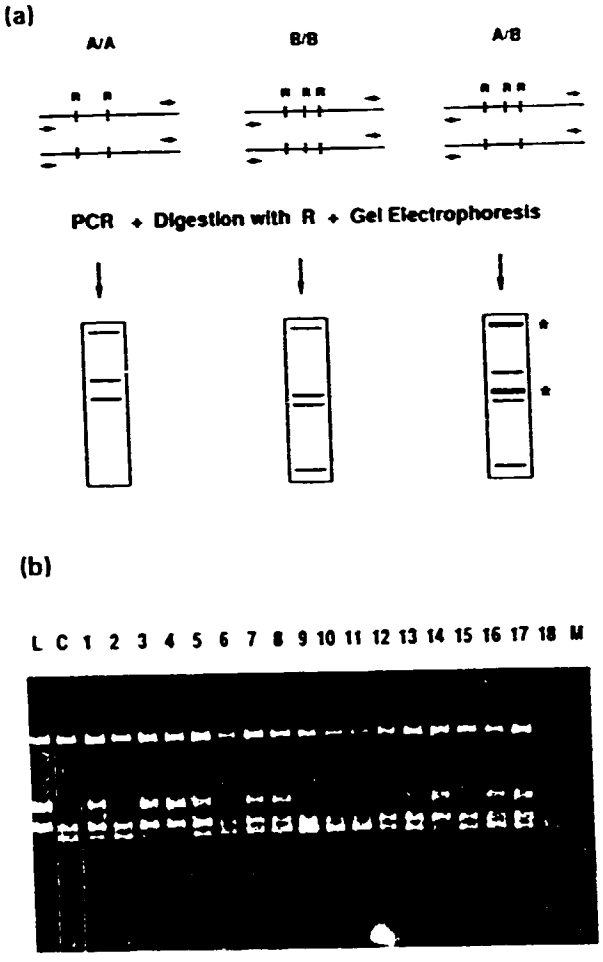

Figure 1. Generation and visualization of *Arabidopsis* ecotype-specific CAPS markers.

(a) Unique sequence primers are used to amplify a mapped DNA sequence from two different inbred ecotypes, A/A and B/B and from the heterozygote A/B. The amplified fragments from ecotypes A/A and B/B contain two and three recognition sites, respectively, for restriction endonuclease R. In the case of the heterozygote A/B, two different PCR products will be obtained, one which is cleaved three times by R and one which is cleaved twice. When fractionated by agarose or acrylamide gel electrophoresis, the PCR products digested by R from individual plants will give readily distinguishable patterns as seen in the lower portion of part (a). The asterisks indicate bands that will appear as doublets.

(b) Segregation of the *PG11* locus in F_3 progeny from a Col *rps2-101C* × Landsberg *erecta* cross. DNA from pooled F_3 plants (lanes 1–18) was amplified using *PG11* primers. PCR products were digested with *Bfa*I, electrophoresed on a 2.5% agarose gel, and stained with ethidium bromide. The two left-most lanes correspond to Ler (L) and Col (C) parents. DNA size markers (510, 390, 344, and 298 bp) are shown in lane M. In general, the doublet bands are readily apparent in the heterozygous plants (lanes 1, 3, 5, 7, 8, 14, 16–18) serving to verify the heterozygous restriction pattern. On the other hand, the observed stochiometry of the two intermediate sized bands for some of the presumptive Col/Col homozygous plants (lanes 9,10,11) was unexpected. Although we have no simple explanation for this result, it is likely due to an artifact of *Bfa*I digestion since unexpected stochiometries were not observed with other restriction endonuclease digests.

Cherry *et al.*, 1992). Among these 26, the following were chosen for further analysis because they were widely dispersed in the *Arabidopsis* genome (Table 1): *GAPB* (Shih *et al.*, 1991), *ADH* (Chang and Meyerowitz, 1986), *GPA1* (Ma *et al.*, 1990), *GAPC* (Shih *et al.*, 1991), *GAPA* (Shih *et al.*, 1991), *GL1* (Oppenheimer *et al.*, 1991), *BGL1* (Dong *et al.*, 1991), *GA1* (Sun *et al.*, 1992), *AG* (Yanofsky *et al.*, 1990), *DHS1* (Keith *et al.*, 1991), *ASA1* (Niyogi and Fink, 1992), *DFR* (Shirley *et al.*, 1992), and *LFY3* (Weigel *et al.*, 1992). Four additional sequences were obtained from other laboratories: *PVV4* (Van der Straeten *et al.*, 1992), *NCC1* (Crawford, personal communication), *m246* (Ma, personal communication), and *PG11* (Galant and Goodman, personal communication). A final DNA sequence, corresponding to RFLP marker *m429* (Chang *et al.*, 1988) was obtained by sequencing 316 bp of an end probe from a 110 kb YAC on chromosome II identified during a walk to the *DET2* gene (Nagpal and Chory, unpublished data; see Experimental procedures for details). These 18 mapped DNA sequences are distributed along all five *Arabidopsis* chromosomes: four on chromosome I, three on chromosome II, four on chromosome III, four on chromosome IV, and three on chromosome V (Table 1).

A pair of oligonucleotide primers (shown in Table 1) was synthesized for each of the DNA sequences listed in Table 1. When possible, primers were chosen such that the PCR products would include introns, to maximize the possibility of finding polymorphisms. When these primers were used to amplify DNA fragments from Columbia (Col) and Landsberg *erecta* (Ler) DNA, each pair of primers amplified a single major PCR product and the size of each PCR product was as predicted from the nucleotide sequence (Table 2), with the single exception of *GAPA*, in which case the Ler product was approximately 10 bp larger than the Col product.

To identify RFLPs between the amplified Ler and Col sequences, the primary PCR products were subjected to digestion with a panel of restriction endonucleases. Enzymes were chosen based on the DNA sequence of the amplified product which, in most cases, was known for at least one of the two ecotypes. Typically, each PCR product was digested with 25 restriction enzymes, which cut at least twice within the sequence. If none of the enzymes detected a polymorphism, a second series of digestions was performed with 25 different enzymes until a polymorphism was found or no more enzymes (out of a panel of 83) were available. The results are shown in Table 2. We named these DNA markers 'CAPS' for cleaved amplified polymorphic sequences.

Figure 1(b) shows the segregation of the CAPS marker *PG11* in the progeny from a Col X Ler cross. In the experiment shown in Figure 1(b), the template DNA for PCR analysis was isolated from pooled F_3 plants derived by selfing individual F_2 plants. However, we also found

Table 1. Map positions and sequences of PCR primers for *Arabidopsis* CAPS markers

Chr.	Gene, marker	Map position[a]	Forward primer[b]	Reverse primer[b]	Enzyme(s)
I	*PW4*	25.2	GTT TGA AAG TGT AGA TGT AAC GAC	GGT TGT GTT TTG CTA GCA TC	*Bsa*AI
	NCC1	47.9	GTC CTA TCT CTA CGA TGT GGA TG	AAG TTA TAA GGC ATT AGA ATC ATA ATC	*Rsa*I
	GAPB	92.6	TCT GAT CAG TTG CAG CTA TG	GGC ACT ATG TTC AGT GCT G	*Bfa*I, *Dde*I
	ADH	13.7	GCG TGA CCA TCA AGA CTA AT	AAA AAT GGC AAC ACT TTG AC	*Xba*I, *Bfa*I
II	*m246*	14.1	TGA AGA GCT ATC CGA GAT GG	GCT TGA ACT CCT CCT CCT TC	*Mae*III
	GPA1	56.2	GGG ATT TGA TGA AGG AGA AC	ATT CCT TGG TCT CCA TCA TC	*Afl*III
	m429	69.6	TGG TAA CAT GTT GGC TCT ATA ATT G	GGC AGT TAT TAT GAA TGT CTG CAT G	*Scr*FI
III	*GAPC*	1.0	CTG TTA TCG TTA GGA TTC GG	ACG GAA AGA CAT TCC AGT C	*Eco*RV
	GAPA	30.9	CAC CGT GAT CTA AGG AGA GCA AG	TGT GCT CAA CCA AAC TTA GCC	*Dde*I
	GL1	44.5	ATA TTG AGT ACT GCC TTT AG	CCA TGA TCC GAA GAG ACT AT	*Taq*I
	BGL1	87.1	TCT TCT CGG TCT ATT CTT CG	TTA TCA CCA TAA CGT CTC CC	*Rsa*I, *Sau*3A, *Afl*III
IV	*GA1*	10.5	AAG CTT CGA ACT CAA GGT TC	CCG GAG AAT CGT ACG GTA C	*Bsa*BI
	AG	47.5	CAA CAG GTT TCT TCT TCT TCT C	CAA ACA CCA TTT AAT CTT GAC A	*Xba*I
	PG11	67.6	CGC AAC TAA CCA CAC ATT AC	AGT GAA ATT CAC CAG CAT G	*Bfa*I
	DHS1	88.7	CAA GTG ACC TGA AGA GTA TCG	AGA GAG AAT GAG AAA TGG AGG	*Dde*I, *Bsa*AI, *Mbo*III
V	*ASA1*	17.2	CTT ACT CCT GTT CTT GCT TAC	CCT CTA GCC TGA ATA ACA GAA C	*Bcl*I
	DFR	63.4	AGA TCC TGA GGT GAG TTT TTC	TGT TAC ATG GCT TCA TAC CA	*Bsa*AI
	LFY3	90.9	TAA CTT ATC GGG CTT CTG C	GAC GGC GTC TAG AAG ATT C	*Rsa*I

[a]The map positions given are those listed in version 1.3 of AAtDB, an *Arabidopsis* data base described in Cherry *et al.* (1992).
[b]Primers are shown 5' to 3'.

Table 2. Restriction enzymes that detect polymorphisms in Landsberg *erecta* (Ler) and Columbia (Col) CAPS amplification products

Chr.	Gene, marker	Size of PCR product (bp)	Enzyme	Restriction fragments in base pairs[a]
I	*PVV4*	1064	*Bsa*AI	Ler: 706; 311; 47 Col: 753; 311
	NCC1	970[b]	*Rsa*I	Ler: 920[b]; 50[b] Col: 870[b]; 50[b]
	GAPB	1481	*Bfa*I	Ler: 850[b]; 360[b]; 212; 58 Col: 1211; 212; 58
			*Dde*I	Ler: 350[b]; 284; 255[b]; 225; 174 Col: 605; 284; 225; 174
	ADH	1291	*Xba*I	Ler: 1097; 262 Col: 1291
			*Bfa*I	Ler: 849[b]; 250[b]; 127; 65 Col: 1099; 127; 65
II	*m246*	1354	*Mae*III	Ler: 1122; 232 Col: 1354
	GPA1	1594	*Aff*III	Ler: 1385; 209 Col: 705[b]; 680; 209
	m429	316	*Scr*FI	Ler: 216[b]; 100[b] Col: 316
III	*GapC*	1148	*Eco*RV	Ler: 713; 390[b]; 340[b] Col: 735; 713
	GAPA	761/771[c]	*Dde*I	Ler: 240[b]; 190[b]; 178; 100; 33; 19; 10 Col: 420; 178; 100; 33; 19; 10
	GL1	519	*Taq*I	Ler: 372; 100; 47 Col: 298[b]; 100; 74[b]; 47
	BGL1	1269	*Rsa*I	Ler: 785; 485 Col: 785; 340[b]; 105[b]
			*Sau*3A	Ler: 1269 Col: 875; 395
			*Aff*III	Ler: 494; 434; 258; 84 Col: 494; 344; 258; 150; 84
IV	*GA1*	1196	*Bsa*BI	Ler: 1196 Col: 707; 527
	AG	1366	*Xba*I	Ler: 1073; 293 Col: 1366
	PG11	1293	*Bfa*I	Ler: 644; 353; 296 Col: 644; 296; 263[b]; 90[b]
	DHS1	1668	*Dde*I	Ler: 1620; 48 Col: 1491; 129; 48
			*Bsa*AI	Ler: 1668 Col: 1188; 480
			*Mbo*II	Ler: 520; 480; 173; 141; 84[b]; 81 Col: 1084; 173; 141; 81
V	*ASA1*	1728	*Bcl*I	Ler: 686; 553[b]; 489[b] Col: 1042; 686
	DFR	1143	*Bsa*AI	Ler: 609, 318, 216 Col: 609; 534
	LFY3	1330	*Rsa*I	Ler: 855; 236; 126; 78; 35 Col: 708; 236, 147; 126; 78, 35

[a]Only those fragments that are large enough to be readily visible are listed.
[b]Denotes approximate size of the fragment.
[c]The Ler product is approximately 10 bp larger than the Col product.

that the DNA isolation procedure described by Dellaporta *et al.* (1983) could be used to isolate DNA from a single *Arabidopsis* leaf that was of sufficient quality to perform the CAPS mapping procedure. We generally obtained 1–5 μg of DNA from a single leaf which is enough DNA for at least 50 PCR reactions. We have successfully amplified the desired PCR products using as little as 5 ng of DNA obtained from this mini-preparation procedure (Glazebrook and Ausubel, unpublished data).

We used the experimental protocol described in detail in the Experimental procedures in developing the CAPS mapping procedure. However, a variety of modifications can be made in the protocol. For example, we have successfully used 40 ng of each PCR primer instead of 200 ng (Glazebrook and Ausubel, unpublished data) and it is possible that even less primer could be used. In addition, instead of using 15 units of each of the restriction endonucleases to digest the PCR products, some of the endonucleases have been used successfully at lower concentrations (10 units of *Eco*RV, *Xba*I, *Rsa*I; 8 units of *Sau*3A; 4 units of *Dde*I; 3 units of *Taq*I; Glazebrook and Ausubel, unpublished data). On the other hand, we found that at least 15 units of *Bfa*I were required to digest the *PG11* PCR product to completion.

Frequency of CAPS polymorphisms

We detected 20 polymorphic changes in approximately 5227 nucleotides that comprised the recognition sites surveyed for polymorphisms in this study. This suggests that on average, Ler and Col genes differ once in every 261 (5227/20) bp. This is an oversimplification, however, because polymorphisms are not randomly distributed in the *Arabidopsis* genome. For example, the *ADH* locus contains 10 bp changes between Ler and Col concentrated in a 220 bp region, but only 13 total changes within a total of 2900 bp that have been sequenced in both ecotypes (Hanfstingl and Ausubel, unpublished data). Most of the polymorphisms (19 out of 20) that we detected were probably due to single base pair changes. One insertion/deletion of approximately 10 nucleotides was found in the *GAPA* gene.

Large insertion/deletion markers and microsatellite-based PCR markers

In addition to CAPS, we also identified PCR-based mapping markers that correspond to relatively large deletions and insertions such as the 5 kb internal region of the retrotransposon *Ta1-1* which is present in single copy on chromosome IV of Landsberg *erecta*, but which is absent from Columbia (Voytas *et al.*, 1990). PCR primers corresponding to *Ta1-1* (ACAGCATCCTGAAGACCTCG and TCGTTGATCGACTTAGTATC) amplified a 435 bp sequence from Ler DNA but failed to amplify any sequence from Col DNA (data not shown). We decided not to pursue this type of PCR marker further because it is not possible to distinguish plants that are homozygous from those that are heterozygous for the amplified sequence.

A third class of PCR markers that we identified was based on PCR amplification across tandem repeats of dinucleotide sequences called microsatellites (Hearne *et al.*, 1992). Microsatellites occur frequently and randomly in most eukaryotic DNAs and display polymorphisms due to variations in the number of repeat units. Screening the *Arabidopsis* sequences deposited in GenBank for different combinations of dinucleotide repeats revealed the presence of at least 10 repeats with a total length of 20 bp or more. We synthesized PCR primers that amplified 150–200 bp fragments that contained the following four microsatellite sequences: a (GA)10 repeat in *GBF3* (Schindler *et al.*, 1992); a $(AC)_8AG(AC)_4$ repeat in the non-coding region of a *myb*-homologous gene (Oppenheimer *et al.*, 1991); a $(AT)_{14}$ repeat in the intron of the gene encoding a basic chitinase (Samac *et al.*, 1990); and a (AT)3AA(AT)10 repeat in the *ATS1A* gene (Krebbers *et al.*, 1988). PCR products were resolved on 8% acrylamide gel. Among these four, only the *ATS1A* repeat was found to be polymorphic between Col and Ler (data not shown). Although we did not pursue the use of microsatellites further, we think that they may serve as highly informative markers in the future once more of them have been identified and mapped (Ecker, personal communication).

Discussion

The 18 sets of PCR-based CAPS primers described in this paper can be used to rapidly assign map positions to newly identified *Arabidopsis* genes. Given the limited number of CAPS markers currently available, however, subsequent analysis using traditional RFLP markers is needed to determine a map position accurate enough to initiate a chromosomal walk. There are several potential sources of new CAPS markers, which would allow the use of CAPS for the generation of high resolution maps. Sources of new CAPS markers include newly identified (and mapped) genes, DNA sequences around mapped RFLPs (where a polymorphic site is already known to exist), or DNA sequences at the ends of YAC clones (e.g. m429 marker on chromosome II). We anticipate that the results presented in this paper will lead others to generate and use additional CAPS. We also anticipate that 'second generation' CAPS markers may eventually supplant some of the original ones. For examples, the *PG11* CAPS marker requires the use of *Bfa*I, an expensive and not particularly robust restriction endonuclease. In the future, *PG11* could be replaced by another CAPS marker that maps nearby.

AAtDB (An *Arabidopsis thaliana* Database, Cherry, *et al.*, 1992) at the Department of Molecular Biology, Massachusetts General Hospital and the *Arabidopsis* Stock Centers at Ohio State University and at The University of Nottingham could serve as clearing houses for the distribution of CAPS-related information.

The maximum distance between any two of the 18 CAPS markers described in this paper is 46.2 cM (*DFR* and *ASA1*). Although it is not possible to determine with certainty the distance between a particular marker and the end of the chromosome on which it resides, the relatively high density of the *Arabidopsis* RFLP map makes it unlikely that any of the flanking CAPS markers on any of the five chromosomes are significantly more than 25.2 cM (in the case of *PVV4*) from an end. Therefore, the farthest away a gene of interest could be from one of the 18 CAPS markers is 25.2 cM.

To carry out the CAPS mapping procedure in the most efficient manner, it is helpful to calculate the minimum number of F_2 progeny from a Col x Ler cross that must be screened for segregation of the CAPS markers to establish linkage to one of them. Based on the binomial distribution, we calculate that a minimum of only 28 F_2 plants are required to establish linkage to one of the 18 CAPS markers at the 95% confidence limit. Because the CAPS markers are co-dominant, analysis of 28 F_2 plants is equivalent to analyzing 56 chromosomes.

To develop a CAPS marker from a known sequence, we routinely designed primers that amplified 1.5–2.5 kb sequences. In some cases, once a polymorphism was identified by testing a panel of restriction enzymes, the primers were redesigned (based on the sequence) to better visualize the polymorphism. For example, the original set of *GAPA* primers preferentially amplified DNA sequences from the Ler ecotype, making it difficult to unambiguously identify heterozygous plants. An additional problem with the original *GAPA* primers was that the polymorphic bands were too close in size to distinguish Ler and Col amplified products with a high degree of confidence. The *GAPA* primers listed in Table 1 eliminated both of these problems.

A major advantage of CAPS is that they are co-dominant genetic markers; that is, different digestion patterns are obtained for plants that are homozygous or heterozygous for the parental alleles. However, as illustrated with the original *GAPA* primers that we designed (see above), a particular set of primers may preferentially amplify only one of the two parental sequences when both are present in a heterozygous plant. Therefore, it is critical, to use an equal mixture of the two homozygous parental DNAs or DNA isolated from a known heterozygous plant as a control.

The CAPS primers described in this paper were specifically designed for the *Arabidopsis* Columbia and Landsberg *erecta* ecotypes. However, six of the PCR markers reported here were tested using the Landsberg *erecta* and Niederzenz ecotypes and three of them detected polymorphisms (Chory, personal communication).

To date, the CAPS method has been used to map several genes including *RPS2* (resistance to *Pseudomonas syringae*; Yu *et al.* 1993), *pad1*, *pad2* and *pad3* (phytoalexin deficient mutants; Glazebrook and Ausubel, unpublished data) and *trp4* (a tryptophan biosynthetic pathway mutant; Keith, personal communication).

Experimental procedures

Plant material

Arabidopsis plants used in this study were the Columbia and Landsberg *erecta* ecotypes. For the cross described in Figure 1(b), Ler was used as the female parent and the Columbia *rps2-101C* mutant (Yu *et al.*, 1993) was used as the pollen donor.

DNA isolation

For large scale PCR reactions, DNA was isolated from whole plants using the procedure described in Ausubel *et al.* (1993). A slightly modified version of the method described by Dellaporta *et al.* (1983) was used to isolate DNA from one to three *Arabidopsis* leaves for CAPS mapping. Briefly, leaves were frozen in liquid nitrogen, ground to a powder in a mortar and pestle, and transferred to 1.5 ml microcentrifuge tubes. Extraction buffer (500 µl) containing 100 mM Tris pH 8, 50 mM EDTA pH 8, 500 mM NaCl and 10 mM mercaptoethanol was added followed by 35 µl of 20% SDS. The samples were incubated at 65°C for 10 min and 130 µl of 5 M potassium acetate was added. After 5 min incubation at 0°C, the precipitate was pelleted for 10 min at 15 000 *g*, the supernatant was transferred to a 2 ml tube containing 640 µl isopropyl alcohol, and 60 µl 3 M sodium acetate, mixed, and incubated at −20°C for 10 min. The precipitated DNA was centrifuged at 15 000 *g* for 15 min and redissolved in 200 µl of 50 mM Tris pH 8.0, 10 mM EDTA. This solution was centrifuged at 15 000 *g* or 5 min to remove insoluble material, and the supernatant was transferred to a tube containing 20 µl 3 M sodium acetate and 440 µl ethanol. After incubation at −20°C for 10 min, DNA was pelleted by centrifugation at 15 000 *g* for 5 min, and washed with 70% ethanol. Pellets were dried and dissolved in 50 µl water.

PCR amplification of Arabidopsis *genomic DNA*

PCR primers were designed using the computer program PRIMER (Version 0.5, May 1991, by Stephen E. Lincoln, Mark J. Daly and Eric S. Lander, Whitehead Institute for Biomedical Research). Large scale PCR reactions were performed using reagents from Boehringer (10× buffer), Pharmacia (deoxynucleotides), and Promega (Taq polymerase). The reactions were carried out in 100 µl that contained 0.125 mM of each deoxynucleotide, 0.5 µg of each primer, 2.5 U of Taq polymerase in 2.5 µl, and 50–100 ng of *Arabidopsis* genomic DNA. Conditions for the amplification were as follows: 30 sec at 95°C; annealing for 30 sec at 56°C; polymerization for 3 min at 72°C. The cycle was repeated 50 times. For mapping purposes, the reaction was scaled down to 10 µl final volume, which contained 0.125 mM each of four deoxynucleotides, 0.2 µg of both primers, 0.25 U of Taq polymerase and approximately 50 ng of *Arabidopsis* DNA (1 µl of the 50 µl obtained from a miniprep). Usually a premix containing deoxy-

nucleotides, Taq polymerase buffer, primers and Taq polymerase was prepared for 30 reactions and transferred to 0.5 ml microcentrifuge tubes to which various DNAs were added. Other PCR conditions were the same as for the large scale reaction.

Restriction enzyme digestions and analysis of PCR products

To identify restriction endonucleases that would generate a polymorphism, reactions were carried out in 10 μl final volume containing 3.5 μl of PCR product, 1 μl of 10× concentrated restriction enzyme buffer, and 1–3 units of the appropriate restriction endonuclease. The digestion products were analyzed on 1.5 % agarose gels for all markers except *m429*, *PG11* and *LFY3*, which were run on 2.5% gels. Ler and Col products were run in adjacent lanes to visualize polymorphisms. For mapping, restriction endonuclease digestion was carried out in the same tube as the PCR reaction. Restriction enzyme mix (10 μl) containing 2 μl of restriction enzyme buffer and 15 U of enzyme was added to each tube and incubated for 2 h at the temperature optimal for the particular enzyme activity. Four microliters of 6× loading dye (Ausubel *et al.*, 1993) was added to each tube prior to electrophoresis.

DNA sequencing

Partial DNA sequence of an end probe from a 110 kb YAC clone (mapped to chromosome II) was obtained by sequencing of an inverse PCR product provided by J. Chory and P. Nagpal (Salk Institute, La Jolla) using the *fmol* DNA Sequencing system (Promega) according to the manufacturer's instructions.

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METHODS IN ARABIDOPSIS RESEARCH

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Chapter 7

Genetic linkage of the *Arabidopsis* genome: Methods for mapping with recombinant inbreds and Random Amplified Polymorphic DNAs (RAPDs)

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1. Introduction

1.1. Genetic maps

Genetically mapped DNA markers are useful in studying the inheritance of genetic traits and the variation among populations, as tools in breeding programs, and in "chromosome walking" approaches to gene cloning.

It is important to realize that genetic maps result from statistical evaluations of large data sets derived from the segregation of markers at many loci. Linkage is derived from this information by two-point (pairwise), three-point, and n-point analyses. Thus, both the order of markers and the genetic distance between them are dependent upon the size and type of population(s) used, the genetic background of the parental strains, and the nature of the molecular markers used. A more reliable, and perhaps different order of existing markers can be obtained by mapping these markers in different populations, in a larger number of individuals of the same population, or both. Addition of new markers may also result in changes of an existing map. It is thus important to remember that genetic maps evolve, become more complete and reliable with time.

The first step in the construction of a map is the selection of a mapping population. Next, one has to select the type of marker that will be used to monitor recombination. We discuss below the alternatives available in *Arabidopsis*.

1.2. Choosing a mapping population

A significant advantage of plant genetics is that sexual crosses can be easily arranged. An additional advantage of *Arabidopsis* is that relatively large populations

can be grown in limited spaces and under controlled envi[illegible]ental conditions over a short period of time.

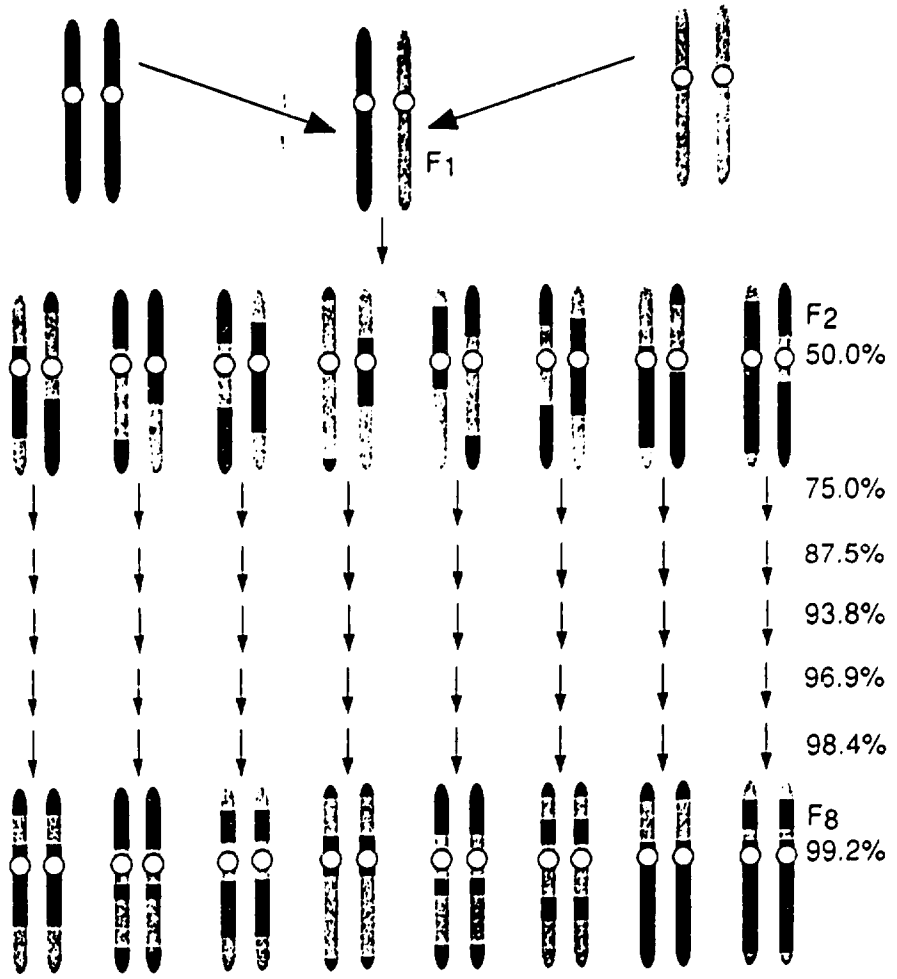

Figure 1. Development of recombinant inbred (RI) lines from F_2 individuals. Following selfing of a single plant per line each generation, the expected level of homozygosity is listed to the right. After only 4 generations of selfing, the resulting F_5 lines are more than 93% homozygous. Approximately one-half of the recombination events observed in the F_2 are fixed and one-half are lost by the next generation, however new recombination events occur and are fixed in the subsequent generations.

To select the parent plants that will be used to develop the mapping population, *Arabidopsis* plants of different ecotypes are selected on the basis of their degree of polymorphism. Depending on the goal of the project, one or both of the parents may carry one or more traits of interest. Crossing the two parent plants will result in an F_1 generation, in which all loci differing in alleles between the parents will be heterozygous (Figure 1). The F_1 plants can be used to generate F_2 progeny by selfing. Alternatively, crossing of F_1 plants to one of the parents gives rise to backcross (BC) progeny. Use of either F_2s or BCs as mapping populations may require increasing the amount of plant material available. Seed pools representing the genotypes of these populations may be created by selfing F_2s or BC_1s and harvesting plants individually. If these pools are large enough, and no segregation distortion occurs, these F_3 or BC_2s families will contain all the alleles present in the individual plant from which they were derived. Alternatively, vegetative propagation may be used to perpetuate F_2s or BC_1s.

Recombinant inbreds (RIs) are another type of mapping population that can be developed in *Arabidopsis*. RIs have been successfully used to map the mouse and corn genomes (Burr and Burr, 1991). To construct an RI population, F_1 plants are selfed to generate F_2s. Individual F_2 plants are then selfed and each plant is individually harvested, creating F_3 families. A few seeds from each F_3 family are drawn at random, germinated, and the resulting plants are randomly selected, leaving a single plant representing each family. Plants are harvested individually, forming the next generation's (F_4) families. The process, called single-seed descent, is repeated until the level of heterozygosity is negligible (F_6 or greater; Figure 1). At this point, selfing will produce progenies which are essentially identical to the previous generations. Thus, RIs constitute permanent populations which can be propagated indefinitely for mapping. Once a map is created using RIs, any new information is additive. Thus, any new marker mapped in an RI population becomes automatically integrated into the preexisting map (see Chapter 3, this volume).

Any trait that can be distinguished between the parent plants can now be scored in the RI collection. For mapping purposes, the proportion of recombinant individuals in the RI population (R) must be converted to the frequency of recombinant gametes in a single meiosis (r) using the equation of Haldane and Waddington (1931):

$$r = R/2 - 2R \qquad [1]$$

In RIs, R approaches 2r for closely linked markers. For example, let's consider the case of two markers separated by a genetic distance of 2 cM. In 100 F_2s there will be 4 recombinant individuals, and a total of 200 gametes. Thus, the frequency of recombinant gametes is 2%. If these same 100 F_2 individuals are used to generate an RI collection, we can expect approximately 4 recombinant lines in 100 RIs. In this case, $r = 0.04/2-(2 \times 0.04) \approx 0.02$.

1.3. Genetic markers

Plant genetic maps can be constructed with either phenotypic or molecular markers. Phenotypic markers affect traits such as leaf or flower morphology, plant size or pigment biosynthesis. Genetic maps based on phenotypic markers are now termed "classical maps" (Koornneef, 1990), to distinguish them from "molecular maps", which are based on protein or DNA markers. Protein markers, generally isozymes, are fast and inexpensive, but have limited usefulness because of their low frequency (Helentjaris *et al.*, 1986). DNA markers arise from variation present in DNA sequences, either base changes or insertions/deletions. These changes are termed DNA polymorphisms.

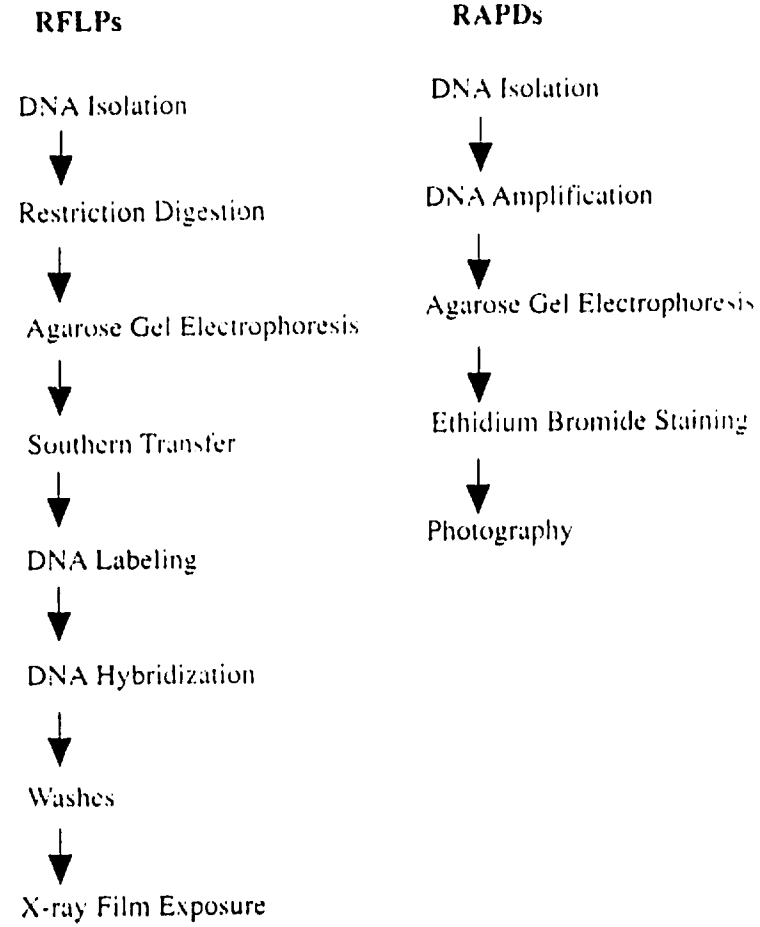

Figure 2. The various steps involved in either RFLP or RAPD analysis.

The two current techniques for detecting DNA polymorphisms are restriction fragment length polymorphisms (RFLP) and random amplified polymorphic DNAs (RAPDs). RFLPs detect differences in the length of specific DNA fragments after digestion of genomic DNA with sequence-specific restriction endonucleases (Botstein *et al.*, 1980). Polymorphic DNA fragment(s) are identified by Southern blot analysis using as probes either complementary DNA (cDNA) or genomic DNA fragments. The DNA sequences used as hybridization probes are generally pre-selected to contain only low-copy DNA (Chang *et al.*, 1989; Nam *et al.*, 1989).

RAPD markers are generated by amplification of genomic DNA with short (9-10 bp) oligonucleotides and thermostable DNA polymerases (Williams *et al.*, 1990). Polymorphisms detected by RAPDs reflect either sequence variation at the sites of priming, small insertions or deletions in the region of the genome which is amplified, or both. Figure 2 shows the steps involved in RFLP and RAPD analysis and Table 1 lists the advantages and disadvantages associated with both types of molecular markers. RFLPs generally are co-dominant, thus allowing the identification of both parental alleles. RAPDs are generally dominant, thus detecting only one of the parental alleles. The main consequence of this difference is that, generally, heterozygotes can be distinguished from the parental classes with RFLPs, but not with RAPDs. An advantage of RAPDs is that they detect polymorphisms in repetitive DNA whereas, as previously mentioned, RFLPs are limited to low-copy DNA.

In *Arabidopsis* approximately one-half of the RAPDs we tested by hybridization to genomic DNA corresponded to less than 4 copies, whereas the remaining RAPDs corresponded to sequences present in more than 10 copies (Reiter *et al.*, 1992).

Table 1. The advantages and disadvantages of RFLPs and RAPDs as genetic markers.

RFLPs	RAPDs
Advantages:	**Advantages:**
-Co-dominant markers -Generally contain low-copy DNA -Well established	-Small amount of DNA required -Assay is very fast -Simple detection -Automatable
Disadvantages:	**Disadvantages:**
-Slower and more laborious -Typically uses radioactivity -Generally assays only regions of the genome containing low-copy DNA	-Many RAPDs contain multiple-copy DNA which limits immediate use as hybridization probes -Less reproducible than RFLPs -Dominant markers

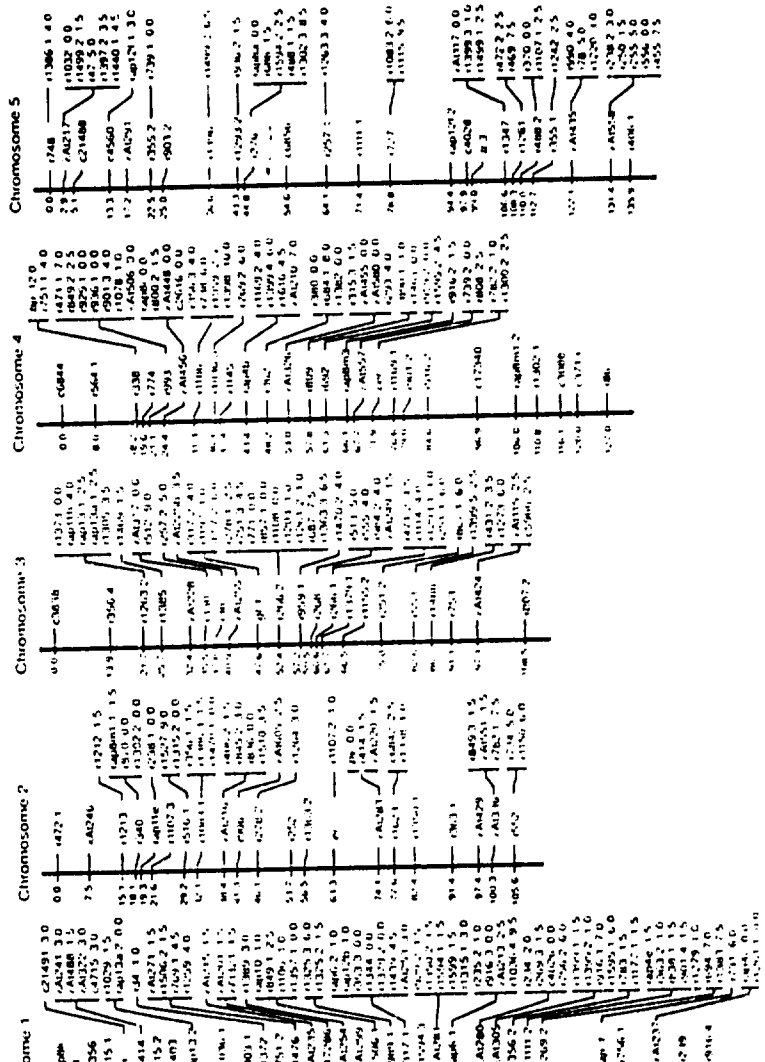

Figure 3. A linkage map of *Arabidopsis*. Markers along vertical bars were ordered with a LOD score difference > 3.0 using MAPMAKER. Their approximate positions are shown in cM to the left of each bar. Marker loci listed to the right of each bar could not be ordered with a LOD score difference > 3.0 and are shown along with their approximate distance in cM from ordered markers. Remaining markers are RAPDs.

1.4. Statistical considerations in choosing mapping populations and markers

In the previous sections we discussed the types of mapping populations and molecular markers that can be used with the *Arabidopsis* genome. Here, we discuss how best to combine populations and markers to obtain the maximum amount of information.

Genetic mapping consists of estimating genetic distances by scoring recombination. The amount of information provided by a mapping population can be defined as the ability of a given combination of population and marker type to permit the identification of the maximum number of recombinant gametes. Factors to be considered are the type (dominant or co-dominant) and phase (coupling or repulsion) of the DNA markers used, and the type of mapping population. The amount of mapping information obtained as a function of these variables has been calculated by Allard (1956) and by Reiter *et al.* (1992). These results can be summarized as follows. F_2s are not effective for mapping dominant markers, particularly when they are in repulsion phase. The main reason for this low efficiency is that these markers cannot distinguish between heterozygous and homozygous loci. Dominant markers, however, can be effectively mapped in BCs (coupling only) or RIs. Thus, one should not use F_2s for mapping dominant markers such as RAPDs, unless heterozygotes can be identified by procedures such as progeny testing. Co-dominant RFLPs can be mapped efficiently in any type of population.

We have mapped the *Arabidopsis* genome by combining RAPD technology with RIs. Our map integrates the classical and RFLP maps to provide a high-density map which will facilitate the cloning of *Arabidopsis* genes by map position (Reiter *et al.*, 1992; Figure 3). We anticipate the use of the RI collection and the accompanying map for two basic purposes: the mapping of cloned genes and the physical linkage of the genome.

In this chapter we offer specific information for mapping with RIs, RAPDs and RFLPs.

2. Growth of RI lines and DNA extraction

Researchers wanting to use our RI collection will receive seed samples of the two parent lines and of approximately 115 RI lines. These lines can be used for either RFLP or RAPD mapping.

2.1. Growth medium for rapid production of plant tissue

Prepare Gamborg's B-5 medium with sucrose (Gamborg *et al.*, 1968) as a liquid medium. Formulated powder can be purchased from Gibco (Grand Island, N. Y., cat. #500-1153EA). Aliquot 50 ml into 250 ml Erlermeyer flasks and autoclave.

The following steps are all carried out in a laminar flow hood. Place up to 2,000 seeds in a scintillation vial. Fill the vial with 50% commercial bleach (2.5 % sodium hypochlorite)/0.02% Tween 20 and incubate for 6-8 min. Be sure to stir or swirl the solution to force most seeds to sink to the bottom and to break up clumped seed. Decant gently and fill the vial with sterile dH_2O. Decant and repeat this step two to three times. One can typically handle up to 20 vials at once. When decanting the final rinse remove as much dH_2O as possible. Add an equal volume of 95% ethanol with a disposable transfer pipet and then transfer immediately the entire EtOH/water seed suspension to a sterile #1 Whatman filter paper disk. The filter paper should be resting on top of an inverted beaker of smaller diameter. This allows the excess EtOH/water to absorb and/or drip off of the filter paper and seeds to cling. Dry the seeds by placing a single fold in the filter paper and leaning it against its corresponding vial. When the seeds and filter paper are completely dry, transfer about 10 seeds to an Erlenmeyer flask containing B5 medium by scraping the filter with a sterile scalpel.

Seal the flasks with foil or caps and incubate the flasks at room temperature on a rotary platform set at 50 rpm under continuous illumination. In 2-3 weeks the culture will grow to form a mass of roots with some leaf development. It is important to harvest the tissue before the leaves turn brown or the yield of DNA will be low. Some RI lines develop significantly less tissue mass than the average. For these lines it may be necessary to grow several flasks.

To harvest tissue, decant the medium and rinse the tissue with dH_2O. Transfer the tissue to paper towels and blot dry. The tissue can be now be placed in disposable 50 ml polypropylene tubes and frozen by placing the tubes in dry ice or liquid nitrogen. Frozen tissue may be stored at -70° C. For ease of handling and improved DNA yield, tissue should be lyophilized. Lyophilized tissue may be stored for years at -20° C.

2.2. DNA extraction (modified from Murray and Thompson, 1980)

Grind lyophilized tissue using a mortar and pestle or coffee grinder. If necessary, the addition of dry ice pellets can aid grinding.

Transfer 200-500 mg lyophilized and ground tissue to a 16 ml polypropylene tube and add 6-9 ml of fresh CTAB extraction buffer that has been prewarmed to 60°C.

If dry ice was used for grinding, be sure it has sublimed completely prior to the addition of extraction buffer.

For fresh tissue extraction, add an equal amount (w/w) of a 2X stock of extraction buffer to frozen and ground tissue.

CTAB extraction buffer: 100 mM Tris-HCl (pH 7.5), 1% CTAB (Cetyl or Mixed alkyl-trimethyl-ammonium bromide), 0.7M NaCl, 10 mM EDTA, 1% β-mercapto ethanol (BME).

Mix the suspension well by rapping the tube upside down on a hard counter to suspend the powder. There is no need to be gentle at this stage. Incubate the mixture at 60°C for 30-90 min with occasional inversion.

Place the tubes at room temperature for 5 min to cool, then add 4.5 ml chloroform:octanol (or isoamyl alcohol) (24:1 v/v). Mix the tubes by gentle inversion for 5-10 min and then spin the tubes for 10 min in a table-top centrifuge at high-speed. Pour or pipet off the upper phase to new tubes and add 1/10th volume of 10% CTAB in 0.7 M NaCl (prewarmed to reduce viscosity).

Repeat the chloroform extraction. Pipet off the aqueous phase to new tubes and add an equal volume of precipitation buffer: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1% CTAB. Mix by inversion and let the tubes sit at room temperature for 30 min.

Spin the tubes in a table-top centrifuge at moderate to high speed. Decant off the supernatant. If a pellet does not form (especially true with fresh tissue extraction), add more precipitation buffer to lower the salt concentration further and respin the tubes. The critical point here is the reduction of the salt concentration which results in the co-precipitation of the DNA with the CTAB.

Resuspend the pellet in 450 μl of 1 M NaCl. Heat the tubes to 56°C if needed to dissolve the pellet. Transfer the entire volume to microfuge tubes and add 900 μl of EtOH. Mix the tubes by inversion to reprecipitate the DNA. Spin the tubes for 5 min at room temperature and decant off the supernatant. Wash the pellets twice with 1 ml each of 70% EtOH.

Redissolve the pellet in 100-400 μl TE and add 1-4 μl of 10 mg/ml RNAse A and incubate at 37°C for 15 min.

Extract with phenol and then, if necessary, with chloroform. Add 1/2 volume 7.5 M NH_4OAc and 3 volumes of EtOH to reprecipitate the DNA. Spin for 5 min. Resuspend the pellet in 100-400 μl TE. Yield should be 20-40 μg of DNA per 50 ml flask (200-300 mg lyophilized tissue).

2.3. Considerations

Remember that the first step is to screen for polymorphisms using the parent lines. Thus, more DNA is needed from parent strains than from RI lines. We recommend growing 10 flasks each of the parent strains and 1-3 flasks each of the RIs.

3. RFLP mapping

To map a DNA clone with RIs, one only needs to perform two Southern blots, one with parental DNA restricted with various enzymes to identify a polymorphism, and a second with DNA from RI lines to determine segregation. The result is a strain distribution pattern which, when integrated with the existing database, provides the location of the marker.

To screen for polymorphism, we routinely digest 1-2 μg of genomic DNA with each of five restriction endonucleases, *Bam*HI, *Eco*RI, *Eco*RV, *Hin*dIII and *Xba*I. Digests are routinely performed for 3-4 h at 37°C using 5-10 U of enzyme per μg of DNA, 12.5 mM spermidine and the appropriate buffer. Restricted DNA is separated in 0.8% agarose gels, denatured for 30 min. in 4 volumes of 0.5 M NaOH, 1.5 M NaCl, neutralized for 30 min. in 4 volumes of 1 M Tris-HCl (pH 7.5), 1.5 M NaCl and blotted onto a nylon membrane of choice (we prefer Immobilon N, from Millipore) using a standard capillary transfer protocol. Membranes are baked for 2 h at 80°C to fix the restricted genomic DNA. The membranes are pre-hybridized at 65°C for 2-4 h in 5X SSPE, 0.5% SDS, 5X Denhardt's, 5% dextran sulfate and 100 μg per ml denatured salmon-sperm DNA. For convenience we pre-hybridize and hybridize the membranes in trays placed in a 65°C air shaker using enough solution to just cover the membranes. The DNA fragment to be mapped should be labeled to high specific activity using random primer labeling (Feinberg and Vogelstein 1983), denatured and added to the trays containing the pre-hybridized membranes.

Hybridization should be allowed to proceed 16-20 h at 65°C. Filters should be washed 2 times at room temperature (5min. each) in 100-200 ml of 2X SSC, 0.5% SDS, then 2-3 times at 65°C (30 min. each) in 400-500 ml of 0.2X SSC, 0.1% SDS. Washed membranes should be blotted briefly on paper towels to remove excess liquid, rapped in plastic wrap and exposed for 1-7 days to X-ray film (Kodak, X-Omat AR) with an intensifying screen. Figure 4 shows examples of polymorphisms detected with this set of enzymes.

Once a polymorphism is identified with a restriction enzyme, use blots containing DNA from the RI lines digested with the same enzyme of choice. After X-ray film exposure, a 1:1 segregation should be observed (Figure 5). Score markers as either A (WS allele), B (W100 allele), or M (heterozygous or missing) (Figure 5).

After the segregation of the marker is scored, the resulting information must be integrated into the existing database containing the segregation data of all the markers mapped previously. Until other arrangements are made we will keep this database in our laboratory. To obtain the linkage information for the probe of interest, mail the segregation data to us and we will provide the resulting map location.

3.1. Considerations

The recommended set of five restriction enzymes for screening parent lines is designed to detect most polymorphisms. On average, 60% of *Arabidopsis* genomic

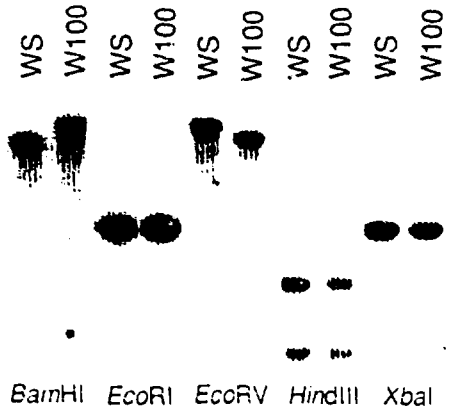

Figure 4. Hybridization pattern observed using an anonymous lambda genomic DNA sequence as a probe against either WS or W100 DNA restriction-digested DNA. Genomic DNA was isolated from both WS and W100 and digested with one of five restriction endonucleases indicated. A polymorphism is observed when the DNA is digested with either *Bam*HI or *Eco*RV.

Figure 5. Separation pattern observed when using an anonymous lambda genomic DNA sequence as a probe against restriction-digested DNA from 21 RI lines. Genomic DNA was isolated from 21 random RI lines derived from a cross between W100 and WS. The DNA was digested using the restriction endonuclease *Bam*HI. The segregation is scored as either A (paternal), B (maternal), or M (missing). No heterozygotes were observed among these 21 RIs. Residual heterozygosity may be present among other RI lines at this locus or at other loci.

or cosmid clones show at least one polymorphism with this set. If a probe is not polymorphic the choices are to increase the number of restriction enzymes tested or to obtain a larger probe. In our experience, it is more productive to obtain larger probes than to test more enzymes. Complementary DNA probes are often not polymorphic. In this case, the recommended course of action is to obtain the corresponding genomic fragment from either bacteriophage lambda or cosmid libraries.

The size of the population used for mapping depends on the resolution desired. For instance, 46 RI individuals provide a 1.1 cM maximum resolution (1 recombinant over 92 chromatids). We recommend a minimum of 46 individual lines. The maximum is determined by the number of available RI lines (115).

4. RAPD mapping

Most of the markers mapped in our RI collection are RAPDs (Reiter *et al.*, 1992). We anticipate a widespread use of this marker set in *Arabidopsis* research. General methods for running RAPD reactions have been extensively discussed by Williams *et al.* (1990, 1992). Here, we provide the protocols optimized in our laboratory for use with *Arabidopsis*. (The reader is encouraged to read the Williams *et al.* references for more general information.)

4.1. Screening for RAPD polymorphisms

As with RFLPs, the first step in RAPD mapping is to screen for polymorphisms between the parental strains WS and W100.

A single RAPD reaction contains:

Tris-HCl (pH 9.0) 80.0 mM
$(NH_4)_2SO_4$ 20.0 mM
$MgCl_2$ 3.5 mM
dNTPs (pH 7.0)100.0 μM/each
Primer 0.4 μM
Ampli*Taq*™ DNA
polymerase 1.0 U
Genomic DNA 25.0 ng

Final volume 25 μl

We purchase dNTPs from Boehringer Mannheim and Ampli*Taq*™ from Perkin Elmer/Cetus. Amplification parameters are: 92°C for 3 min, 45 cycles at 95°C for 30 sec, 34°C for 15 sec, and 74°C for 1 min. As a final step, we included a 72°C

RAPD reaction will have to obtain a copy of this "book of patterns" and use it to insure that the RAPD pattern obtained is identical to the one obtained in our laboratory. Furthermore, we advise researchers to map this RAPD band in the RI population, thus confirming genetically that the correct RAPD band is being used.

4.4. Troubleshooting

All of our RAPD reactions were carried out using a Biocycler oven. The advantage of this machine is its low cost and the ability to run multiple microtiter plates at one time. The disadvantage is the ability to reproduce reactions using other "more standard" solid block machines. Because the BIOS oven uses forced-air to perform the heating and cooling functions, it cycles very slowly. The average cycle takes 14 min to complete because ramping times are long. Most RAPD reactions can be repeated by using a more rapid 45 cycle amplification protocol: 94°C for 30 sec, 35°C for 30 sec, 72°C for 2 min and maximal ramp times. To more closely duplicate the BIOS oven we recommend the following program:

94°C for 30 sec
ramp-down from 94°C to 35°C for 6 min
35°C for 30 sec
ramp-up from 35°C to 72°C for 4 min
72°C for 1 min
ramp-up from 72°C to 94°C for 2 min

The other most critical parameter affecting RAPD pattern reproducibility is primer quality and primer concentration. Raising or lowering the primer concentration 2-4 fold can significantly affect the observed pattern. Lowering the concentration appears to enhance the amplification of larger products and increasing the concentration enhances the appearance of smaller products.

It is inevitable that a few RAPDs will be difficult to reproduce in other laboratories. If, after a few attempts, the same patterns cannot be reproduced, don't panic because the beauty of RAPDs is that you can quickly and easily screen for new ones as described below.

4.5. Targeting new RAPD bands

In many cases the researcher is interested in having high marker density for a specific location(s) in the genome. This would be true especially for cloning based upon map position. By using DNA pooling strategies one can rapidly identify new markers which will map to targeted segments of the genome. DNA pools may be constructed based upon plant phenotype or upon plant genotype. The first case in plants has been described recently by Michelmore *et al.* (1991) and has been termed bulk segregant analysis (Figure 7). Using this strategy, plants are placed into two

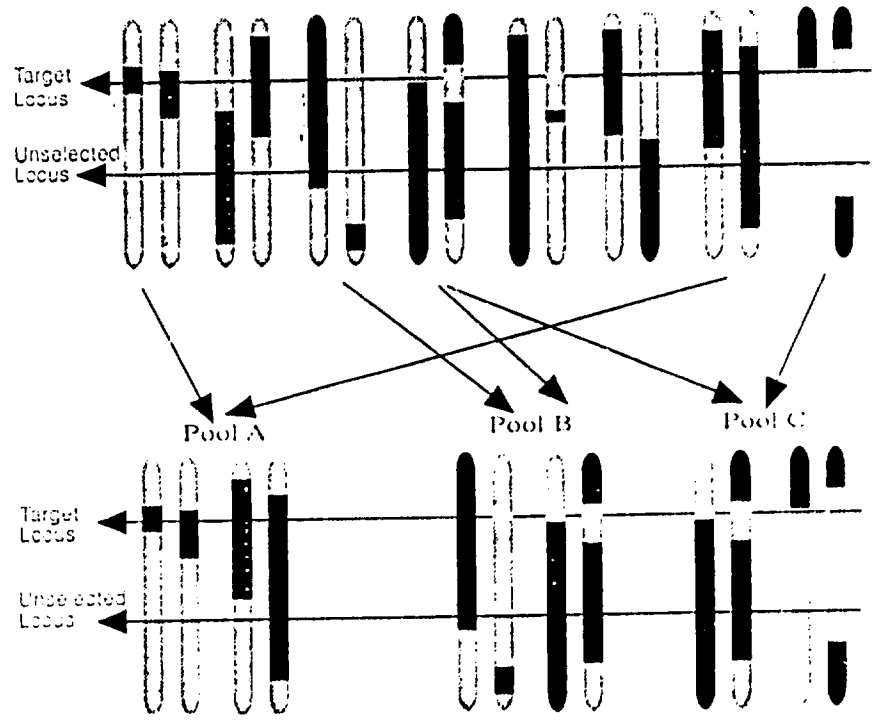

Figure 7. Theoretical display of pooling strategy based upon phenotype. Upper portion shows the genotype of 8 F2 individuals. Black bars represent genome regions inherited from the male parent and grey bars represent genome regions inherited from the female parent. Individuals are chosen for pooling based solely upon the phenotype coded by alleles at the target locus. Pool A contains individuals with a mutant phenotype (homozygous recessive at the target locus). Note the DNA at or near the target locus is inherited from the male parent only. Pools B and C contain individuals with a wild-type phenotype at the target locus. If no heterozygotes are selected the pool would contain genotypes shown in pool C, however if heterozygotes cannot be identified then the pool would contain genotypes shown in pool B. Note the DNA composition of each pool at the target locus. Polymorphism would only be observed between pools A and either B or C with a marker located at or near the target locus. No polymorphism would be observed between pools with a marker located at an unselected locus. Dominant alleles (like RAPD products) must originate from maternal DNA in order to observe a polymorphism between pool A and pool B. If heterozygotes are absent (pool C) then dominant alleles will identify polymorphisms regardless of origin.

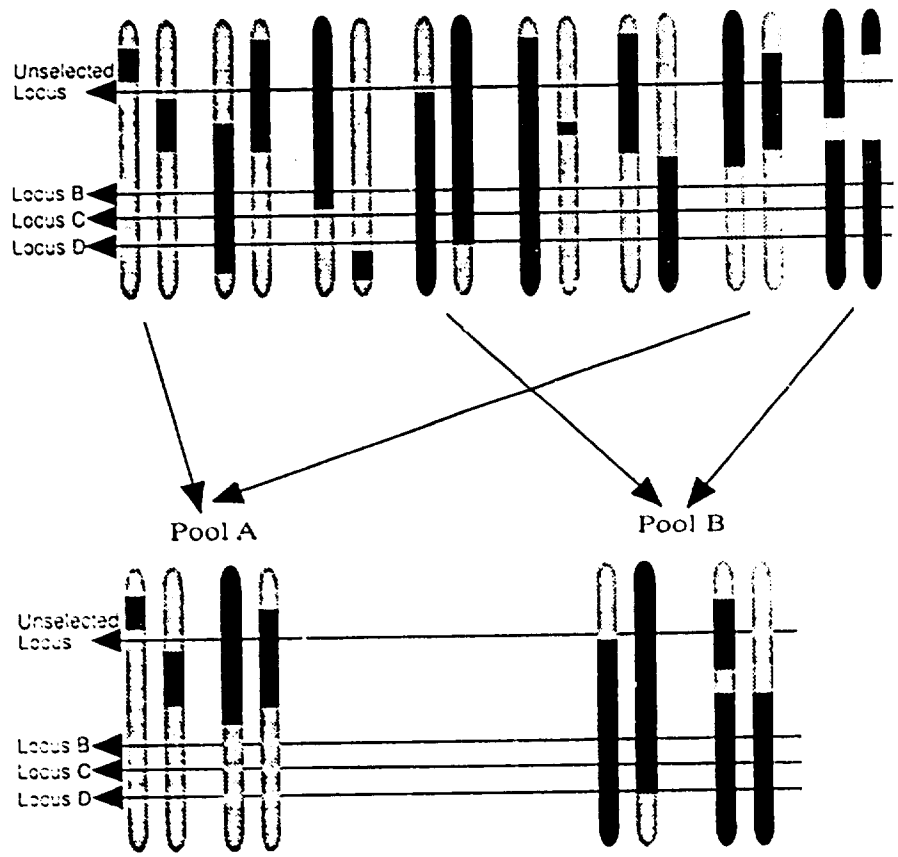

Figure 8. Theoretical display of pooling strategy based upon genotype. Upper portion shows the genotype of 8 F2 individuals. Black bars represent genomic regions inherited from the male parent and grey bars represent genomic regions inherited from the female parent. Individuals are chosen for pooling based solely upon the genotype coded by alleles at 3 marker loci (locus B, C and D). Pool A contains individuals with the maternal genotype for all 3 marker loci. Pool B contains individuals scored for the paternal genotype at each of the target loci. Note the DNA composition of each pool at the target loci. Polymorphism would only be observed between pools A and B with a marker located at or near the target loci. No polymorphism would be observed between pools with a marker located at an unselected locus. Depending upon the genotypic information available, the size and location of target segment(s) may be easily varied.

pools based upon the segregation of a phenotype. Michelmore *et al.* (1991) created plant pools in lettuce by scoring F_2 plants segregating at a locus which confers resistance to downey mildew. DNA was isolated from a pool of resistant plants and a pool of sensitive plants. The pools were screened for polymorphisms using RAPDs and new markers linked to the resistance locus identified. The implications of pooling are obvious when one is interested in finding additional markers at or near a locus which one plans to clone based upon map position.

We have extended the strategy to pooling based upon plant genotype. We have 115 RI lines which have been scored by 80 RFLP loci throughout the genome. A single region or regions of the genome may be chosen for targeting and 2 DNA pools constructed based solely upon the genotypic information provided by RFLPs at or adjacent to the target site(s) (Figure 8). A new application of pooling DNA to establish the relative order of RFLP loci linked to a phenotypic marker has been described for the *Arabidopsis bio1* mutation by Patton *et al.* (1991).

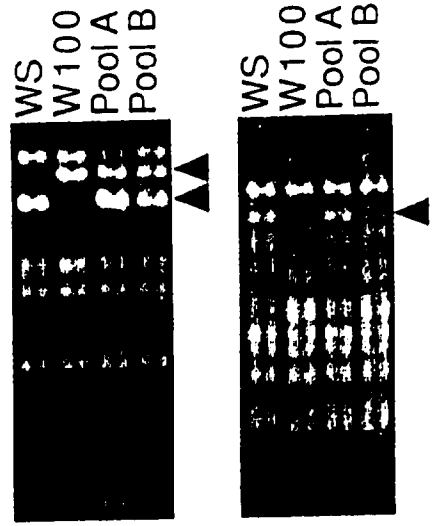

Figure 9. Example of pooling by genotype for detection of polymorphisms. Two RAPD primers identify polymorphic products between parental lines. Only the second primer (right panel) identifies a polymorphism between the pools. Arrows indicate polymorphic bands.

To create pools based upon phenotype, plants must be chosen from a population segregating for the phenotype of interest, such as an F_2, BC or RI population. Again, it is important that the parents used to create the population are potentially polymorphic. Ten or more plants should be chosen for each pool. Pool A would be composed of plants, all possessing the appropriate phenotype (and concomitantly carrying the corresponding DNA). Pool B would be composed of plants, with a wild-type allele at the locus of interest.

DNA is then extracted from the 2 pools and used in RAPD reactions as described. In addition, reactions using parental DNA should be performed simultaneously. Figure 9 shows the expected results using different primers, one which is not polymorphic between pools and one which is polymorphic between pools. Linkage to the phenotype of interest should be confirmed by performing RAPD reactions with a randomly drawn set of individuals from the population.

To create pools based upon genotype, RI lines must be chosen which carry a specific genotype (based upon the available RFLP data provided for each RI line) for the target region. By example, a WS pool would be created by selected RI lines which inherited the WS alleles of RFLP loci at or near a target site. A W100 pool would be similar except the RI lines chosen would have inherited the W100 alleles at the same RFLP loci examined. Again, DNA would be extracted from each pool and RAPD reactions performed. Confirmatory mapping is again required. The advantage to using genotype-selected RI lines is that any part of the genome may be targeted using the same RI population.

4.6. Considerations

The DNA pooling approach to mapping is a new, relatively untested approach. We are currently examining the feasibility of this approach to the mapping of several mutations throughout the *Arabidopsis* genome. Interested researchers are encouraged to contact the authors for further information.

5. Linkage analysis

The segregation results must be analyzed with the aid of computer programs designed to process recombination information and to provide linkage data. We performed a multipoint linkage analysis of our RI data using MAPMAKER (Lander *et al.*, 1987). This program is available in either Macintosh or UNIX versions. Unfortunately, MAPMAKER is designed to analyze either F2 or BC, but not RI data sets. Initially, one conducts a two-point analysis with MAPMAKER to determine the maximum likehood recombination and the corresponding LOD score. The LOD score is defined as the $\log_{10}$ of the ratio of the probability that the data would arise if the two loci considered were actually linked, divided by the probability that the data would arise if the loci were unlinked (Lander *et al.*, 1987). The traditional threshold

value for accepting linkage is 3.0. In the analysis of our [illegible], all markers were linked at a LOD score greater than 3.0, and recombination fraction (R) less than 0.15. By increasing the LOD threshold to 4.5, five linkage groups were identified (Reiter *et al.*, 1992). These linkage groups were assigned to the five *Arabidopsis* chromosomes based on the segregation of the phenotypic markers. Next, three-point and multipoint analyses are performed on each linkage group to determine the most likely order. With RIs the recombination frequencies from the MAPMAKER output represent the proportion of recombinant individuals (R), and the actual recombination frequency (r) must be calculated from these values.

6. Conclusions

The aim of current efforts in the *Arabidopsis* genome is to reach a point at which cloning a gene by map position will not be significantly more difficult than similar experiments in prokaryotes or lower eukaryotes. This challenging task will require technological breakthroughs in mutagenesis, DNA cloning, detection of alterations at the DNA or mRNA level, and better and faster genetic complementation tests. Our contributions to genetic mapping have already improved the accuracy and speed of this process, and we hope that the *Arabidopsis* community will make extensive use of these tools.

Acknowledgement

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Operon 10-mer Kits — Product Information

Product Description

Operon 10-mer Kits contain 10-base oligonucleotide primers for use in genetic mapping (Williams *et al.* [1]) and DNA fingerprinting (Welsh and McClelland [2]). Operon Technologies presently has 1000 different 10-base primers in stock. These primers are sold in kits of 20 sequences each and are designated "Kit A" through "Kit Z" and "Kit AA" through "Kit AX". The sequences were selected randomly, with the requirement that their (G+C) content is 60% to 70%, and that they have no self-complementary ends.

Principle of Technique

A single 10-base oligonucleotide primer is used to amplify genomic DNA. A DNA amplification product is generated for each genomic region that happens to be flanked by a pair of 10-base priming sites (in the appropriate orientation), which are within 5,000 base pairs of each other. Amplification products are analyzed by electrophoresis. Genomic DNA from two different individuals often produce different amplification fragment patterns. A particular DNA fragment which is generated for one individual but not for another represents a DNA polymorphism and can be used as a genetic marker. These markers are inherited in a Mendelian fashion (1). In mapping studies, the segregation of these markers among the progeny of a sexual cross can be used to construct a genetic map. In fingerprinting studies, the banding patterns are compared directly to allow strain determination, usually without the need to correlate band differences with particular properties.

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Methods

Each Operon 10-mer sample tube contains enough primer for approximately 1000 amplification reactions. Each 10-mer sample tube should be resuspended in sterile water or a sterile buffer such as Tris. For long term stability, we recommend that you subdivide each 10-mer sample into several aliquots, dry each aliquot, and store at -20°C. Use clean disposable plasticware for all transfers.

For DNA amplification, the following conditions are those originally recommended by Williams *et al.* (1):

> Amplification reactions are performed in a volume of 25 ul containing 10 mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 uM each of dATP, dCTP, dGTP, and dTTP, 5 picamoles of a single 10-base primer, 25 ng of genomic DNA, and 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus). Amplification should be performed on a top quality thermal cycler programmed for 45 cycles of 1 minute at 94°, 1 minute at 36°, and 2 minutes at 72°. Amplification products are analyzed by electrophoresis in 1.4% agarose gels and detected by staining with ethidium bromide.

More recent published methods now recommend an annealing temperature between 33° and 35°.

Number of Amplification Products

The number of different amplification products for each reaction depends upon the primer sequence, the genomic sequence, and the genome size. Assuming that the priming sites are randomly distributed throughout a genome, simple probability theory predicts that the number of amplification products per haploid genome should be approximately $2.5 \times 10^{-9} \times G$, where G is the size of the haploid genome in base pairs. For example, lettuce has a haploid genome size of approximately 2×10^9 base pairs, so the above formula predicts that a typical reaction should yield between 5 and 10 bands, depending on the extent of genetic heterozygosity. This prediction is in close agreement with actual results in lettuce obtained by Michelmore *et al.*(5). However, for much smaller genome sizes, such as that of *E. coli* ($G = 4 \times 10^6$ base pairs), the above formula predicts that

most primers should generate no bands at all. Nevertheless, several laboratories have reported multiple amplification products from prokaryotic DNA. Such results can only be explained on the basis of mismatch between the primer and the DNA template (1).

Naming of Primers and Markers

In order to prevent ambiguity in the naming of primers from different sources, Operon is now attaching the prefix "OP" to the names of all of its primers. For example, the fourth sequence in Kit H is now labelled "OPH-04". To refer to a specific polymorphic amplification product, we recommend the convention used by Paran *et al.* (3) which is to add a subscript denoting its size to the primer name. For example, an 800 bp amplification product produced by primer OPAC-01 would be called "OPAC-01_{800}".

Trouble-Shooting

Not all amplifications products arise from perfect pairing between primer and DNA template. Amplification products arising from mis-pairing may still be reproducible and may be useful genetic markers. However, we suspect that these mismatched markers are more sensitive to slight changes in the temperature cycle, so we strongly suggest using identical amplification conditions when comparing results.

The DNA amplification method described above is unusual in that it uses very short (10-base) primers, which have less specificity than longer primers. As a result, this method is quite sensitive to small variations in the temperature cycle, particularly the annealing temperature (recommended to be 34°). Since the actual temperatures delivered to the tubes by different thermal cyclers may differ significantly, it is often necessary to refine our recommended temperature program in order to optimize this method to your particular thermal cycler. If no amplification products are seen, it may be necessary to adjust temperatures downward. If too many products are seen, it may be necessary to adjust temperatures upward.

Occasionally, a "smear" of amplification products is observed, and this may be converted to discretely sized bands by adjusting the concentration of the polymerase, the primer, or the genomic DNA.

It is important that your genomic DNA is relatively free of single strand breaks since such breaks will prevent amplification. Avoid repeated boiling of your genomic DNA samples.

Although we have chosen oligonucleotide sequences which do not have self-complementary ends, some of our 10-mers are still capable of forming complicated secondary structures which can lead to the production of amplification artifacts. These artifacts tend to appear if one intentionally omits the genomic DNA to test a "reagent blank". Fortunately, such artifacts are not produced when the genomic DNA is included.

Uses in Genetic Mapping

Of those kits already tested, our customers report getting useful genetic markers from about 50% to 98% of our 10-mer sequences, depending on the species.

A variety of mapping and fingerprinting strategies which employ this technique have appeared in the scientific literature and are listed below (1-8). This technology has recently been reviewed by Rafalski *et al.* (6).

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Total Price: $_____

Payment Method: Payment is required within 30 days of shipping your order. Payment can be made by any of the following methods: international money order, Visa or Mastercard, or by check drawn on funds from an American bank. **Orders over $500 must be approved by Operon.** For payment by credit card, please provide us with the name of the cardholder, the card number, and the expiration date of the card. Bank wires should be sent to: Civic Bank of Commerce, 1814 Franklin, Oakland CA 94612. Account number: 1050208385. Please add $20 for bank processing charges.

Orders will be shipped the same day payment is received.

Shipping Information: Orders are shipped via Federal Express. Transit time to most countries is approximately 3 to 4 working days, barring delays in customs.

Duties, Import Taxes: Please note that all duties and import taxes are the responsibility of the recipient. We mark all shipments "FOR RESEARCH PURPOSES ONLY", and suggest you inform your customs agent of the nature of our product in advance of shipment so that you avoid any extra duties.

OPERON

OPERON TECHNOLOGIES, INC

Operon 10-mer Kits

Each kit contains twenty 10-mer primers, as listed below, and contains 0.5 O.D. (approx. 15 µgm) per tube. The list price is $150.00 per kit.

KIT A

code	5' to 3'	code	5' to 3'	code	5' to 3'	code	5' to 3'
OPA-01	CAGGCCCTTC	OPA-06	GGTCCCTGAC	OPA-11	CAATCGCCGT	OPA-16	AGCCAGCGAA
OPA-02	TGCCGAGCTG	OPA-07	GAAACGGGTG	OPA-12	TCGGCGATAG	OPA-17	GACCGCTTGT
OPA-03	AGTCAGCCAC	OPA-08	GTGACGTAGG	OPA-13	CAGCACCCAC	OPA-18	AGGTGACCGT
OPA-04	AATCGGGCTG	OPA-09	GGGTAACGCC	OPA-14	TCTGTGCTGG	OPA-19	CAAACGTCGG
OPA-05	ACGGGTCTTG	OPA-10	GTGATCCCAG	OPA-15	TTCCGAACCC	OPA-20	GTTGCGATCC

KIT B

OPB-01	GTTTCGCTCC	OPB-06	TGCTCTGCCC	OPB-11	GTAGACCCGT	OPB-16	TTTGCCCGGA
OPB-02	TGATCCCTGG	OPB-07	GGTGACGCAG	OPB-12	CCTTGACGCA	OPB-17	AGGGAACGAG
OPB-03	CATCCCCCTG	OPB-08	GTCCACACGG	OPB-13	TTCCCCCGCT	OPB-18	CCACAGCAGT
OPB-04	GGACTGGAGT	OPB-09	TGGGGGACTC	OPB-14	TCCGCTCTGG	OPB-19	ACCCCCGAAG
OPB-05	TGCGCCCTTC	OPB-10	CTGCTGGGAC	OPB-15	GGAGGGTGTT	OPB-20	GGACCCTTAC

KIT C

OPC-01	TTCGAGCCAG	OPC-06	GAACGGACTC	OPC-11	AAAGCTGCGG	OPC-16	CACACTCCAG
OPC-02	GTGAGGCGTC	OPC-07	GTCCCGACGA	OPC-12	TGTCATCCCC	OPC-17	TTCCCCCCAG
OPC-03	GGGGGTCTTT	OPC-08	TGGACCGGTG	OPC-13	AAGCCTCGTC	OPC-18	TGAGTGGGTG
OPC-04	CCGCATCTAC	OPC-09	CTCACCGTCC	OPC-14	TGCGTGCTTG	OPC-19	GTTGCCAGCC
OPC-05	GATGACCGCC	OPC-10	TGTCTGGGTG	OPC-15	GACGGATCAG	OPC-20	ACTTCGCCAC

KIT D

OPD-01	ACCGCGAAGG	OPD-06	ACCTGAACGG	OPD-11	AGCGCCATTG	OPD-16	AGGGCGTAAG
OPD-02	GGACCCAACC	OPD-07	TTGGCACGGG	OPD-12	CACCGTATCC	OPD-17	TTTCCCACGG
OPD-03	GTCGCCGTCA	OPD-08	GTGTGCCCCA	OPD-13	GGGGTGACGA	OPD-18	GAGAGCCAAC
OPD-04	TCTGGTGAGG	OPD-09	CTCTGGAGAC	OPD-14	CTTCCCCAAG	OPD-19	CTGGGGACTT
OPD-05	TGAGCGGACA	OPD-10	GGTCTACACC	OPD-15	CATCCGTGCT	OPD-20	ACCCGGTCAC

KIT E

OPE-01	CCCAAGGTCC	OPE-06	AAGACCCCTC	OPE-11	GAGTCTCAGG	OPE-16	GGTGACTGTG
OPE-02	GGTGCGGGAA	OPE-07	AGATGCAGCC	OPE-12	TTATCGCCCC	OPE-17	CTACTGCCGT
OPE-03	CCAGATGCAC	OPE-08	TCACCACGGT	OPE-13	CCCGATTCGG	OPE-18	GGACTGCAGA
OPE-04	GTGACATGCC	OPE-09	CTTCACCCGA	OPE-14	TGCGGCTGAG	OPE-19	ACGGCGTATG
OPE-05	TCAGGGAGGT	OPE-10	CACCAGGTGA	OPE-15	ACGCACAACC	OPE-20	AACGGTGACC

KIT F

OPF-01	ACGGATCCTG	OPF-06	GGGAATTCGG	OPF-11	TTGGTACCCC	OPF-16	GGAGTACTGG
OPF-02	GAGGATCCCT	OPF-07	CCGATATCCC	OPF-12	ACGGTACCAG	OPF-17	AACCCGGGAA
OPF-03	CCTGATCACC	OPF-08	GGGATATCGG	OPF-13	GGCTGCAGAA	OPF-18	TTCCCGGGTT
OPF-04	GGTGATCAGG	OPF-09	CCAAGCTTCC	OPF-14	TGCTGCAGGT	OPF-19	CCTCTAGACC
OPF-05	CCGAATTCCC	OPF-10	GGAAGCTTGG	OPF-15	CCAGTACTCC	OPF-20	GGTCTAGAGG

1000 Atlantic Ave., Suite 108 · Alameda CA 94501 · Tel. (510) 865-8644 Fax (510) 865-5255

KIT G

code	5' to 3'	code	5' to 3'	code	5' to 3'	code	5' to 3'
OPG-01	CTACGGAGGA	OPG-06	GTGCCTAACC	OPG-11	TGCCCGTCGT	OPG-16	AGCGTCCTCC
OPG-02	GGCACTGAGG	OPG-07	GAACCTGCGG	OPG-12	CAGCTCACGA	OPG-17	ACGACCGACA
OPG-03	GAGCCCTCCA	OPG-08	TCACGTCCAC	OPG-13	CTCTCCGCCA	OPG-18	GGCTCATGTG
OPG-04	AGCGTGTCTG	OPG-09	CTGACGTCAC	OPG-14	GGATGAGACC	OPG-19	GTCAGGGCAA
OPG-05	CTGAGACGGA	OPG-10	ACGGCCGTCT	OPG-15	ACTGGGACTC	OPG-20	TCTCCCTCAG

KIT H

OPH-01	GGTCGGAGAA	OPH-06	ACGCATCGCA	OPH-11	CTTCCGCAGT	OPH-16	TCTCAGCTGG
OPH-02	TCGGACGTGA	OPH-07	CTGCATCGTG	OPH-12	ACGCGCATGT	OPH-17	CACTCTCCTC
OPH-03	AGACGTCCAC	OPH-08	GAAACACCCC	OPH-13	GACGCCACAC	OPH-18	GAATCGGCCA
OPH-04	GGAAGTCGCC	OPH-09	TGTAGCTGGG	OPH-14	ACCAGGTTGG	OPH-19	CTGACCAGCC
OPH-05	AGTCGTCCCC	OPH-10	CCTACGTCAG	OPH-15	AATGGCGCAG	OPH-20	GGGAGACATC

KIT I

OPI-01	ACCTGGACAC	OPI-06	AAGGCGGCAG	OPI-11	ACATGCCGTG	OPI-16	TCTCCGCCCT
OPI-02	GGAGGAGAGG	OPI-07	CAGCGACAAG	OPI-12	AGAGGGCACA	OPI-17	GGTGGTGATG
OPI-03	CAGAAGCCCA	OPI-08	TTTGCCCGGT	OPI-13	CTGGGGCTGA	OPI-18	TGCCCAGCCT
OPI-04	CCGCCTAGTC	OPI-09	TGGAGAGCAG	OPI-14	TGACGGCGGT	OPI-19	AATGCGGGAG
OPI-05	TGTTCCACGG	OPI-10	ACAACGCGAG	OPI-15	TCATCCGAGG	OPI-20	AAAGTGCCGG

KIT J

OPJ-01	CCCGGCATAA	OPJ-06	TCGTTCCGCA	OPJ-11	ACTCCTGCGA	OPJ-16	CTGCTTAGGG
OPJ-02	CCCGTTGGGA	OPJ-07	CCTCTCGACA	OPJ-12	GTCCCGTGGT	OPJ-17	ACGCCAGTTC
OPJ-03	TCTCCGCTTG	OPJ-08	CATACCGTGG	OPJ-13	CCACACTACC	OPJ-18	TGGTCGCAGA
OPJ-04	CCGAACACGG	OPJ-09	TGAGCCTCAC	OPJ-14	CACCCGGATG	OPJ-19	GGACACCACT
OPJ-05	CTCCATGGGG	OPJ-10	AAGCCCGAGG	OPJ-15	TGTAGCAGGG	OPJ-20	AAGCGGCCTC

KIT K

OPK-01	CATTCGAGCC	OPK-06	CACCTTTCCC	OPK-11	AATGCCCCAG	OPK-16	GAGCGTCGAA
OPK-02	GTCTCCGCAA	OPK-07	AGCGAGCAAG	OPK-12	TGGCCCTCAC	OPK-17	CCCAGCTGTG
OPK-03	CCAGCTTAGG	OPK-08	GAACACTGGG	OPK-13	GGTTGTACCC	OPK-18	CCTAGTCGAG
OPK-04	CCGCCCAAAC	OPK-09	CCCTACCGAC	OPK-14	CCCGCTACAC	OPK-19	CACAGGCGGA
OPK-05	TCTGTCGAGG	OPK-10	GTGCAACGTG	OPK-15	CTCCTGCCAA	OPK-20	GTGTCGCGAG

KIT L

OPL-01	GGCATGACCT	OPL-06	GAGGGAAGAG	OPL-11	ACGATGAGCC	OPL-16	AGGTTGCAGG
OPL-02	TGGGCGTCAA	OPL-07	AGGCGGGAAC	OPL-12	GGGCGGTACT	OPL-17	AGCCTGAGCC
OPL-03	CCAGCAGCTT	OPL-08	AGCAGGTGGA	OPL-13	ACCGCCTGCT	OPL-18	ACCACCCACC
OPL-04	GACTGCACAC	OPL-09	TGCGAGAGTC	OPL-14	GTGACAGGCT	OPL-19	GAGTGGTGAC
OPL-05	ACGCAGGCAC	OPL-10	TGGGAGATGG	OPL-15	AAGAGAGGGG	OPL-20	TGGTGGACCA

KIT M

OPM-01	GTTGGTGGCT	OPM-06	CTGGGCAACT	OPM-11	GTCCACTGTG	OPM-16	GTAACCAGCC
OPM-02	ACAACGCCTC	OPM-07	CCGTGACTCA	OPM-12	GGGACGTTGG	OPM-17	TCAGTCCGGG
OPM-03	GGGGGATGAG	OPM-08	TCTGTTCCCC	OPM-13	GGTGGTCAAG	OPM-18	CACCATCCGT
OPM-04	GGCGGTTGTC	OPM-09	GTCTTGCGGA	OPM-14	AGGGTCGTTC	OPM-19	CCTTCAGGCA
OPM-05	GGGAACGTGT	OPM-10	TCTGGCGCAC	OPM-15	GACCTACCAC	OPM-20	AGGTCTTGGG

KIT N

OPN-01	CTCACGTTGG	OPN-06	GAGACCCACA	OPN-11	TCGCCGCAAA	OPN-16	AAGCGACCTG
OPN-02	ACCAGCGGCA	OPN-07	CAGCCCAGAG	OPN-12	CACAGACACC	OPN-17	CATTGGCGAG
OPN-03	GGTACTCCCC	OPN-08	ACCTCAGCTC	OPN-13	AGCGTCACTC	OPN-18	GGTGAGGTCA
OPN-04	GACCGACCCA	OPN-09	TGCCCGCTTG	OPN-14	TCGTGCGGGT	OPN-19	GTCCGTACTG
OPN-05	ACTGAACGCC	OPN-10	ACAACTGGGG	OPN-15	CAGCGACTGT	OPN-20	GGTGCTCCGT

KIT O

code	5' to 3'	code	5' to 3'	code	5' to 3'	code	5' to 3'
OPO-01	GGCACGTAAG	OPO-06	CCACGGGAAG	OPO-11	GACAGGAGGT	OPO-16	TCGGCGGTTC
OPO-02	ACGTAGCGTC	OPO-07	CAGCACTGAC	OPO-12	CAGTGCTGTG	OPO-17	GGCTTATGCC
OPO-03	CTGTTGCTAC	OPO-08	CCTCCAGTGT	OPO-13	GTCAGAGTCC	OPO-18	CTCGCTATCC
OPO-04	AAGTCCGCTC	OPO-09	TCCCACGCAA	OPO-14	AGCATGGCTC	OPO-19	GGTGCACGTT
OPO-05	CCCAGTCACT	OPO-10	TCAGAGCGCC	OPO-15	TGGCGTCCTT	OPO-20	ACACACGCTG

KIT P

OPP-01	GTAGCACTCC	OPP-06	GTGGGCTGAC	OPP-11	AACGCGTCGG	OPP-16	CCAAGCTGCC
OPP-02	TCGGCACGCA	OPP-07	GTCCATGCCA	OPP-12	AAGGGCGAGT	OPP-17	TGACCCGCCT
OPP-03	CTGATACGCC	OPP-08	ACATCGCCCA	OPP-13	GGAGTGCCTC	OPP-18	GGCTTGGCCT
OPP-04	GTGTCTCAGG	OPP-09	GTGGTCCGCA	OPP-14	CCAGCCGAAC	OPP-19	GGGAAGGACA
OPP-05	CCCCGGTAAC	OPP-10	TCCCGCCTAC	OPP-15	GGAAGCCAAC	OPP-20	GACCCTAGTC

KIT Q

OPQ-01	GGGACGATGG	OPQ-06	GAGCGCCTTG	OPQ-11	TCTCCGCAAC	OPQ-16	AGTGCAGCCA
OPQ-02	TCTGTCGGTC	OPQ-07	CCCCGATGGT	OPQ-12	AGTAGGGCAC	OPQ-17	GAAGCCCTTG
OPQ-03	GGTCACCTCA	OPQ-08	CTCCAGCGGA	OPQ-13	GGAGTGGACA	OPQ-18	AGGCTGGGTG
OPQ-04	AGTGCGCTGA	OPQ-09	GGCTAACCGA	OPQ-14	GGACGCTTCA	OPQ-19	CCCCCTATCA
OPQ-05	CCGCGTCTTG	OPQ-10	TGTGCCCGAA	OPQ-15	GGGTAACGTC	OPQ-20	TCGCCCAGTC

KIT R

OPR-01	TGCGGGTCCT	OPR-06	GTCTACGGCA	OPR-11	GTAGCCGTCT	OPR-16	CTCTGCGCGT
OPR-02	CACAGCTGCC	OPR-07	ACTGGCCTGA	OPR-12	ACAGGTGCGT	OPR-17	CCGTACGTAG
OPR-03	ACACAGAGGG	OPR-08	CCCGTTGCCT	OPR-13	GGACGACAAG	OPR-18	GGCTTTGCCA
OPR-04	CCCGTAGCAC	OPR-09	TGAGCACGAG	OPR-14	CAGGATTCCC	OPR-19	CCTCCTCATC
OPR-05	GACCTAGTGG	OPR-10	CCATTCCCCA	OPR-15	GGACAACGAG	OPR-20	ACGGCAAGGA

KIT S

OPS-01	CTACTGCGCT	OPS-06	GATACCTCGG	OPS-11	AGTCGGGTGG	OPS-16	AGGGGGTTCC
OPS-02	CCTCTGACTG	OPS-07	TCCGATGCTG	OPS-12	CTGGGTGAGT	OPS-17	TGGGGACCAC
OPS-03	CAGAGGTCCC	OPS-08	TTCAGGGTGG	OPS-13	GTCGTTCCTG	OPS-18	CTGGCGAACT
OPS-04	CACCCCCTTG	OPS-09	TCCTGGTCCC	OPS-14	AAAGGGGTCC	OPS-19	GAGTCAGCAG
OPS-05	TTTGGGGCCT	OPS-10	ACCGTTCCAG	OPS-15	CAGTTCACGG	OPS-20	TCTGGACGGA

KIT T

OPT-01	GGGCCACTCA	OPT-06	CAAGGGCAGA	OPT-11	TTCCCCGCGA	OPT-16	GGTGAACGCT
OPT-02	GGAGAGACTC	OPT-07	GGCAGGCTGT	OPT-12	GGGTGTGTAG	OPT-17	CCAACGTCGT
OPT-03	TCCACTCCTG	OPT-08	AACGGCGACA	OPT-13	AGGACTGCCA	OPT-18	GATGCCAGAC
OPT-04	CACAGAGGGA	OPT-09	CACCCCTGAG	OPT-14	AATGCCGCAG	OPT-19	GTCCGTATGG
OPT-05	GGGTTTGGCA	OPT-10	CCTTCGGAAG	OPT-15	GGATGCCACT	OPT-20	GACCAATGCC

KIT U

OPU-01	ACGGACGTCA	OPU-06	ACCTTTGCGG	OPU-11	AGACCCAGAG	OPU-16	CTGCGCTGGA
OPU-02	CTGAGGTCTC	OPU-07	CCTGCTCATC	OPU-12	TCACCAGCCA	OPU-17	ACCTGGGGAG
OPU-03	CTATGCCGAC	OPU-08	GGCGAAGGTT	OPU-13	GGCTGGTTCC	OPU-18	GAGGTCCACA
OPU-04	ACCTTCGGAC	OPU-09	CCACATCGGT	OPU-14	TGGGTCCCTC	OPU-19	GTCAGTGCGG
OPU-05	TTGGCGGCCT	OPU-10	ACCTCGGCAC	OPU-15	ACGGGCCAGT	OPU-10	ACCTCGGCAC

KIT V

OPV-01	TGACGCATGG	OPV-06	ACGCCCAGGT	OPV-11	CTCGACAGAG	OPV-16	ACACCCCACA
OPV-02	AGTCACTCCC	OPV-07	GAAGCCAGCC	OPV-12	ACCCCCCACT	OPV-17	ACCGGCTTGT
OPV-03	CTCCCTGCAA	OPV-08	GGACGGCGTT	OPV-13	ACCCCCTGAA	OPV-18	TGGTGGCGTT
OPV-04	CCCCTCACGA	OPV-09	TGTACCCGTC	OPV-14	AGATCCCGCC	OPV-19	GGGTGTGCAG
OPV-05	TCCGAGAGGG	OPV-10	GGACCTGCTG	OPV-15	CAGTGCCGGT	OPV-20	CAGCATGGTC

KIT W

code	5' to 3'	code	5' to 3'	code	5' to 3'	code	5' to 3'
OPW-01	CTCAGTGTCC	OPW-06	ACGCCCGATG	OPW-11	CTGATGCGTG	OPW-16	CAGCCTACCA
OPW-02	ACCCCGCCAA	OPW-07	CTGGACGTCA	OPW-12	TGGGCAGAAG	OPW-17	GTCCTGCGTT
OPW-03	GTCCGGAGTG	OPW-08	GACTGCCTCT	OPW-13	CACAGCGACA	OPW-18	TTCAGGGCAC
OPW-04	CAGAAGCGGA	OPW-09	GTGACCGAGT	OPW-14	CTGCTGAGCA	OPW-19	CAAAGCGCTC
OPW-05	GGCGGATAAG	OPW-10	TCGCATCCCT	OPW-15	ACACCGGAAC	OPW-20	TGTGGCAGCA

KIT X

OPX-01	CTGGGCACGA	OPX-06	ACGCCAGAGG	OPX-11	GGAGCCTCAG	OPX-16	CTCTGTTCGG
OPX-02	TTCCGCCACC	OPX-07	GAGCGAGGCT	OPX-12	TCGCCAGCCA	OPX-17	GACACGGACC
OPX-03	TGGCGCAGTG	OPX-08	CAGGGGTGGA	OPX-13	ACGGGAGCAA	OPX-18	GACTAGGTGG
OPX-04	CCGCTACCGA	OPX-09	GGTCTGGTTG	OPX-14	ACAGGTGCTG	OPX-19	TGGCAAGGCA
OPX-05	CCTTTCCCTC	OPX-10	CCCTAGACTG	OPX-15	CAGACAAGCC	OPX-20	CCCAGCTAGA

KIT Y

OPY-01	GTGGCATCTC	OPY-06	AAGGCTCACC	OPY-11	AGACGATGGG	OPY-16	GGGCCAATGT
OPY-02	CATCGCCGCA	OPY-07	AGAGCCGTCA	OPY-12	AAGCCTGCGA	OPY-17	GACGTGGTGA
OPY-03	ACAGCCTGCT	OPY-08	AGGCAGAGCA	OPY-13	GGGTCTCGGT	OPY-18	GTGGAGTCAG
OPY-04	GGCTGCAATG	OPY-09	AGCAGCCGCAC	OPY-14	GGTCGATCTG	OPY-19	TGAGGGTCCC
OPY-05	GGCTGCGACA	OPY-10	CAAACGTGGG	OPY-15	AGTCGCCCTT	OPY-20	AGCCGTGGAA

KIT Z

OPZ-01	TCTGTGCCAC	OPZ-06	GTGCCGTTCA	OPZ-11	CTCAGTCGCA	OPZ-16	TCCCCATCAC
OPZ-02	CCTACGGGGA	OPZ-07	CCAGGAGGAC	OPZ-12	TCAACGGGAC	OPZ-17	CCTTCCCACT
OPZ-03	CAGCACCGCA	OPZ-08	GGGTGGGTAA	OPZ-13	GACTAAGCCC	OPZ-18	AGGGTCTGTG
OPZ-04	AGGCTGTGCT	OPZ-09	CACCCCAGTC	OPZ-14	TCGGAGGTTC	OPZ-19	GTGCGAGCAA
OPZ-05	TCCCATGCTG	OPZ-10	CCGACAAACC	OPZ-15	CAGGGCTTTC	OPZ-20	ACTTTGGCGG

KIT AA

OPAA-01	AGACGGCTCC	OPAA-06	GTGGGTGCCA	OPAA-1	ACCCGACCTG	OPAA-16	GGAACCCACA
OPAA-02	GAGACCAGAC	OPAA-07	CTACGCTCAC	OPAA-12	GGACCTCTTG	OPAA-17	GAGCCCGACT
OPAA-03	TTAGCGCCCC	OPAA-08	TCCGCAGTAG	OPAA-13	GAGCGTCGCT	OPAA-18	TGGTCCAGCC
OPAA-04	AGGACTGCTC	OPAA-09	AGATGGGCAG	OPAA-14	AACGGGCCAA	OPAA-19	TGAGGCGTGT
OPAA-05	GGCTTTAGCC	OPAA-10	TGGTCGGGTG	OPAA-15	ACGGAAGCCC	OPAA-20	TTGCCTTCGG

KIT AB

OPAB-01	CCGTCGGTAG	OPAB-06	GTGGCTTGGA	OPAB-11	GTGCGCAATG	OPAB-16	CCCGGATGGT
OPAB-02	CGAAACCCCT	OPAB-07	GTAAACCGCC	OPAB-12	CCTGTACCCA	OPAB-17	TCGCATCCAG
OPAB-03	TGGCGCACAC	OPAB-08	GTTACGGACC	OPAB-13	CCTACCGTGG	OPAB-18	CTGGCGTGTC
OPAB-04	GGCACGCGTT	OPAB-09	GGGCGACTAC	OPAB-14	AAGTGCGACC	OPAB-19	ACACCGATGG
OPAB-05	CCCGAAGCGA	OPAB-10	TTCCCTCCCA	OPAB-15	CCTCCTTCTC	OPAB-20	CTTCTCGGAC

KIT AC

OPAC-01	TCCCAGCAGA	OPAC-06	CCAGAACGGA	OPAC-11	CCTGGGTCAG	OPAC-16	CCTCCTACGG
OPAC-02	GTCGTCGTCT	OPAC-07	GTGGCCGATG	OPAC-12	GGCGAGTGTG	OPAC-17	CCTGGAGCTT
OPAC-03	CACTGGCCCA	OPAC-08	TTTGGGTGCC	OPAC-13	GACCCGATTG	OPAC-18	TTGGGGGAGA
OPAC-04	ACGGGACCTG	OPAC-09	AGAGCGTACC	OPAC-14	GTCGGTTGTC	OPAC-19	AGTCCGCCTG
OPAC-05	GTTAGTGCGG	OPAC-10	AGCAGCGAGG	OPAC-15	TGCCGTGAGA	OPAC-20	ACGGAAGTGG

KIT AD

OPAD-01	CAAAGGGCGG	OPAD-06	AAGTGCACGG	OPAD-11	CAATCGGGTC	OPAD-16	AACGGGCGTC
OPAD-02	CTGAACCGCT	OPAD-07	CCCTACTGGT	OPAD-12	AAGAGGGCGT	OPAD-17	GGCAAACCCT
OPAD-03	TCTCGCCTAC	OPAD-08	GGCAGGCAAG	OPAD-13	GGTTCCTCTG	OPAD-18	ACGAGAGGCA
OPAD-04	GTAGGCCTCA	OPAD-09	TCGCTTCTCC	OPAD-14	GAACGAGGGT	OPAD-19	CTTGGCACGA
OPAD-05	ACCGCATCGG	OPAD-10	AAGAGGCCAG	OPAD-15	TTTGCCCCGT	OPAD-20	TCTTCGGAGG

KIT AE

code	5' to 3'	code	5' to 3'	code	5' to 3'	code	5' to 3'
OPAE-01	TGAGGGCCGT	OPAE-06	GGGGAAGACA	OPAE-11	AAGACCGGGA	OPAE-16	TCCGTGCTGA
OPAE-02	TCGTTCACCC	OPAE-07	GTGTCAGTGG	OPAE-12	CCGAGCAATC	OPAE-17	GGCAGGTTCA
OPAE-03	CATAGAGCGG	OPAE-08	CTGGCTCAGA	OPAE-13	TGTGGACTGG	OPAE-18	CTGGTGCTGA
OPAE-04	CCAGCACTTC	OPAE-09	TGCCACGAGG	OPAE-14	GAGAGGCTCC	OPAE-19	GACAGTCCCT
OPAE-05	CCTGTCAGTG	OPAE-10	CTGAAGCGCA	OPAE-15	TGCCTGGACC	OPAE-20	TTGACCCCAG

KIT AF

OPAF-01	CCTACACGGT	OPAF-06	CCGCAGTCTG	OPAF-11	ACTGGGCCTC	OPAF-16	TCCCGGTGAG
OPAF-02	CAGCCGAGAA	OPAF-07	GGAAAGCGTC	OPAF-12	GACGCAGCTT	OPAF-17	TGAACCGAGG
OPAF-03	GAAGGAGGCA	OPAF-08	CTCTGCCTGA	OPAF-13	CCGAGGTGAC	OPAF-18	GTGTCCCTCT
OPAF-04	TTGCGGCTGA	OPAF-09	CCCCTCAGAA	OPAF-14	GGTGCGCACT	OPAF-19	GGACAAGCAG
OPAF-05	CCCGATCAGA	OPAF-10	GGTTGGAGAC	OPAF-15	CACGAACCTC	OPAF-20	CTCCGCACAG

KIT AG

OPAG-01	CTACGGCTTC	OPAG-06	GGTGGCCAAG	OPAG-11	TTACGGTGGG	OPAG-16	CCTGCGACAG
OPAG-02	CTGAGGTCCT	OPAG-07	CACAGACCTG	OPAG-12	CTCCCAGGGT	OPAG-17	AGCGGAAGTG
OPAG-03	TGCGGGAGTG	OPAG-08	AAGAGCCCTC	OPAG-13	GGCTTGGCGA	OPAG-18	GTCGGCATAC
OPAG-04	GGAGCGTACT	OPAG-09	CCGAGGGGTT	OPAG-14	CTCTCGGCGA	OPAG-19	AGCCTCGGTT
OPAG-05	CCCACTAGAC	OPAG-10	ACTGCCCGAC	OPAG-15	CCCACACGCA	OPAG-20	TGCGCTCCTC

KIT AH

OPAH-01	TCCGCAACCA	OPAH-06	GTAAGCCCCT	OPAH-11	TCCGCTGAGA	OPAH-16	CAAGGTGGGT
OPAH-02	CACTTCCGCT	OPAH-07	CCCTACGGAG	OPAH-12	TCCAACGGCT	OPAH-17	CAGTGGGGAG
OPAH-03	GGTTACTGCC	OPAH-08	TTCCCGTGCC	OPAH-13	TGAGTCCGCA	OPAH-18	GGGCTAGTCA
OPAH-04	CTCCCCAGAC	OPAH-09	AGAACCGAGG	OPAH-14	TGTGGCCGAA	OPAH-19	GGCAGTTCTC
OPAH-05	TTGCAGGCAG	OPAH-10	GGGATGACCA	OPAH-15	CTACAGCGAG	OPAH-20	GGAAGGTGAG

KIT AI

OPAI-01	GGCATCGGCT	OPAI-06	TGCCCGCACTT	OPAI-11	ACGGCGATGA	OPAI-16	AAGGCACGAG
OPAI-02	AGCCGTTCAG	OPAI-07	ACGAGCATGG	OPAI-12	GACGCGAACC	OPAI-17	CCTCACGTCC
OPAI-03	GGGTCCAAAG	OPAI-08	AAGCCCCCCA	OPAI-13	ACGCTGCGAC	OPAI-18	TCGCGGAACC
OPAI-04	CTATCCTGCC	OPAI-09	TCGCTGGTGT	OPAI-14	TGGTGCACTC	OPAI-19	GGCAAAGCTG
OPAI-05	GTCGTAGCGG	OPAI-10	TCGGGGCATC	OPAI-15	GACACAGCCC	OPAI-20	CCTGTTCCCT

KIT AJ

OPAJ-01	ACGGGTCAGA	OPAJ-06	GTCGGAGTGG	OPAJ-11	GAACGCTGCC	OPAJ-16	TCTGGACCGA
OPAJ-02	TCGCACAGTC	OPAJ-07	CCCTCCCTAA	OPAJ-12	CAGTTCCCGT	OPAJ-17	ACCCCCTATG
OPAJ-03	AGCACCTCGT	OPAJ-08	GTGCTCCCTC	OPAJ-13	CAGCCGTTCC	OPAJ-18	GGCTAGGTGG
OPAJ-04	GAATGCGACC	OPAJ-09	ACGGCACGCA	OPAJ-14	ACCGATGCTG	OPAJ-19	ACAGTGGCCT
OPAJ-05	CAGCGTTGCC	OPAJ-10	GTTACCGCGA	OPAJ-15	GAATCCGGCA	OPAJ-20	ACACGTGGTC

KIT AK

OPAK-01	TCTGCTACGG	OPAK-06	TCACGTCCCT	OPAK-11	CAGTGTGCTC	OPAK-16	CTGCGTGCTC
OPAK-02	CCATCGGAGG	OPAK-07	CTTGGGGGAC	OPAK-12	AGTGTAGCCC	OPAK-17	CAGCGGTCAC
OPAK-03	GGTCCTACCA	OPAK-08	CCGAAGGGTG	OPAK-13	TCCCACGAGT	OPAK-18	ACCCCGAAAC
OPAK-04	AGGGTCGGTC	OPAK-09	AGGTCGGCGT	OPAK-14	CTGTCATGCC	OPAK-19	TCGCAGCGAG
OPAK-05	GATGGCAGTC	OPAK-10	CAAGCGTCAC	OPAK-15	ACCTGCCGTT	OPAK-20	TGATGGCGTC

KIT AL

OPAL-01	TGTGACGAGG	OPAL-06	AAGCGTCCTC	OPAL-11	GTCACGTCCT	OPAL-16	CTTTCGAGGG
OPAL-02	ACCCTGTGGG	OPAL-07	CCGTCCATCC	OPAL-12	CCCAGGCTAC	OPAL-17	CCGCAAGTGT
OPAL-03	CCCACCCTTG	OPAL-08	GTCGCCCTCA	OPAL-13	GAATGGCACC	OPAL-18	GGAGTGGACT
OPAL-04	ACAACGGTCC	OPAL-09	CAGCGAGTAG	OPAL-14	TCGCTCCGTT	OPAL-19	TCTGCCAGTG
OPAL-05	GACTGCGCCA	OPAL-10	AAGGCCCCTG	OPAL-15	AGGGGACACC	OPAL-20	AGGAGTCGGA

KIT AM

code	5' to 3'	code	5' to 3'	code	5' to 3'	code	5' to 3'
OPAM-01	TCACGTACGG	OPAM-06	CTCGGGATGT	OPAM-11	AGATGCGCGG	OPAM-16	TGGCGGTTTG
OPAM-02	ACTTGACGGG	OPAM-07	AACCGCGGCA	OPAM-12	TCTCACCGTC	OPAM-17	CCTAACGTCC
OPAM-03	CTTCCCTGTG	OPAM-08	ACCACGAGTG	OPAM-13	CACGGCACAA	OPAM-18	ACGGGACTCT
OPAM-04	GAGGGACCTC	OPAM-09	TGCCGGTTCA	OPAM-14	TGGTTGCGGA	OPAM-19	CCAGGTCTTC
OPAM-05	GGGCTATGCC	OPAM-10	CAGACCGACC	OPAM-15	GATGCGATGG	OPAM-20	ACCAACCAGG

KIT AN

OPAN-01	ACTCCACGTC	OPAN-06	GGGAACCCGT	OPAN-11	GTCCATGCAG	OPAN-16	GTGTCGAGTC
OPAN-02	CACCGCAGTT	OPAN-07	TCGCTGCGGA	OPAN-12	AACGGCGGTC	OPAN-17	TCAGCACAGG
OPAN-03	AGCCAGGCTG	OPAN-08	AAGGCTGCTG	OPAN-13	CTTCCAGGAC	OPAN-18	TGTCCTGCGT
OPAN-04	GGCGTAAGTC	OPAN-09	GGGGGAGATG	OPAN-14	AGCCGGGTAA	OPAN-19	ACCACGCCTT
OPAN-05	GGGTGCAGTT	OPAN-10	CTGTGTGCTC	OPAN-15	TGATGCCGCT	OPAN-20	GAGTCCTCAC

KIT AO

OPAO-01	AAGACGACGG	OPAO-06	AGGCAGCCTG	OPAO-11	GGGGGCTTGA	OPAO-16	CACAACGGGA
OPAO-02	AATCCGCTGG	OPAO-07	GATGCGACGG	OPAO-12	TCCCGGTCTC	OPAO-17	CCCATGTGTG
OPAO-03	AGTCGGCCCA	OPAO-08	ACTGGCTCTC	OPAO-13	CCCACAGGTG	OPAO-18	GGGAGCGCTT
OPAO-04	AACAGGGCAG	OPAO-09	CCAGATGGGG	OPAO-14	CTACTGGGGT	OPAO-19	GTTCTCGGAC
OPAO-05	TGGAAGCACC	OPAO-10	GACATCGTCC	OPAO-15	GAAGGCTCCC	OPAO-20	GGCTTGCCTG

KIT AP

OPAP-01	AACTGGCCCC	OPAP-06	GTCACGTCTC	OPAP-11	CTGGCTTCTG	OPAP-16	GGGCAGATAC
OPAP-02	TGGTCATCCC	OPAP-07	ACCACCCGCT	OPAP-12	GTCTTACCCC	OPAP-17	ACGGCACTCC
OPAP-03	GTAAGGCGCA	OPAP-08	ACCCCCACAC	OPAP-13	TGAAGCCCCT	OPAP-18	GTCGTCGACA
OPAP-04	CTCTTGGGCT	OPAP-09	GTGGTCCAGA	OPAP-14	TGCCATGCTG	OPAP-19	GTGTCTGCCT
OPAP-05	GACTTCAGGG	OPAP-10	TGGGTGATCC	OPAP-15	GGGTTGGAAG	OPAP-20	CCCGGATACA

KIT AQ

OPAQ-01	GGCAGGTGGA	OPAQ-06	ACGGATCCCC	OPAQ-11	GACGCCTCCA	OPAQ-16	CCCGGAAGAG
OPAQ-02	ACCCTCGGAC	OPAQ-07	GGAGTAACGG	OPAQ-12	CAGCTCCTGT	OPAQ-17	TTCGCCTGTC
OPAQ-03	GAGGTGTCTG	OPAQ-08	TCGGTAGACC	OPAQ-13	GAGTCGGCTG	OPAQ-18	GGGAGCGAGT
OPAQ-04	GACGGCTATC	OPAQ-09	AGTCCCCCTC	OPAQ-14	CCCGTGTAGG	OPAQ-19	AGTAGGGCCT
OPAQ-05	ACGGAGCTGA	OPAQ-10	CATACCCTCC	OPAQ-15	TGCGATGCGA	OPAQ-20	GTGAACGCTC

KIT AR

OPAR-01	CCATTCCGAG	OPAR-06	TGGGGCTCAA	OPAR-11	GGGAAGACGG	OPAR-16	CCTTGCGCCT
OPAR-02	CACCTGCTGA	OPAR-07	TCCTTCGGTG	OPAR-12	GGATCGTCGG	OPAR-17	CCACCACGAC
OPAR-03	GTGAGGCGCA	OPAR-08	GTGAATGCGG	OPAR-13	GGGTCGGCTT	OPAR-18	CTACCGGCAC
OPAR-04	CCAGGAGAAG	OPAR-09	GGGGTGTTCT	OPAR-14	CTCACAGCAC	OPAR-19	CTGATCGCGG
OPAR-05	CATACCTGCC	OPAR-10	TGGGGCTGTC	OPAR-15	ACACTCTGCC	OPAR-20	TGCGCCATCC

KIT AS

OPAS-01	CACACCGTGT	OPAS-06	GGCGCGTTAG	OPAS-11	ACCGTGCCGT	OPAS-16	AACCCTTCCC
OPAS-02	GTCCTCGTGT	OPAS-07	GACGAGCAGG	OPAS-12	TGACCAGGCA	OPAS-17	AGTTCCGCGA
OPAS-03	ACGGTTCCAC	OPAS-08	GGCTGCCAGT	OPAS-13	CACGGACCGA	OPAS-18	GTTGCGCAGT
OPAS-04	GTCTTGGGCA	OPAS-09	TGGAGTCCCC	OPAS-14	TCGCAGCGTT	OPAS-19	TGACAGCCCC
OPAS-05	GTCACCTGCT	OPAS-10	CCCGTCTACC	OPAS-15	CTGCAATGGG	OPAS-20	TCTGCCTGGA

KIT AT

OPAT-01	CAGTGGTTCC	OPAT-06	CCGTCCCTGA	OPAT-11	CCAGATCTCC	OPAT-16	CTCTCCGTAG
OPAT-02	CAGGTCTAGG	OPAT-07	ACTGCGACCA	OPAT-12	CTGCCTAGCC	OPAT-17	AGCGACTGCT
OPAT-03	GACTGGGAGG	OPAT-08	TCCTCGTGGG	OPAT-13	CTGGTGGAAG	OPAT-18	CCAGCTGTGA
OPAT-04	TTGCCTCGCC	OPAT-09	CCGTTAGCGT	OPAT-14	GTGCCGCACT	OPAT-19	ACCAAGGCAC
OPAT-05	ACACCTGCCA	OPAT-10	ACCTCCGGTC	OPAT-15	TGACGCACGG	OPAT-20	ACATCAGCCC

KIT AU

code	5' to 3'	code	5' to 3'	code	5' to 3'	code	5' to 3'
OPAU-01	GGGATGGAAC	OPAU-06	TCTCTAGGGG	OPAU-11	CTTCTCGGTC	OPAU-16	TCTTAGGCGG
OPAU-02	CCAACCCGCA	OPAU-07	AGACCCTTGG	OPAU-12	CCACTCGTGT	OPAU-17	TTGGCATCCC
OPAU-03	ACGAAACGGG	OPAU-08	CACCGATCCA	OPAU-13	CCAAGCACAC	OPAU-18	CACCACTAGG
OPAU-04	GGCTTCTGTC	OPAU-09	ACGGCCAATC	OPAU-14	CACCTCGACC	OPAU-19	AGCCTGGGGA
OPAU-05	GAGCTACCGT	OPAU-10	GGCGTATGGT	OPAU-15	TGCTGACGAC	OPAU-20	GTCGAAACCC

KIT AV

OPAV-01	TGAGGGGGAA	OPAV-06	CCCGAGATCC	OPAV-11	GACCCCGACA	OPAV-16	GACAAGGACC
OPAV-02	TCACCGTGTC	OPAV-07	CTACCAGGGA	OPAV-12	AGCCGTCGAA	OPAV-17	CTCGAACCCC
OPAV-03	TGTAGCCGTG	OPAV-08	TGAGAAGCGG	OPAV-13	CTGACTTCCC	OPAV-18	TTGCTCACGG
OPAV-04	TCTGCCATCC	OPAV-09	GAGGTCCTAC	OPAV-14	CTCCGGATCA	OPAV-19	CTCGATCACC
OPAV-05	GTGAGCGTGG	OPAV-10	ACCCCTGGCA	OPAV-15	GGCAGCAGGT	OPAV-20	TCATGCGCAC

KIT AW

OPAW-01	ACCTAGGGGA	OPAW-06	TTTGGGCCCC	OPAW-11	CTGCCACGAG	OPAW-16	TTACCCCGCT
OPAW-02	TCGCAGGTTC	OPAW-07	AGCCCCCAAG	OPAW-12	GAGCAAGGCA	OPAW-17	TGCTGCTGCC
OPAW-03	CCATGCGGAG	OPAW-08	CTGTCTGTGG	OPAW-13	CTACGATGCC	OPAW-18	GGCGCAACTG
OPAW-04	AGGAGCGACA	OPAW-09	ACTGGGTCGG	OPAW-14	GGTTCTGCTC	OPAW-19	GGACACAGAG
OPAW-05	CTGCTTCGAG	OPAW-10	GGTGTTTGCC	OPAW-15	CCAGTCCCAA	OPAW-20	TGTCCTAGCC

KIT AX

OPAX-01	GTGTGCCGTT	OPAX-06	AGGCATCGTG	OPAX-11	TGATTGCGGG	OPAX-16	GTCTGTGCGG
OPAX-02	GGGAGGCAAA	OPAX-07	ACGCGACAGA	OPAX-12	GGTCGGGTCA	OPAX-17	TGGGCTCTGG
OPAX-03	CCAAGAGGCT	OPAX-08	AGTATGGCGG	OPAX-13	GAGCACTGCT	OPAX-18	GTGTGCAGTG
OPAX-04	TCCCCAGGAG	OPAX-09	GGAAGTCCTG	OPAX-14	CACGGGCTTG	OPAX-19	CCCTGTCGCA
OPAX-05	AGTGCACACC	OPAX-10	CCAGGCTGAC	OPAX-15	CAGCAATCCC	OPAX-20	ACACTCGGCA

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The RED BOOK BULLETIN

Supplement 13
Winter 1991

"Never Clone Alone"

Subscriber's Notebook

Protocols in this column are submitted by subscribers to CPMB. They have not been tested in the labs of our editorial board, but have been selected as useful addenda to existing protocols in CPMB and edited accordingly.

PCR to Rapidly Detect Cloned Insert DNA

We have used the following adaptation of PCR (*UNIT 15.1*) to detect quickly the presence and orientation of inserted DNA in plasmid or phage clones. Bacterial cells containing plasmid are suspended directly in the PCR mixture. This method requires ~10 min to set up, 2 to 3 hr to amplify, 30 min to analyze, and costs ~50¢/sample (including *Taq* and primers). The procedure significantly speeds analysis and avoids the tedium of "traditional" minipreps.

1. Prepare a 110-µl reaction cocktail for PCR (this recipe is appropriate for screening five colonies; scale up accordingly as desired, including a slight volume excess):

   11 µl 10× amplification buffer
   11 µl 2.5 mM 4dNTP mix
   1 µl 2.5 U/µl *Taq* DNA polymerase
   1 µl oligo primer 1 (1 µg/µl)
   1 µl oligo primer 2 (1 µg/µl)
   85 µl H_2O

   Distribute 20 µl of PCR cocktail into each of five microcentrifuge tubes.

   To check for inserts in pUC-derived vectors (e.g., Bluescript, pGEM, pTZ), M13 universal and reverse sequencing primers can be used as primers 1 and 2. We use modified M13 primers that have been lengthened to 25 bp, so that "Turbo" PCR can be used. To check for orientation, a primer complementary to the vector and flanking the cloning site can be used with a primer that is complementary to sequences in the insert. Alternatively, a primer complementary to the insert can be used with both universal and reverse primers. This way, a unique PCR product will be generated for the insert in either orientation and also for clones that lack any insert.

2. Insert a sterile toothpick into the edge of a transformant colony (putative clone) to pick up a few cells. Swirl toothpick in the PCR cocktail in microcentrifuge tube to suspend cells in the reaction mixture. Overlay the reaction mixture with 30 ml mineral oil. Drop the toothpick into 2 ml culture media and grow shaking at 37°C during the PCR analysis. Repeat for each colony to be analyzed.

3. Proceed with rapid PCR cycling as follows: heat samples 1 min at 95°C; incubate samples 30 sec at 55°C; incubate samples 1 min at 75°C. It is usually sufficient to continue with 15 to 20 cycles.

   The use of primers 20 to 25 bp in length allows elimination of the annealing step and shortening of the procedure.

4. Add 30 µl chloroform to each tube; this will cause mineral oil to settle beneath the aqueous layer. Add 6 µl of 4× sample buffer to each tube and load and run samples on a 6% polyacrylamide or 1% agarose gel. Upon completion of electrophoresis, stain gel with ethidium bromide.

   Clones carrying the desired insert will display a DNA band of the expected length. When using two vector primers to detect the presence of an insert, negative clones will display a smaller fragment (230 bp for Bluescript), confirming lysis and amplification of vector sequences. For determining orientation of the insert, absence of product indicates that the insert is in the undesired or incorrect orientation. The presence of a DNA product of expected size (which equals the distance between the internal and external primers used) indicates that the insert is in the correct orientation.

5. After PCR analysis, perform miniprep or large-scale plasmid prep using 2-ml liquid cultures from step 2.

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focus

Using RAPD markers for crop improvement

Robbie Waugh and Wayne Powell

The detection and exploitation of naturally occurring DNA sequence polymorphisms represent one of the most significant recent developments in molecular biology. Restriction endonuclease digestion of total genomic DNA followed by hybridization with a radioactively labelled probe reveals differently sized hybridizing fragments. This form of polymorphism, termed Restriction Fragment Length Polymorphism (RFLP), has been used extensively for genetic studies[1]. However, the technical complexity of performing RFLP analysis, coupled with the widespread use of short-lived radioisotopes in the detection method, has prompted a debate on whether the routine application of RFLPs in large-scale crop improvement programmes is feasible.

Since its development, the polymerase chain reaction (PCR)[2] has revolutionized many standard molecular biological techniques, with modifications of the original procedure designed to suit a range of needs. One such variation generates a specific class of molecular marker termed Randomly Amplified Polymorphic DNAs (RAPDs)[3,4]. This procedure has the advantages of being technically simple, quick to perform, requires only small amounts of DNA and involves no radioactivity (Table 1). RAPDs are well suited for use in the large sample-throughput systems required for plant breeding, population genetics and studies of biodiversity.

Molecular basis of polymorphism

The modification of the basic PCR technique which allows RAPDs to be generated is remarkably simple. Instead of using a pair of carefully designed and fairly long oligonucleotide primers to amplify a specific target sequence, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template, such as a plant genome. Theoretically, the number of amplified fragments generated by PCR depends on the length of the primer and the size of the target genome, and is based on the probability that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR. For most plants, primers that are 9–10 nucleotides long are predicted to generate, on average, 2–10 amplification products. The primers are generally of random sequence, biased to contain at least 50% Gs and Cs, and to lack internal inverted repeats. The products are easily separated by standard electrophoretic techniques and visualized by ultraviolet (UV) illumination of ethidium-bromide stained gels. Polymorphisms result from changes in either the sequence of the primer binding site (e.g. point mutations), which prevent stable association with the primer, or from changes which alter the size or prevent the successful amplification of a target DNA (e.g. insertions, deletions, inversions). As a rule, size variants are only rarely detected and individual amplification products represent one allele per locus. In inheritance studies, the amplification products are transmitted as dominant markers. (An outline of the approach is shown in Box 1.)

Locating and manipulating genes of interest

RFLPs have been used extensively for the creation of linkage maps, and this has enabled indirect selection strategies for crop-improvement programmes to be developed[5,6]. The overall objective is to manipulate both monogenic and polygenic traits with greater speed and precision. Success depends on identifying the appropriate segregating population, and having the resources to create a detailed linkage map. While RAPDs can be exploited as markers that segregate in a mendelian manner (Fig. 1)[7], identifying useful linked genes using this approach is a laborious and time-consuming process (although a recent report by Reiter *et al.*[8] would question this statement).

Combining the use of RAPDs and near-isogenic lines (NILs) provides a route for quickly identifying markers linked to a trait of interest. NILs are generated by a process of repeated backcrossing with selection for the desirable character at each round of crossing. This procedure results in the production of genotypes that are essentially identical at all genetic loci with the exception of the region surrounding the gene under selection. The high probability that any polymorphism detected will be in the DNA surrounding the introduced gene, provides a powerful means of identifying markers that are linked to the trait of interest. Using the RAPD approach, Martin *et al.*[9] screened two tomato NILs, that differed for the presence or absence of a gene (*pto*) conferring resistance to *Pseudomonas*, with 144 random primers. A total of seven polymorphic amplification products were identified between the two lines. Four of these products were investigated further by segregation analyses, and three were confirmed to be tightly linked to the *pto* gene. The entire procedure took four weeks, compared with a projected time of approximately two

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Table 1. Properties of restriction fragment length polymorphisms (RFLPs) and randomly amplified polymorphic DNAs (RAPDs)

	RFLPs	RAPDs
Distribution	Ubiquitous	Ubiquitous
Inheritance	Simple mendelian, stable, co-dominant	Simple mendelian, stable, dominant/null
Level of polymorphism	High	High
Detect allelic variants?	Yes	No
No. of loci detected	1–3	1–10
Part of genome surveyed	Generally low-copy, coding regions	Whole genome
Detectable in all tissues?	Yes	Yes
Technical difficulty	Intermediate	Low
Reliability	High	Intermediate/high[a] (occasional spurious products)
Quality of DNA required	Relatively pure	Crude
Quantity of DNA required	2–10 μg	10–50 ng
Use radioisotopes?	Yes	No
Type of probes required	Species-specific low-copy gDNA[b] or cDNA clones	Random 9- or 10-mer oligonucleotides
Recurring cost	High	High

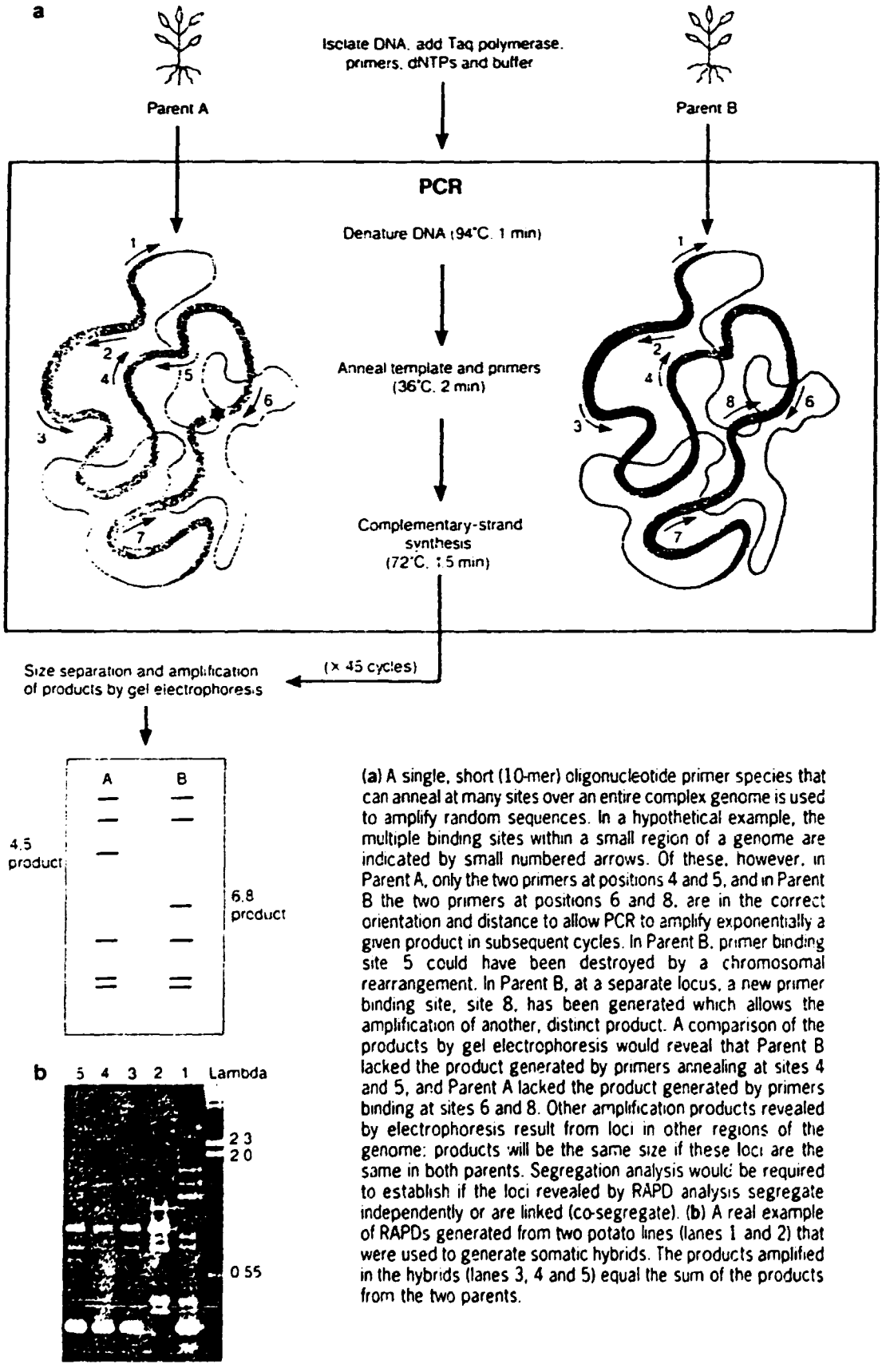
[a] There is currently some debate among scientists from different laboratories as to the reliability of the RAPD approach. In our hands, RAPD markers are both reliable and reproducible. An obvious cause of unreliability could be a variation in RAPD reaction conditions.
[b] gDNA = genomic DNA

years using RFLPs. As NILs are often the product of co-ordinated plant breeding programmes, it is likely that, in the near future, markers linked to many major genes (particularly those conferring resistance to plant pathogens) will be quickly assigned.

Based on similar principles, an alternative approach using bulked DNAs from the homozygous individuals of an F_2 population was recently described by Michelmore *et al.*[30]. A lettuce population segregating for downy-mildew resistance genes (*Dm*) was classified on the basis of the inheritance of *Dm5/8* type resistance. After progeny testing the F_2 plants to determine their genotype at the *Dm5/8* locus (i.e. heterozygous or homozygous) the two groups were subdivided, and two separate samples of DNA were prepared for RAPD analysis, one by pooling DNA from at least ten of the homozygous resistant plants, and the other by pooling DNA from the same number of homozygous susceptible plants. These two bulked DNA samples, while homozygous for alternative alleles at the *Dm5/8* locus (and a certain amount of flanking DNA), are present in a randomized heterozygous background because of the contribution of all other unselected alleles from each of the individuals in the bulk. Thus, any polymorphic products generated by RAPD analysis must arise from DNA linked closely to the target locus as it is the only region in the DNA samples that differs significantly between the two bulks. In total, only 300 PCR reactions were required by Michelmore *et al.* to obtain three markers linked to the target locus, emphasizing the efficiency of RAPDs for locating genes of interest.

Giovannoni *et al.*[31] described an extension of this approach, where the individual plants used to construct the bulked DNA samples were chosen on the basis that they contained alternative alleles of closely linked RFLP markers. The RFLP markers, therefore, define an interval which should differ between the bulks and thus polymorphic products generated by PCR should be derived from either within the defined

Box 1. The generation of RAPDs



(a) A single, short (10-mer) oligonucleotide primer species that can anneal at many sites over an entire complex genome is used to amplify random sequences. In a hypothetical example, the multiple binding sites within a small region of a genome are indicated by small numbered arrows. Of these, however, in Parent A, only the two primers at positions 4 and 5, and in Parent B the two primers at positions 6 and 8, are in the correct orientation and distance to allow PCR to amplify exponentially a given product in subsequent cycles. In Parent B, primer binding site 5 could have been destroyed by a chromosomal rearrangement. In Parent B, at a separate locus, a new primer binding site, site 8, has been generated which allows the amplification of another, distinct product. A comparison of the products by gel electrophoresis would reveal that Parent B lacked the product generated by primers annealing at sites 4 and 5, and Parent A lacked the product generated by primers binding at sites 6 and 8. Other amplification products revealed by electrophoresis result from loci in other regions of the genome: products will be the same size if these loci are the same in both parents. Segregation analysis would be required to establish if the loci revealed by RAPD analysis segregate independently or are linked (co-segregate). (b) A real example of RAPDs generated from two potato lines (lanes 1 and 2) that were used to generate somatic hybrids. The products amplified in the hybrids (lanes 3, 4 and 5) equal the sum of the products from the two parents.

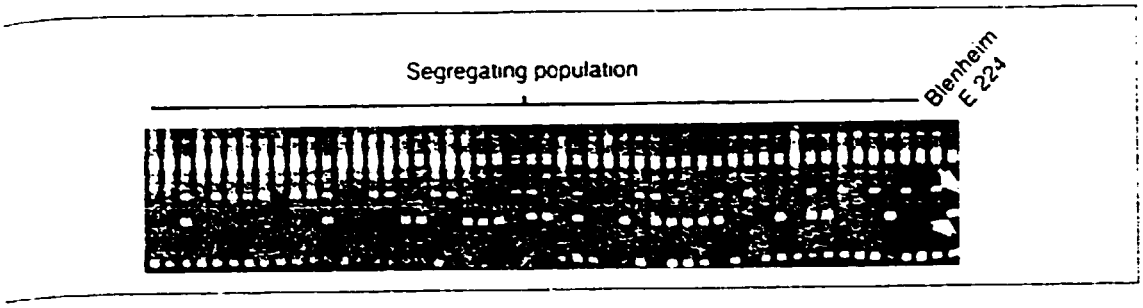


Figure 1

The polymorphic RAPD markers (arrowed) segregate independently and in a mendelian manner in a population derived from crossing two barley lines, Blenheim and E224.

interval or immediately adjacent to it. A population used previously to construct a high-density genetic-linkage map of tomato[12], was further investigated by screening with 200 random primers, for two genetic intervals – one of 15 cM (see Glossary) and one of 0.5 cM – which contained the genes responsible for regulating pedicle abscission and fruit ripening. Three polymorphic products were identified and subsequently shown, by segregation analysis, to be tightly linked to the selected intervals. The technique can be applied to any sexually reproducing organism where segregating populations are being used to construct genetic-linkage maps. The approach is particularly attractive for focusing on particular chromosomal regions where there are few existing markers (e.g. centromeres, ends of linkage groups), but where the interval can be defined by flanking markers. Furthermore, different DNA bulks can be made from the same segregating population to target different chromosomal intervals.

Finally, while these examples are particularly relevant to efficient methods of identifying markers linked to desirable targets, it must be stressed that formal genetic proof of linkage in all cases has to be obtained by segregation analysis which demands appropriate mapping populations.

Perhaps the most exciting use of RAPDs will be in the mapping of polygenic sources of variation. Traits controlled by polygenic systems include yield and quality factors, and resistances to biotic and abiotic stresses. In addition to their biological and economic importance, these traits are often the most difficult to manipulate in breeding programmes since their genetic basis is poorly understood. Polygenically or quantitatively determined sources of variation are characterized by a continuous range of variation (Fig. 2). Thus the members at the extremes of the distribution are the individual genotypes expressing high or low phenotypic scores for the traits measured; are expected to differ at most of the loci controlling the characters. These genotypes are likely to be the most informative since the alleles (increasing and decreasing) are expected to be highly associated. Bulked samples comprising the extreme members of the distribution may be screened with RAPDs and markers linked to loci controlling quantitative traits quickly identified. The efficiency of this approach will be improved if recombinant inbred lines (RILs)[12] are used in the analysis. In particular, double haploid families[13,14] (see Glossary) are well suited to mapping with RAPDs since dominance-related effects are absent and, hence, linkage data can be obtained directly. Such families can be extensively replicated and this facilitates the partitioning of the total phenotypic variation into heritable and non-heritable components. This approach (called 'localized mapping') has already been used to map qualitative traits in RILs of *Arabidopsis thaliana*[9].

Identification of somatic hybrids

Somatic hybridization by protoplast fusion allows the transfer of potentially useful traits between sexually incompatible plant species, and is an important method for gene introgression in crop improvement programmes. However, a major limitation to the more

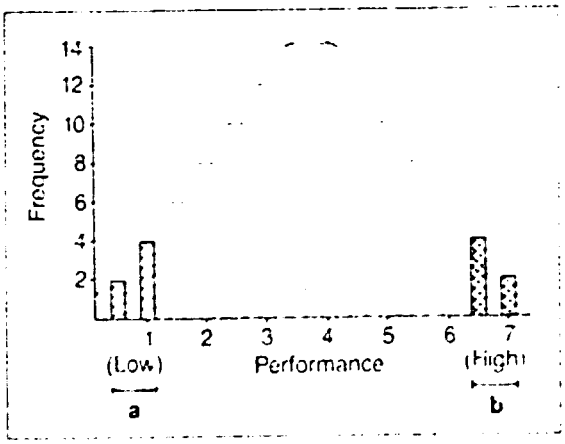


Figure 2

Schematic representation of a quantitative trait controlled by a polygenic system and exhibiting continuous variation. (Frequency = the number of individual genotypes. Performance = phenotypic score, i.e. the performance of the quantitative trait under study.) In order to identify RAPDs linked to the genetic loci controlling the trait, DNA from the six individual genotypes identified in the 'tails' of the distribution (**a** and **b**) are pooled to form a bulk. The bulked samples are screened with RAPDs and putative markers linked to the quantitative trait quickly identified. The segregation of these RAPD loci are then monitored in each individual genotype and the intensity of linkage between the RAPD marker and the quantitative trait established.

Glossary

Alleles – Two or more genes are said to be alleles when they: (1) occupy the same relative position (locus) on a pair of homologous chromosomes and undergo pairing during meiosis; (2) produce different effects on the same set of developmental processes; and (3) can mutate one to another.

Backcross – The generation derived from crossing a hybrid with one of its original parents (usually the female).

Centimorgan (cM) – A measurement of length on a genetic map (1 cM = the distance that separates two genes between which there is a 1% chance of recombination) dependent on the size of the genome (e.g. *Arabidopsis*: 1cM = 139 kb; human: 1cM = 1108 kb).

Doubled haploid – See **Haploid** below.

F_1 – The offspring derived from crossing plants or animals of the parental generation (P_1).

F_2 – The offspring derived from crossing the members of the F_1 among themselves (**selfing**).

Gene introgression – The introduction of genes of one species into the genepool of another.

Haploid – A sporophyte that contains the gametic number of chromosomes. Doubling the chromosome number to produce a **doubled haploid** results in complete homozygosity.

Heterozygote – Having two different alleles at the corresponding loci of a pair of chromosomes.

Homozygote – Having identical genes at the two corresponding loci of a pair of chromosomes.

Locus – The position occupied in all homologous chromosomes by a particular gene or one of its alleles.

Monogenic – Discrete character differences controlled by alleles at one genetic locus.

Polygenic – Character differences that are controlled by more than one genetic locus, and are influenced by both environmental and genetic factors. Polygenically controlled traits tend to show a continuous range of phenotypes.

Progeny testing – A method for assessing the genotype of an individual by a study of its offspring.

Recombinant inbred line (RIL) – A genotype created by repeated self pollination.

Segregation analysis – Monitoring the separation and distribution of alleles to different cells, usually following meiosis.

Somatic cells – The cells of an organism other than the germ cells.

Somatic hybridization – Fusion of two somatic cells.

extensive exploitation of somatic hybridization is the lack of appropriate methods for the unequivocal identification of nuclear hybrids. Several methods are currently used. These include combining light-bleached protoplasts with green leaf mesophyll protoplasts and manual isolation of the heterokaryons, genetic complementation of auxotrophic mutants or the fusion of different antibiotic- and/or herbicide-resistant lines which have first been derived by plant transformation. In the absence of a selection system, hybrid vigour, isozymes, proteins and RFLPs have all been used. However, these methods are both time consuming and costly. Recently, Bird *et al.*[5] described a method based on RAPDs that requires only a small sample of a developing regenerant to determine its hybridity. A range of 10-mer primers was screened to identify those capable of generating dominant polymorphic amplification products from each of the fusion parents. Putative inter- and intraspecific hybrids were screened with the informative primers and the heterokaryons unequivocally identified by their molecular profiles which represented a combination of the profiles of the donor genotypes (see example in Box 1.b). By identifying somatic hybrids at an early stage in the regeneration process, RAPDs can make a significant contribution to the efficiency of somatic hybridization programmes.

Optimizing strategies for the evaluation and conservation of genetic resources

The genetic improvement of any organism depends upon the existence, nature and extent of the genetic variability available for manipulation. Current and future plant-breeding programmes will not only require access to this variability, but also will be dependent upon the conservation and management of biodiversity. Traditionally, genetic resources have been characterized by a combination of morphological and agronomic traits. The effectiveness of this approach is limited, and requires accurate information on (1) the amount of diversity present in gene pools, and (2) the spatial distribution of diversity in relation to eco-geographic factors. RAPDs provide a useful system to monitor the levels of diversity detected between and within populations.

Despite their ecological and agricultural importance, little information is available on the extent, distribution and nature of the genetic variability available in tree species of the tropics. Chalmers *et al.*[6] used RAPD markers to partition the genetic variation in *Gliricidia sepium* and *G. maculata* into 'between'- and 'within'-population components. Five individuals from 10 geographically and ecologically distinct populations were examined with 11 primers (see Fig. 3). Overall, 60% of the genetic variation was attributed to between-population and 40% to within-population components. Information of this nature will allow breeding programmes and strategies to be designed for maximum gain from artificially imposed selection regimes. Collection missions can also be directed to areas possessing maximum levels of diversity.

RAPDs have also been successfully used to 'fingerprint' individual accessions of *Theobroma cacao* (Cocoa). As with many tropical tree crops, effective conservation of the cocoa gene pool hinges on optimizing strategies for the identification, collection and preservation of existing natural variation. Since living collections are difficult and expensive to maintain, methods of eliminating duplicate accessions would be invaluable. Although RFLPs have been used successfully for this purpose in other crops, a major obstacle to this approach is the recalcitrance of cocoa material to routine, established DNA-extraction procedures. Nevertheless, DNA of sufficient quality and quantity can be obtained for PCR-based analysis. Wilde *et al.*[7] performed RAPD analysis on a range of cocoa accessions representing a diverse spectrum of available

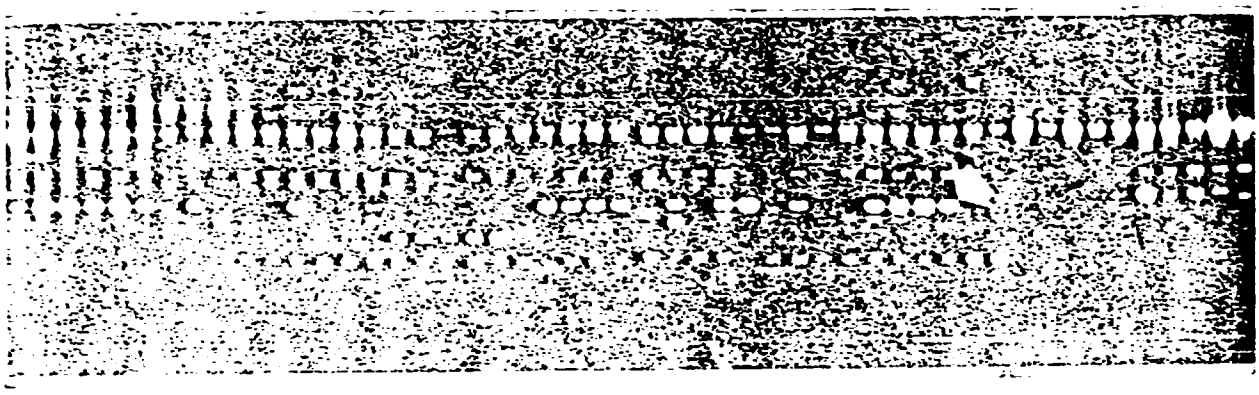


Figure 3

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Prospects

Acknowledgements

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Update on Plant Breeding

Genetic Analysis with Random Amplified Polymorphic DNA Markers

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For many years, the principles of genetics have been applied to crop variety improvement with great success. Several crop species, notably corn, wheat, and tomato, have been used as model genetic systems because of their central importance to food production. Until recently, virtually all progress in both breeding and model genetic systems has relied on a phenotypic assay of genotype. Because the efficiency of a selection scheme or genetic analysis based on phenotype is a function of the heritability of the trait, factors like the environment, multigenic and quantitative inheritance, or partial and complete dominance often confound the expression of a genetic trait. Many of the complications of a phenotype-based assay can be mitigated through direct identification of genotype with a DNA-based diagnostic assay. For this reason, DNA-based genetic markers are being integrated into several plant systems and are expected to play an important role in the future of plant breeding.

The utility of DNA-based diagnostic markers is determined to a large extent by the technology that is used to reveal DNA-based polymorphisms. Currently, the technology of choice for many species is the RFLP assay. RFLP assays detect DNA polymorphisms through restriction endonuclease digestions, coupled with DNA blot hybridizations, and are, in general, time consuming and labor intensive. Over the last few years, polymerase chain reaction technology has led to the development of several novel genetic assays based on selective DNA amplification (Krawetz, 1989; Innis et al., 1990). One of the strengths of these new assays is that they are more amenable to automation than conventional techniques. They are also simple to perform and are preferable in experiments where the genotype of a large number of individuals is to be determined at a few genetic loci. Unfortunately, because of a prerequisite for DNA sequence information, these assays are limited in their application.

Nearly 2 years ago, a new genetic assay was developed independently by two different laboratories (Welsh and McClelland, 1990; Williams et al., 1990). This procedure, which we have called the RAPD assay, detects nucleotide sequence polymorphisms in a DNA amplification-based assay using only a single primer of arbitrary nucleotide sequence. In this reaction, a single species of primer binds to the genomic DNA at two different sites on opposite strands of the DNA template. If these priming sites are within an amplifiable distance of each other, a discrete DNA product is produced through thermocyclic amplification (Fig. 1). The presence of each amplification product identifies complete or partial nucleotide sequence homology, between the genomic DNA and the oligonucleotide primer, at each end of the amplified product. On average, each primer will direct the amplification of several discrete loci in the genome, making the assay an efficient way to screen for nucleotide sequence polymorphism between individuals. For example, the frequency of finding RAPD polymorphisms has been shown to be 0.3 per primer in *Arabidopsis thaliana*, 0.5 per primer in soybean, 1 per primer in corn, and 2.5 per primer in *Neurospora crassa*. The major advantage of this assay is that there is no requirement for DNA sequence information. The protocol is also relatively quick and easy to perform and uses fluorescence in lieu of radioactivity (Williams et al., 1992). Because the RAPD technique is an amplification-based assay, only nanogram quantities of DNA are required, and automation is feasible.

APPLICATIONS OF THE RAPD ASSAY

There are several applications of the RAPD assay that have been developed over the past 2 years. Each of these techniques exploits the efficiency of detection of DNA sequence-based polymorphisms in the RAPD assay. The RAPD technology has quickly gained widespread acceptance and application because it has provided a tool for genetic analysis in biological systems that have not previously benefitted from the use of molecular markers.

Development of Genetic Maps

One of the first practical uses of RAPD markers was in the creation of high-density genetic maps. By using a more efficient assay, Reiter et al. (1992) were able to place over 250 new genetic markers on a recombinant inbred population of *A. thaliana* in only 4 person-months, clearly demonstrating the utility of RAPD markers for quickly saturating both a global and local genetic map.

Historically, many important crop systems have suffered from a lack of genetic markers. For example, genetic linkage analysis in conifers has been slow primarily due to the large size of the genome and the inherent difficulty involved in producing a segregating F_2 population (Carlson et al., 1991).

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Abbreviations: RAPD, random amplified polymorphic DNA; RFLP, restriction fragment-length polymorphism.

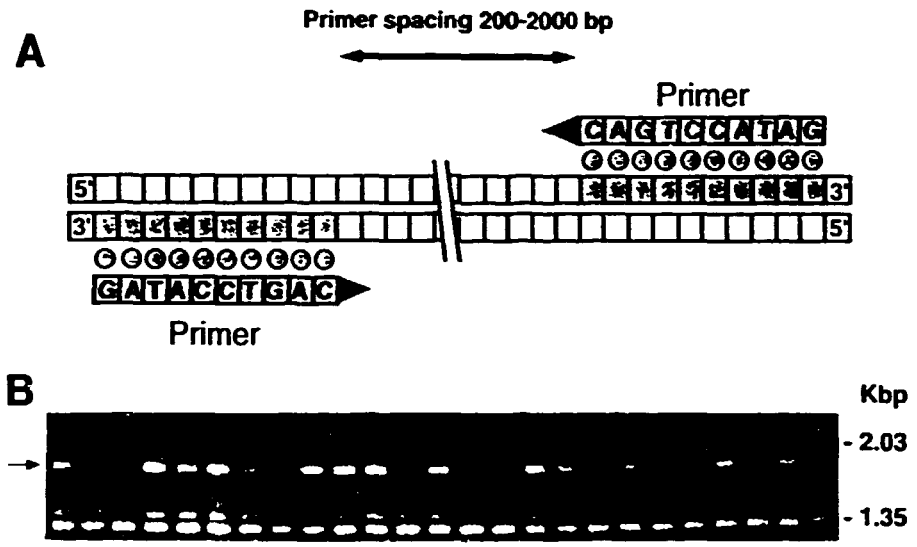

Figure 1. A, Schematic representation of the amplification of DNA with a single oligodeoxynucleotide primer. B, Amplification products from F_2 individuals segregating from a cross between *Glycine max* cv Bonus and *Glycine soja* PI81762. The arrow points to a segregating locus, and the size standards are shown to the right of the gel. The RAPD reaction was performed as described in Williams et al. (1990), and the products of the amplification reaction were visualized by separation on a 1.4% agarose gel stained with ethidium bromide.

Carlson et al. (1991) and Chaparro et al. (1992) have recently shown that the speed and efficiency of RAPD analysis has made mapping in conifers a reasonable endeavor. For example, Chaparro et al. (1992) were able to create a 191-marker RAPD map of loblolly pine in only 6 person-months. Both the *A. thaliana* and loblolly pine genetic maps were synthesized with unprecedented speed, taking advantage of the unique reproductive biology of each system.

Because RAPD polymorphisms are the result of either a nucleotide base change that alters the primer binding site, or an insertion or deletion within the amplified region (Williams et al., 1990; Parks et al., 1991), polymorphisms are usually noted by the presence or absence of an amplification product from a single locus. This also means that the RAPD technique tends to provide only dominant markers. Individuals containing two copies of an allele are not distinguished quantitatively from those containing only one copy of the allele. The disadvantage of mapping with dominant markers is that markers linked in repulsion, for example markers residing on separate chromatids, such as could be found in an F_2 population, provide little information for the estimate of genetic distance (Allard, 1956). Therefore, when mapping with dominant markers, it is necessary to work with markers that are only linked in coupling, i.e. markers residing on a single chromatid as can be found in a backcross or recombinant inbred population, in haploid or gametophytic tissue, or alternatively in an F_2 population where only RAPD markers amplified from a single parent are mapped (Williams et al., 1992). Genetic simulations show that dominant markers linked in coupling are as efficient for mapping as codominant markers on a per gamete basis (Hanafey Maize Genetics Meeting).

Targeting Genetic Markers

Several groups have used the RAPD assay as an efficient tool to identify molecular markers that lie within regions of a genome introgressed during the development of near isogenic lines (Klein-Lankhorst et al., 1991; Martin et al., 1991; Paran et al., 1991). By definition, any region of the genome that is polymorphic between two near-isogenic plants is potentially linked to the introgressed trait. Thus, Klein-Lankhorst et al. (1991) were able to identify RAPD markers specific to chromosome 6 of tomato by screening a *Lycopersicon esculentum* substitution line, and Martin et al. (1991) were able to confirm linkage of RAPD markers to the *Pto* locus in tomato after screening two near-isogenic lines. Paran et al. (1991) used two different sets of near-isogenic lettuce lines to identify RAPD markers linked to the *Dm1*, *Dm3*, and *Dm11* locus. RAPD markers were 4 to 6 times more efficient, on a per assay basis, than was screening for these polymorphisms using RFLP technology, and were over 10-fold more efficient in time and labor. Another advantage of this technology is that a genetic map of the entire genome is not required to identify markers linked to a trait of interest; instead, specific regions of the genome can be focused on.

There are, however, two disadvantages to using near-isogenic lines to identify markers linked to a genetic trait. The first is that it takes several generations of backcrossing to create a near-isogenic line. The second is that frequently there are several regions of the donor genome that are inadvertently co-introgressed into the near-isogenic line (Young and Tanksley, 1989). This results in the identification of marker polymorphisms between near-isogenic lines that are not necessarily linked to the trait that is being studied.

Pooling Strategies

Recently, another technology has been developed that is designed to identify genetic markers linked to very specific regions of the genome. Arnheim et al (1985) outlined a genome pooling strategy that allows RFLP markers to be targeted to a region of the genome that is not in linkage equilibrium as a result of selection at a particular locus. The strategy requires pooling genomic DNA from individuals that are known to be genetically fixed at a particular locus. Markers linked to that locus are identified by their linkage disequilibrium with respect to the rest of the population. The limitation of this approach is that it relies on RFLP technology, which is relatively inefficient for identifying polymorphic regions of a genome, and, more importantly, on the premise that linkage disequilibrium exists at the locus of interest within the source population.

Recently, Michelmore et al. (1991) have described the use of RAPD markers to screen efficiently for markers linked to specific regions of the genome. This method, called bulk segregant analysis, uses two bulked DNA samples gathered from individuals segregating in a single population. Each bulk is composed of individuals that differ for a specific phenotype or genotype, or individuals at either extreme of a segregating population. For simple genetic traits, all loci in the genome should appear to be in linkage equilibrium except in the region of the genome linked to the selected locus. Markers linked to this locus should appear polymorphic between the pools for alternate parental alleles. Because many segregating individuals are used to generate the pools, there is only a minimal chance that regions of the genome unlinked to the target locus will also be polymorphic between the pools. Random primers can then be used efficiently to amplify loci from each pool and to identify RAPD polymorphisms linked to the trait of interest. Michelmore et al. (1991) have successfully used this technique to target markers to the *Dm5/8* locus in lettuce.

The advantage of this technology is that markers are targeted to a much smaller locus within the genome, and the likelihood of identifying false positive markers is small (Michelmore et al., 1991) compared with near-isogenic line analysis. Selections made from an F_2 population will always be in linkage disequilibrium with respect to selected regions of the genome, and markers can be targeted to any locus where any form of selection can be applied, either phenotypic or genotypic.

Giovannoni et al. (1991) demonstrated the use of a pooling strategy, based on known RFLP genotypes from existing mapping populations, to create pools of DNA from individuals homozygous for opposite parental alleles in a targeted chromosomal interval. This method was used to target RAPD markers to regions of the tomato genome responsible for fruit ripening and pedicel abscission. Reiter et al. (1992) used this pooling strategy to identify 100 RAPD markers specific to chromosome 1 of *A. thaliana*. As genetic maps approach saturation, pooling on phenotype or genotype will allow researchers to move away from a random approach to map saturation and focus more efficiently on specific regions of the genome.

Population Genetics

The area of research that has shown the most growth with respect to the use of RAPD technology is that of population genetics (Hedrick, 1992). RAPD markers have been used to create DNA fingerprints for the study of individual identity and taxonomic relationship in both eukaryotic and prokaryotic organisms (Caetano-Anollés et al., 1991a; Hu and Quiros, 1991; Welsh et al., 1991; Wöstemeyer et al., 1991; Hadrys et al., 1992; Kresovich et al., 1992; Lark et al., 1992; Stiles et al., 1992; Wilde et al., 1992). An important question is whether RAPD bands of equal mol wt that are shared between individuals are homologous characters (characters inherited from a common ancestor) or homoplastic characters (characters that arise independently within a population). It seems likely that closely related individuals would co-inherit a shared character state from a common ancestor and unlikely that they would acquire the same character independently. Williams et al. (1992) demonstrated this to be the case by using single RAPD bands as hybridization probes to detect homologous characters on a DNA blot of RAPD products. Within the limits of the resolution of an agarose gel, RAPD bands that were amplified from different species of the genus *Glycine*, and scored as homologous by relative mobility, were also shown to be homologous by hybridization.

Several groups have reported on the utility of RAPD markers as a source of phylogenetic information. Arnold et al. (1991) were successful in using RAPD markers to test for interspecific nuclear gene flow between *Iris fulva* and *I. hexagona*, and to study the presumed hybrid origin of *I. nelsonii*. Hu and Quiros (1991) were able to show that the amplification products from only four random primers were sufficient to discriminate between 14 different broccoli and 12 different cauliflower cultivars (*Brassica oleracea* L.). RAPD markers have also been used effectively to assess the amount of genetic diversity in germplasm collections. Using only 25 different decamer oligonucleotide primers, Kresovich et al. (1992) collected information on 140 different polymorphic characters in a "test array" of individuals representing *B. oleracea* L. and *B. rapa* L. They showed the utility of the assay for discriminating between different individuals in a germplasm collection and that the ability to distinguish between closely related individuals was simply a function of the number of RAPD bands that were observed. RAPD markers provided an efficient technology for discovering these polymorphic characters.

Using an arbitrary primer as short as five nucleotides, combined with silver staining to increase the sensitivity of DNA band detection, Caetano-Anollés et al. (1991a) produced a detailed and relatively complex DNA fingerprint for several different species. This approach, termed DNA amplification fingerprinting, has been reviewed recently (Caetano-Anollés et al., 1992b) and promises to generate more genetic information from each amplification.

CONCLUSION

DNA-based diagnostics are now well established as a means to assay diversity at the locus, chromosome, and whole genome levels. As technology has advanced, DNA sequence

based assays have become easier to use, more efficient at screening for nucleotide sequence-based polymorphisms, and available to a wider cross-section of the genetics research community. The ultimate genetic assay would be based on the determination of the complete DNA sequence at any locus of interest. Increased genetic resolution would be obtained simply by sequencing a larger contiguous segment of DNA at the locus. As a prelude to this, there are now several good examples of DNA sequence-based diagnostic assays that are designed to identify the presence or absence of a specific nucleotide bp at a discrete locus (Landegren et al., 1988; Korner and Livak, 1989; Newton et al., 1989; Wu et al., 1989; Barany, 1991; Dockhorn-Dworniczak et al., 1991; Kuppuswamy et al., 1991; Suzuki et al., 1991). Today these assays are limited in their application only by the high cost of DNA sequence determination. As DNA sequencing technology becomes more cost efficient and automated, genetic assays may be based directly on DNA sequence analysis.

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Fingerprinting genomes using PCR with arbitrary primers*

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ABSTRACT

Simple and reproducible fingerprints of complex genomes can be generated using single arbitrarily chosen primers and the polymerase chain reaction (PCR). No prior sequence information is required. The method, arbitrarily primed PCR (AP-PCR), involves two cycles of low stringency amplification followed by PCR at higher stringency. We show that strains can be distinguished by comparing polymorphisms in genomic fingerprints. The generality of the method is demonstrated by application to twenty four strains from five species of *Staphylococcus*, eleven strains of *Streptococcus pyogenes* and three varieties of *Oryza sativa* (rice).

INTRODUCTION

It is important in epidemiology and ecology to be able to identify bacterial species and strains accurately. Rapid identification and classification of bacteria is normally carried out by morphology, nutritional requirements, antibiotic resistance, isoenzyme comparisons, phage sensitivity (1,2,3,4) and, more recently, DNA based methods, particularly rRNA sequences (5), strain-specific fluorescent oligonucleotides (6,7) and the polymerase chain reaction (8,9,10). Each of these methods has specific applications and advantages. We demonstrate here a complementary method that is simple and fast, and can be applied to any species for which DNA can be prepared. This method, which we call Arbitrarily Primed Polymerase Chain Reaction (AP-PCR) 'fingerprinting' has the further merit of requiring little knowledge of the biochemistry or molecular biology of the species being studied.

As a first application, and to demonstrate the ease and utility of this method, we tested strains from five species of *Staphylococcus*, the relationships for which had been determined by DNA-DNA hybridization (11,12,13,14), eleven strains of the human pathogen *Streptococcus pyogenes*, and three varieties of rice, an agriculturally important plant.

METHODS

Strains

S. aureus, ISP-8, isolated from human. ATCC 8432, from bird. ATCC 15564, from human. ATCC 6538, from human. Sau3A, from human. ATCC 12600, from human.

S. cohnii, JL 143, from human. CM 89, from human. SS 521, from Squirrel monkey.

Staphylococcus haemolyticus, C 12J2, isolated from Mangabey. PAY 9F2, from Chimpanzee. AW 263, from human. MID 563, from Mouse lemur. ATCC 29970, from human.

S. hominis, ATCC 27844, from human. ATCC 27846, from human. Ful, questionable origin.

S. warneri, CPB 10E2, isolated from Cercopithecus. GAD 473, from Bush-baby. MCY 3E6, from Rhesus monkey. PBNZP 4D3, from Langur. LED 355, from Lemur.

All *Staphylococcus* strains were kindly provided by W.E. Kloos of North Carolina State Univ. except ISP-8 from Peter Pattee, Iowa State, Ful, and those from the American Type Culture Collection.

Streptococcus pyogenes. DNAs from the human pathogenic strains D471, TI/195/2, 40RS15, 52RS15, 47RS15, 55RS15, 1/E9, T28/51/4, K58Hg, SM6, UAB 092 and *Enterococcus faecalis*, OGI X, were all kindly supplied by Susan Hollingshead (Univ. of Alabama, Birmingham, AL).

Oryza sativa, (Rice). Genomic DNAs from ssp. *indica* IR54, ssp. *japonica* Calsorse 76 and *O. sativa* cv. 'Lemont' were kindly provided by Thomas Hodges (Purdue Univ., West Lafayette, IN) and Timothy Croughan (Louisiana State U., Rice Expt. Station, Crowley, LA).

Staphylococcus genomic DNA preparation

Staphylococcus strains were grown overnight at 37°C in 2–5 ml of brain heart infusion media. The cells were pelleted, resuspended in 0.2 ml of 1× TE, with 0.2 mg/ml lysostaphin and incubated at 37°C for one hour. 0.2 ml proteinase K solution (0.5 mg/ml proteinase K, 1% sarkosyl, 200 mM EDTA, 1 mM calcium chloride) was added to each, followed by digestion at 50°C for 1 hr. The cleared lysates were extracted with phenol, then chloroform, then ethanol precipitated. The DNA was dissolved in TE, and the final concentration was estimated by agarose gel electrophoresis and ethidium staining.

Primers

The primer Kpn-R (CCAAGTCGACATGGCACRTGTATA-CATAYGTAAC), and the pBS reverse sequencing primer (GG-AAACAGCTATGACCATGA), designed for other purposes and chosen arbitrarily for these experiments, were obtained from Genosys, Houston, TX.

*Publication of this paper was delayed by the authors to allow simultaneous publication with a paper submitted later by another group. *Nucleic Acids Research* regrets that due to administrative errors the other paper, by Williams *et al.*, was published on pages 6531–6535 of issue 22. Both sets of authors agree that the two papers should be considered as published simultaneously and should be referred to together.

AP-PCR amplification

10 μl reactions were prepared using 0.025 U Taq pol and 1× buffer (Stratagene) adjusted to 4 mM with $MgCl_2$, 0.2 mM of each dNTP, 10 μM Kpn-R primer and DNA at various concentrations as indicated in the figure legends. A high Mg^{2+} concentration was selected to enhance the stability of primer/template interactions. The reaction was overlaid with oil and cycled through the following temperature profile: 94°C for 5 min. to denature, 40°C for 5 min. for low stringency annealing of primer and 72°C for 5 min. for extension for two cycles. This temperature profile was followed by ten high stringency cycles: 94°C for 1 min., 60°C for 1 min. and 72°C for 2 min. for 10 cycles. At the end of this reaction, 90 μl of a solution containing 2.25 U Taq pol in 1× buffer, 0.2 mM dNTPs and 50 μCi α-[^{32}P] dCTP was added and the high stringency cycles were continued for an additional 20 or 30 rounds. This protocol was designed to allow for high primer concentration during the low stringency steps.

RESULTS

We observed that by using a single primer and two cycles of low stringency PCR followed by many cycles of high stringency PCR we were able to produce a discrete and reproducible set of products characteristic of genomes. We have found that between three and twenty products predominate from most bacterial and eukaryotic genomes. The fingerprints we have produced are reminiscent of those produced by *specific* priming at the *Alu* repeat in human genomic DNA (15), except that AP-PCR does not rely on high homology to abundant dispersed repeats.

Our rationale for the phenomenon of AP-PCR is as follows: At a sufficiently low temperature, primers can be expected to anneal to many sequences with a variety of mismatches. Some of these will be within a few hundred base pairs of each other and on opposite strands. Sequences between these positions will be PCR amplifiable. The extent to which sequences amplify will depend on the efficiency of priming at each pair of primer annealing sites and the efficiency of extension. At early cycles, those that prime most efficiently will predominate. At later times, those that amplify most efficiently will predominate.

We investigated the parameters that affected the production of reproducible, species- and strain-specific, AP-PCR fingerprints. The number, reproducibility and intensity of bands in a fingerprint should be a function of several parameters,

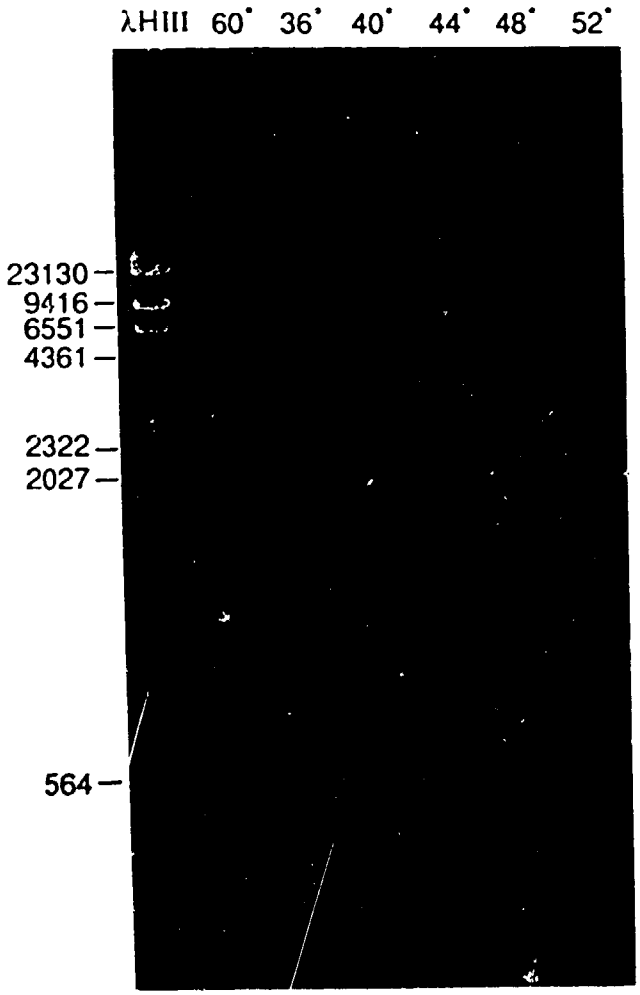

Figure 1. Variation of temperature in first two steps: Discrete AP-PCR products are produced over a wide range of temperatures. PCR was performed using 1 ng of *S. aureus* ISP8 DNA with the Kpn-R primer and the standard protocol (methods) except the two initial low temperature steps were varied. DNA was electrophoresed in 1% agarose 1× TBE gel and visualized by ethidium staining. The low stringency annealing temperature was 36° (lane 2), 40° (lane 3), 44° (lane 4), 48° (lane 5) and 52° (lane 6). In lane 1, the low stringency step was 60° which is typical for conventional PCR. Lane M is *Hin*dIII-digested bacteriophage lambda DNA.

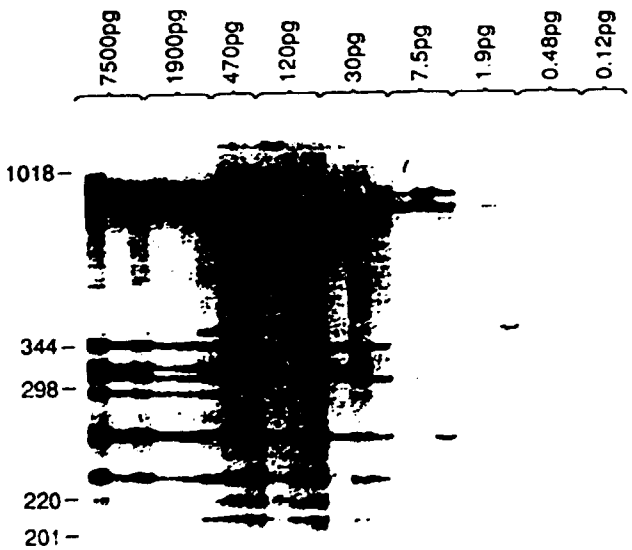

Figure 2. Template titration: The pattern of AP-PCR products depends on the template DNA concentration. AP-PCR was performed using *S. aureus* ISP8 DNA with the Kpn-R primer and the standard protocol (methods) except the template concentration was varied. Each series of nine different concentrations was from three independent dilutions (amount of template per reaction is shown above each set of three lanes. Size markers are the 1 kb ladder from BRL/Gibco. The DNA was separated on a 5% acrylamide, 50% urea 1× TBE gel and visualized by autoradiography.

including the concentration of salts, primer annealing temperature, template concentration, primer length and primer sequence.

Temperature

Initially, we determined the effect of temperature on AP-PCR using the genome of the bacteria *Staphylococcus* as a template. If the stringency is kept high (at least 60°C) throughout the thermo-cycling reaction and the primer is sufficiently different from any sequence in the template, no specific product will be observed (lane 1, Figure 1). Cycling at lower temperatures for two cycles (annealing at 35–50°C) followed by 40 cycles of standard PCR is sufficient to generate a pattern of bands which is characteristic of the species from which the template is derived (lanes 2–5, Figure 1). The pattern of bands changes only slightly as the temperature is raised, until, at some point, the temperature is too high for this set of matches to predominate (lane 6, Figure 1). We conclude that consistent AP-PCR can be achieved over a fairly broad range of temperatures. Other primers, ranging in size from 20 to 34 bases, give a different pattern but have similar temperature dependent characteristics (data not shown). However, the temperature that results in reproducible PCR products may need to be adjusted if very large genomes or much shorter or much longer primers are employed. In preliminary experiments we have recently performed with primers that have considerable sequence redundancy, very low temperatures for the initial two steps were not required, presumably because some of the sequences in the mixture inevitably anneal quite well to any complex genome (data not shown).

Template concentration

Figure 2 shows the effect of varying template concentration on AP-PCR patterns. *S. aureus* ISP8 genomic DNA was varied through nine different concentrations in the range (7.5 ng to 0.12 pg). When run on a high resolution polyacrylamide gel, the pattern generated by AP-PCR with the 34 base Kpn-R primer is very consistent for template concentrations between 7.5 ng and 30 pg. Below this level the PCR is more sporadic. However, the products produced are also almost all represented at higher template concentrations. In other experiments 10 pg has been the lower limit of reliable AP-PCR for genomes of this size (approx 3×10^6 bp) (16). Ten pg of template DNA equals approximately 3000 molecules. One interpretation of this data is that even the best priming events are quite inefficient and, at this low template concentration, the probability of initiating each AP-PCR event during each of the two low temperature cycles is about one in 55 (sq root of 3000). We expect that the efficiency of priming can be improved by altering our present protocol.

Intra-specific patterns of AP-PCR products in *S. aureus*

We compared the pattern of AP-PCR products from six *S. aureus* strains over a concentration range of 7.5 ng to 30 pg of genomic DNA (Figure 3). In this range the patterns are highly reproducible and also very similar between strains. The differences between strains should be diagnostic of specific strains and their relatedness. Each primer of similar length but of different sequence gives a different set of patterns, as expected since the template/primer interactions are different. Intra-specific variation in the pattern is observed for each primer (data not shown). Such data should be of value in the construction of phylogenetic trees.

Species specific AP-PCR products

Genomic DNA of strains from five different species of *Staphylococcus* were each amplified at two different DNA concentrations (Figure 4). In general, there is a species-specific pattern of PCR products, for instance, the *S. warneri* strains (lanes

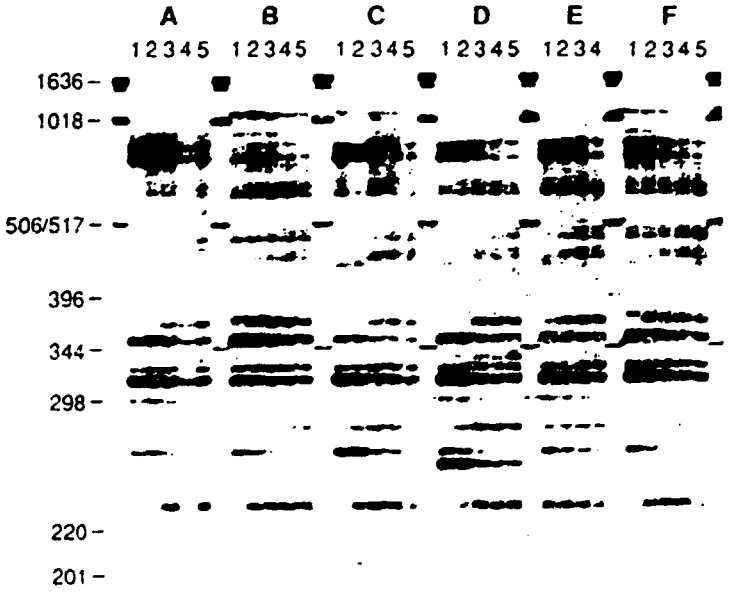

Figure 3. Genomic fingerprints of six strains of *Staphylococcus aureus*: AP-PCR patterns are similar between strains of the same species. Six strains of *S. aureus* were fingerprinted by AP-PCR with the Kpn-R primer and the standard protocol and five different amounts of template, 7.5 ng, 1.9 ng, 470 pg, 120 pg and 30 pg per reaction. The highest concentrations are shown on the left for each strain. Lanes 1, ISP8; lanes 2, ATCC 8432; lanes 3, ATCC 15564; lanes 4, ATCC 6538; lanes 5, *Sau*3A; lanes 6, ATCC 12600. The resulting amplified material was resolved by electrophoresis through 5% polyacrylamide containing 50% urea and 1× TBE and visualized by autoradiography. Size markers are the 1 kb ladder from BRL/Gibco.

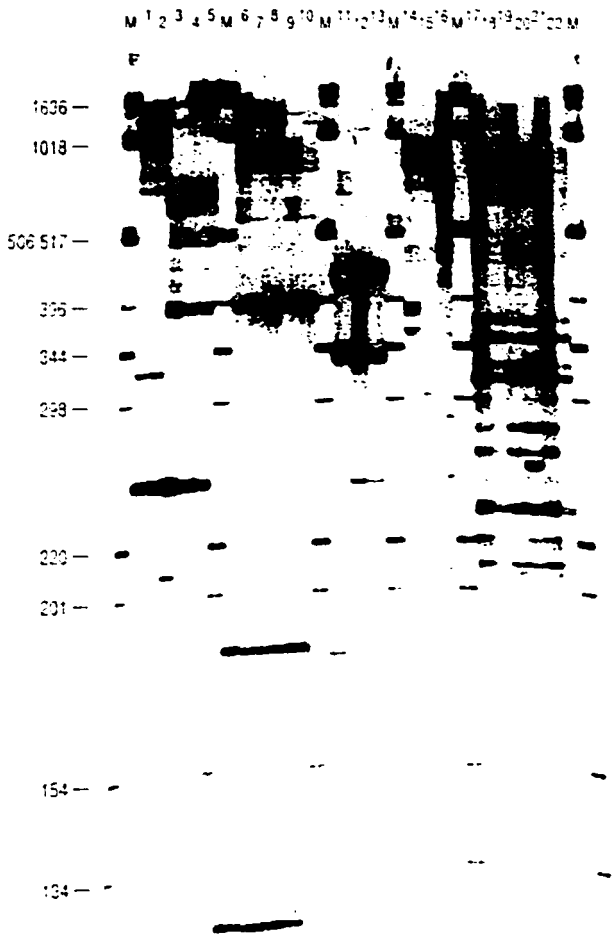

Figure 4. Genomic fingerprints of several strains from five species of *Staphylococcus*. AP-PCR patterns are characteristic of the species. Fingerprints of seventeen isolates representing four different species of *Staphylococcus* were fingerprinted by AP-PCR with the Kpn-R primer and the standard protocol with 16 ng of template per reaction. The resulting amplified material was resolved by electrophoresis through 5% polyacrylamide containing 50% urea and 1 × TFE and visualized by autoradiography. Size markers are the 1 kb ladder from BRL Gibco. The species fingerprinted were lane 1, *S. haemolyticus* CC 12J2; lane 2, PAY 9F2; lane 3, AW 263; lane 4, MID 563; lane 5, ATCC 29970, lane 6, *S. warneri* CPB 10E2; lane 7, GAD 473; lane 8, MCY 3E6; lane 9, PBNZP 4D3; lane 10, LED 355; lane 11, *S. hominis* ATCC 27844; lane 12, ATCC 27846; lane 13, Fu1; lane 14, *S. cohnii* JL 143; lane 15, CM 89; lane 16, SS 521; lane 17, *S. aureus* ISP-8; lane 18, ATCC 8432; lane 19, ATCC 15564; lane 20, ATCC 6538; lane 21, Sau 3A; lane 22, ATCC 12600. Size markers are the 1 kb ladder from BRL/Gibco.

6 – 10) give an almost identical pattern of AP-PCR products. However, the *S. haemolyticus* group consists of two distinct patterns that share only one AP-PCR product and have at least three prominent products that are not shared (lanes 1 – 5). The differences in products were dramatic and are presumably the result of a large amount of sequence difference between strains. The most likely possibility seems to be that the *S. haemolyticus* species has two distinct 'sub-species'. It is interesting to note that one 'sub-species' AP-PCR pattern is produced by *S. haemolyticus* strains (AW 263, ATCC 29970 and PAY 9F2) that live on the closely related primate species human and chimpanzee, the other strains of *S. haemolyticus* (MID 563 and CC 12J2) are from Mangabey and Lemur (Kloos, unpublished).

The utility of the method was further demonstrated when AP-PCR revealed the incorrect classification of a strain. Using AP-PCR, strains that are misassigned are very rapidly uncovered.

The AP-PCR products are species or sub-species specific and not conserved even between relatively closely related species, such as *S. haemolyticus*, *S. warneri*, and *S. hominis*. Nevertheless, a few AP-PCR products may be shared between species. For instance, the product at about 400 base pairs in *S. warneri* may be the same as in the *S. haemolyticus* strains PAY9F2, AW263 and ATCC29970, however, we cannot exclude the possibility of coincident migration.

It should be noted that occasionally a plasmid may contribute one of the prominent AP-PCR products. There are small plasmids in some of the *Staphylococcus* strains. The best way to avoid being confused by the lateral transfer of plasmids between unrelated strains is to perform separate AP-PCR reactions with a few different primers (data not shown). Small plasmids, because of their low sequence complexity, are extremely unlikely to contribute to the AP-PCR patterns of most arbitrary selected primers.

The AP-PCR method can, in principle, be applied to detect polymorphisms in a wide variety of organisms using a variety of different primers. To illustrate this, we examined several *Streptococcus* strains and a plant, rice, by amplification using the twenty base pBS reverse sequencing primer and genomic DNA. Figure 5 shows the polymorphic fingerprints that are produced for these organisms. For instance, *Streptococcus pyogenes* strains (genome size 2 – 3 megabases) have some AP-PCR products in common, whereas, others are shared by only some strains and yet others are unique to the strain. Consistent with our results in *Staphylococcus*, the one strain from another species, *E. faecalis*, (Figure 5, lane b11) shares no common PCR products with *S. pyogenes* strains. We are currently constructing a phylogenetic tree for *S. pyogenes* using data accumulated from AP-PCR with three separate primers (manuscript in prep.).

Rice, despite having a genome size of about 700 – 1000 megabases (17,18,19), gave a simple AP-PCR pattern (with a 48°C low stringency step) which included a mix of species-specific and strain-specific products. Strain specific polymorphisms can, in principle, be used as markers for genetic mapping. Experiments with an assortment of primers and with the maize and human (each about 3,000 megabases) genomes (data not shown) indicate that AP-PCR will work with most species and most primers.

Selection of primers

AP-PCR for species level identification of strains does not require any particular primer sequence. Investigators may wish to choose a standard set of primers so that data can be compared between labs. Primers that are already in extensive use seem to be the logical choice. We propose the 20 base pair sequencing primers, TTATGTAAAACGACGGCCAGT (Universal M13 – 20), GGAAACAGCTATGACCATG, (M13 reverse sequencing), GTAATACGACTCACTATAG (T7), and GCAATTAACCCTCACTAAAG (T3). These primers have been very successful in our subsequent AP-PCR studies. These primers will also have other unrelated uses in most labs: for sequencing projects and for the specific PCR of clones as labelled probes for Southern blots.

Lysis by boiling

For bacteria, the data presented here involved the purification of DNA by cell wall lysis, proteinase K digestion and phenol

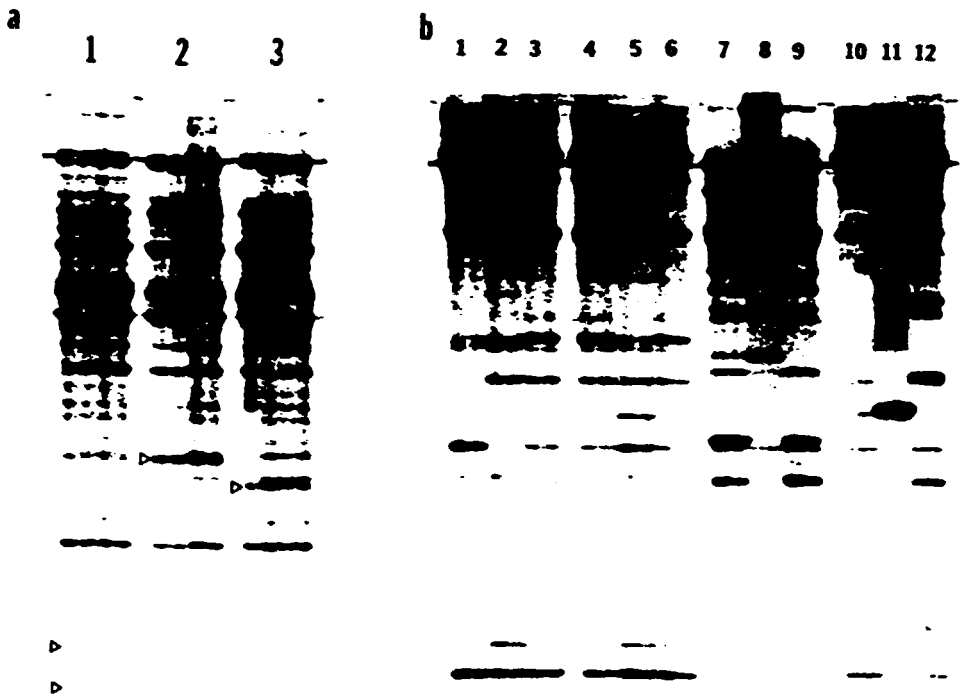

Figure 5. Genomic fingerprints of rice and *Streptococcus* strains: AP-PCR patterns can be generated for any species. Fingerprints of three inbred strains of rice and eleven strains of *Streptococcus* were fingerprinted by AP-PCR with the standard protocol and the pBS reverse sequencing primer. The low stringency annealing step was performed at 48°C for rice and at 40°C for *Streptococcus*. In the case of rice a serial dilution of 51 ng, 12 ng, 3 ng and 0.8 ng of genomic template DNA is presented for each strain. In the case of *Streptococcus*, 19 ng and 5 ng of genomic template were used. The resulting amplified material was resolved by electrophoresis through 5% polyacrylamide containing 50% urea and 1 × TBE and visualized by autoradiography. Size markers are the 1 kb ladder from BRL/Gibco. The strains fingerprinted were: Lanes a1, *O. sativa* ssp. *indica* IR54; lanes a2, ssp. *japonica* Caisorse 76; lanes a3, Lemont. Lanes b1, *S. pyogenes* D471; lanes b2, TI/195/2; lanes b3, 40RS15; lanes b4, 52RS15; lanes b5, 47RS15; lanes b6, 55RS15; lanes b7, 1/E9; lanes b8 T28/51/4; lanes b9, K58 Hg; lanes b10, SM6; lanes b11, *E. faecalis* OGI X; lanes b12, *S. pyogenes* UAB 092. Size markers are the 1 kb ladder from BRL/Gibco.

extraction. We have found that equivalent data can be generated by a much simpler DNA preparation in which the cells from a colony on a petri dish are simply boiled, the debris pelleted and the supernatant diluted for AP-PCR (data not shown). This allows the time from petri dish to strain identification to be reduced to 36 hours.

DISCUSSION

The classification of bacterial species or strains is an area of active research in population biology and medicine. Recent progress has included the use of oligonucleotides for the direct staining of cells using fluorescent probes to species or strain specific genes (eg 6,7) and the Polymerase Chain Reaction (PCR) (8). This latter method may revolutionize epidemiology and population biology. Specific PCR primer pairs can be used to identify genes characteristic of a particular species or even strain. PCR also obviates the need for cloning in order to compare the sequences of genes from related organisms (eg 10), allowing the very rapid construction of DNA sequence based phylogenies. For epidemiological purposes, specific primers to informative pathogenic features can be used in conjunction with PCR to assign identity.

While fluorescent probes and PCR are both very powerful methods, conventional primers for PCR and oligos for *in situ* hybridization require sequence information from the relevant genes and must be custom built for each location within the genome of a species or strain. We have developed an alternative method, arbitrarily primed PCR (AP-PCR), which is very rapid and simple and generates a fingerprint of PCR products. AP-PCR does not require a particular set of primers. Instead, this method uses primers chosen without regard to the sequence of the genome to be fingerprinted. Thus, AP-PCR requires no prior knowledge of the molecular biology of the organisms to be investigated. Each primer gives a different pattern of AP-PCR products, each with the potential of detecting polymorphisms

between strains. Thus, the data produced allows the differentiation of even closely related strains of the same species. In this respect it is similar to isozyme studies (3,4).

We have defined a window of conditions for AP-PCR which results in a reproducible and effective method for preliminary identification of any strain. This method may be very useful when the particular aim is to survey a large number of individuals in a population, for epidemiological or population studies, for example. As a first application of the method we compared strains from various species of *Staphylococcus*, *Streptococcus*, and rice. We were able to generate specific patterns of PCR products for each strain and these were generally quite similar within species. However, the three *S. cohnii* strains were quite divergent, but recognizably related, consistent with previous data from DNA renaturation (11,12,13,14). Further, five *S. haemolyticus* isolates could be grouped into two distinct AP-PCR patterns, indicating that this species has a distinct population structure or two distantly related sub-species or perhaps even species.

It is possible to extend the method to other species. Using similar conditions and various primers, we have generated discrete fingerprints from genomes 50,000 to 3,000,000,000 base pairs in size, including the genomes of viruses, humans and plants (including the rice results shown). We believe a characteristic pattern could be obtained for any genome. The method would then be useful in breeding programs, genetic mapping, population genetics or epidemiology.

It was initially surprising to us that arbitrary primers would give discrete patterns in low stringency PCR. However, it should be noted that when Wesley et al., dissected polytene chromosomes of drosophila and amplified with a primer that had a string of redundant bases at the 3' end (20), they unexpectedly observed discrete DNA products rather than amplification of the whole fragment. Our initial observations, presented here, indicate that a degenerate primer is unnecessary for the production of multiple discrete products in PCR.

In the Wesley et al. study (20), PCR products could be removed from the gel and used to hybridize back to genomic digests, clones or chromosomes. Since polymorphic AP-PCR bands can be used as probes, a link between the genetic and physical map can be envisioned.

For reproducible AP-PCR, many hundreds of template molecules were required under our present conditions. We are investigating conditions that may allow more efficient priming. In future experiments we would like to define conditions that generate common patterns between closely related species by making the AP-PCR less sensitive to sequence divergence. Conversely, we are attempting to develop conditions that will increase the ability of AP-PCR to detect differences in DNA sequence and, thereby, distinguish strains within a species. We are currently experimenting with shorter oligonucleotides and different PCR conditions. A more variable AP-PCR pattern within a species would allow a more detailed picture of intra-specific population structure.

Williams et al. have independently shown that arbitrary primers, ten bases long, can be used to generate polymorphic genomic fingerprints by the polymerase chain reaction (21).

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DNA polymorphisms amplified by arbitrary primers are useful as genetic markers

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ABSTRACT

Molecular genetic maps are commonly constructed by analyzing the segregation of restriction fragment length polymorphisms (RFLPs) among the progeny of a sexual cross. Here we describe a new DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. These polymorphisms, simply detected as DNA segments which amplify from one parent but not the other, are inherited in a Mendelian fashion and can be used to construct genetic maps in a variety of species. We suggest that these polymorphisms be called RAPD markers, after Random Amplified Polymorphic DNA.

INTRODUCTION

Genetic maps comprising closely-spaced DNA markers are useful for genome analysis. DNA markers that are shown to be genetically linked to a trait of interest can be used for gene cloning, medical diagnostics, and for trait introgression in plant and animal breeding programs (1, 2). In many organisms, however, saturated genetic maps are not available. The DNA markers most commonly used are restriction fragment length polymorphisms (RFLP, 3). Anonymous low copy number genomic clones are frequently used to visualize polymorphisms. Detection of RFLPs by Southern blot hybridizations are laborious and incompatible with the high analytical throughput required for many applications (4). Other polymorphism assays (5) that are based on the polymerase chain reaction (PCR), require target DNA sequence information for the design of amplification primers. The time and cost of obtaining this sequence information is prohibitive for many large scale genetic mapping applications. Here we describe a simple process, distinct from the PCR process, which is based on the amplification of genomic DNA with single primers of arbitrary nucleotide sequence. These primers detect polymorphisms in the absence of specific nucleotide sequence information, and the polymorphisms function as genetic markers, and can be used to construct genetic maps.

MATERIALS AND METHODS

Primer synthesis

Oligodeoxynucleotide primers were synthesized by standard phosphoramidate chemistry on a DuPont Coder 300 DNA synthesizer. After removal of protecting groups in 30% ammonium hydroxide at 55° for 5 h, the samples were dried under vacuum, dissolved in 200 μl of water, and purified by gel filtration on Sephadex G25 (NAP-5 disposable columns, Pharmacia).

Sources of genomic DNA

Human DNA samples from anonymous individuals were obtained from Drs. John Gilbert and Allen Roses of Duke Medical Center, Duke University. Soybean DNA was isolated (6) from the inbred cultivars *Glycine max* variety Bonus, and *Glycine soja* accession PI 81762, and from 88 F_2 individuals segregating from a cross of these two parents obtained from Dr. Theodore Hymowitz, University of Illinois. Corn DNA was isolated (7) from the *Zea mays* lines CM37 and T232 obtained from Dr. Ben Burr, Brookhaven National Laboratory. *Neurospora crassa* DNA samples were isolated from the strains Oak Ridge FGSC 4488 and Mauriceville FGSC 2225 (8), obtained from Dr. R.L. Metzenberg, University of Wisconsin. Bacterial DNA samples were obtained from Dr. John Webster, DuPont Co.

Amplification conditions

Amplification reactions were performed in volumes of 25 μl containing 10mM Tris-Cl, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dCTP, dGTP and TTP (Pharmacia), 0.2 μM primer, 25 ng of genomic DNA, and 0.5 unit of *Taq* DNA polymerase (Perkin Elmer Cetus). Amplification was performed in a Perkin Elmer Cetus DNA Thermal Cycler programmed for 45 cycles of 1 min at 94°, 1 min at 36°, 2 min at 72°, using the fastest available transitions between each temperature. Amplification products were analyzed by electrophoresis in 1.4% agarose gels and detected by staining with ethidium bromide.

Annealing temperatures above 40° in the thermal cycling profile prevented amplification by many of the 10 base

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oligonucleotides tested (data not shown). With some combinations of primer and genomic DNA template a non-discrete size range of amplification products, appearing as a 'smear' as visualized on a gel, could be converted to discretely sized bands by reducing the concentration of either the polymerase or the genomic DNA.

Genetic analysis of amplified DNA polymorphisms.

Amplified polymorphic DNA fragments and RFLPs were mapped in the context of a 436 marker RFLP map of soybean (manuscript in preparation), a 110 marker RFLP map of corn (9), or an 80 marker RFLP map of *N. crassa* (10), by scoring marker segregation in the respective populations used to create these maps. Multipoint maps and LOD scores were calculated using the Mapmaker program (11).

Use of amplified segments as RFLP probes

Polymorphic DNA segments AP3.1 and AP11a.1, amplified (as described above) from the soybeans Bonus and PI 81762 respectively, were resolved by electrophoresis in a 1.4% agarose gel and excised from the gel. A 5 μl slice of gel containing DNA was added to a 100 μl reaction mixture and was amplified under the conditions described above, using primers AP3 and AP11a, respectively. The re-amplified DNA samples were labeled with ^{32}P (BRL Random Primers DNA Labeling System, Life Technologies, Inc.) and used as hybridization probes (12) to detect RFLPs with the restriction enzymes *Pst* I for AP3.1 and *Eco*RI for AP11a.1. These RFLPs were mapped in soybean as described above.

RESULTS

Polymorphism and sequence specificity.

Figure 1A shows the results of an experiment in which single primers were used to amplify segments of genomic DNA from humans, corn, soybean, and *N.crassa*. Primers were designed in the absence of any nucleotide sequence information for the species tested. The nucleotide sequence of each primer was chosen within the constraints that the primer was 9 or 10 nucleotides in length, between 50 and 80% G+C in composition, and contained no palindromic sequences (4). Several DNA segments were amplified in each sample. While most of these segments were common to both individuals of a given species, some segments were amplified from one individual but not the other. For example, a 1.4-kb DNA segment was amplified by primer AP9 from one human sample but not from the other (Figure 1A, lanes 1 and 2, respectively). At least one such polymorphism was apparent in each of the species examined. To confirm that the observed bands were amplified genomic DNA, and not primer artefacts (13), genomic DNA was omitted from control reactions for each primer. No amplification products were seen for any primer except for primer AP12h (Fig 1, lane 9); this artefact is not significant, however, since it is not produced when genomic DNA is included in the reaction mixture (Fig 1, lanes 7–8). These results show that single primers of arbitrary sequence can be used to amplify genomic DNA segments, and that polymorphisms can be detected between the amplification products of different individuals. Several samples of bacterial DNA were also assayed to determine whether these short primers could be used to amplify DNA segments from small genomes. The results shown in Figure 1B indicate that genomes as small as *E. coli* (4×10^3 kbp) will support amplification, and that bacteria can be distinguished according to the banding patterns of their DNA on an agarose gel.

A set of eleven related oligonucleotide 10-mers was synthesized to determine the contribution of each nucleotide to the specificity of the amplification reaction. Each primer differed from the oligonucleotide 10-mer, 5'-TGGTCACTGA, by substitution of a single nucleotide at a successive position in the sequence. The G+C content of all primers was maintained at 50%, and each primer was used to amplify DNA from two different species of soybean, *Glycine max* and *Glycine soja*. Following amplification, DNA samples were analyzed by agarose gel electrophoresis (Figure 2). Several DNA segments were amplified in each sample, and polymorphisms were apparent for many of the primers (e.g., a 0.65-kb band is present in Figure 2 lane 21, but absent in lane 22). In this experiment, most of the nucleotide substitutions in the primer caused a complete change in the pattern of amplified DNA as compared to the original primer, and in

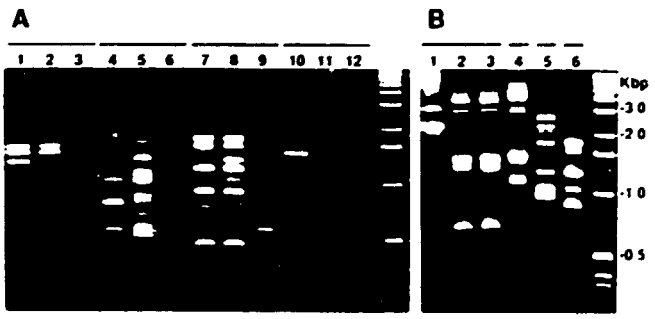

Figure 1a. Amplification of eukaryotic DNA. DNA was amplified from a variety of species using primers of arbitrary nucleotide sequence (Materials and Methods). Amplification products were resolved by electrophoresis in a 1.4% agarose gel which was stained with ethidium bromide and photographed. Molecular weight markers (kilobase pairs, kbp) are as indicated. Lane 1 and 2, human DNA Hu2 and Hu3, respectively, amplified with primer 5'-ACGGTACACT. Lane 4 and 5, corn CM37 and T232, respectively, amplified with GCAAGTAGCT. Lanes 7 and 8, soybean *G. max* and *G. soja*, respectively, amplified with CGGCCCCTGT. Lanes 10 and 11, *N. crassa* Oakridge and Mauriceville, respectively, amplified with CACATGCTTC. Genomic DNA was omitted in control reactions (lanes 3, 6, 9, and 12) to determine whether any of the bands seen with genomic DNA are actually primer artefacts. **Figure 1b.** Amplification of prokaryotic DNA. Lanes 1–3, *Escherichia coli* (strains 037, 641, 642, respectively). Lane 4, *Listeria monocytogenes* (strain 681). Lane 5, *Staphylococcus aureus* (strain 684). Lane 6, *Salmonella typhimurium* (strain 706). All genomic DNA samples were amplified with primer 5' TCACGATGCA.

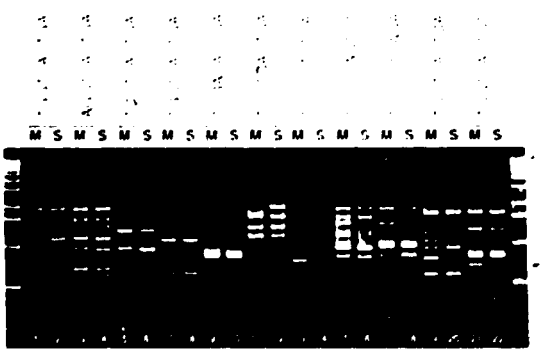

Figure 2. The effect of nucleotide substitutions in the primer on amplification. Genomic DNA from soybean *G. max* (M) and *G. soja* (S) was amplified with the indicated primers. Squares indicate nucleotide substitutions relative to the primer 5' TGGTCACTGA (lanes 1–2). The arrow points to a polymorphic 0.65 kb band (see Results).

many cases revealed new polymorphisms. For example, the pattern obtained with the primer 5'-TGGTCACTGA (Figure 1, lanes 1 and 2) differs from that carying the G-to-C substitution 5'-TGCTCACTGA (Figure 2, lanes 7 and 8). Less dramatic differences in patterns are seen for substitutions at the 5'-most position (Figure 2, lanes 1–4). These results show that an oligonucleotide 10-mer can act as a primer in the DNA amplification reaction, that polymorphisms can be detected among the amplification products, and that nucleotide changes in the primer (and by inference, the template) determine whether a given DNA segment will be amplified.

Genetic segregation

To learn whether these amplification polymorphisms are useful as genetic markers, and to assess whether the assay is reproducible, 11 polymorphisms generated with various primers were mapped in soybean, using 66 segregating F2 individuals. Each polymorphism was scored as a dominant marker and correlated with the segregation data for 430 soybean RFLP markers, derived from the same 66 individuals (manuscript in preparation). Segregation of the AP11a.1 polymorphism is shown for 16 of these F2 individuals in Fig 3A. Analysis of the data (11) indicates that AP11a.1 maps to linkage group 5 at the position shown in Figure 4. The probability that the marker is at the position indicated is $10^{16.8}$ times greater than the probability that AP11a.1 is unlinked, indicating the certainty of the map assignment. This also shows that the assay is robust, permitting reliable scoring of a polymorphism in a segregating population. The map positions and probabilities for 10 other markers are indicated in Figure 4. Primers AP4c and AP10b each revealed two different and unlinked polymorphisms (markers AP4c.1 and AP4c.2, and AP10b.1 and AP10b.2, respectively), demonstrating that single primers can be used to amplify DNA from dispersed polymorphic loci. The RAPD markers mapped (Figure 4) increased the saturation of the soybean map by filling in some gaps (for example markers AP4 and AP12h1 in LG27), and by extending the map in the telomeric direction (markers AP5a2 in LG21b, AP4c3 in LG 3a, see Figure 4).

Many different random sequence primers were used to assess the quality and frequency of polymorphisms in corn, soybean, and *N. crassa* (data not shown). This was accomplished by determining what percentage of primers could be used to detect polymorphisms that could be mapped with confidence (ie. LOD scores in support of linkage greater that 4.0). The frequencies of polymorphism detection was 1 per primer for corn (number of tested primers, p = 34), 0.5 per primer for soybean (p = 45), and 2.5 per primer for *N. crassa* (p = 88).

Comparison to RFLPs

RFLP analysis was used to confirm the map positions of RAPD markers. Several polymorphic amplified DNA segments that had been previously mapped were excised from an agarose gel, labeled with ^{32}P, and used as hybridization probes to detect RFLPs. Two RFLPs were found using the amplified DNA segments AP3.1 and AP11a.1. Co-segregation of the RFLP

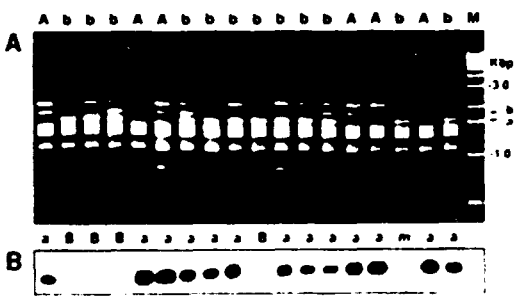

Figure 3. panel A. Segregation of an amplification polymorphism in soybean. Genomic DNA samples from 66 F2 progeny were amplified with primer AP11a (5'-ACCTCGAGCACTGTCT). Amplification products from the parents *G. max*, and *G. soja* (lanes 1 and 2, respectively), and sixteen F2 individuals are shown. Arrow 'a' points to a segregating polymorphic band that is clear to score, while arrow 'b' points to a band that appears to be polymorphic in the parents, but cannot be confidently scored among the progeny (see Discussion). **Panel B.** Segregation of a RFLP detected by a polymorphic amplified DNA probe. The AP11a.1 polymorphism amplified in *G. soja* (Figure 3a, lane 2 band 'a') was used as a hybridization probe to detect a *Bcl II* RFLP. Panel B shows the hybridization of this probe to a Southern blot of *Bcl II* digested genomic DNA from the same individuals shown in panel A. Segregation scores for the amplification polymorphism and the RFLP are shown above panel A and B, respectively. Scores are interpreted in the following way: 'A' = genotype of parent A (lane 1), 'B' = genotype of parent B (lane 2), 'a' = either A or a heterozygote, 'b' = either B or a heterozygote, 'm' = missing data. 'M' identifies the molecular weight markers.

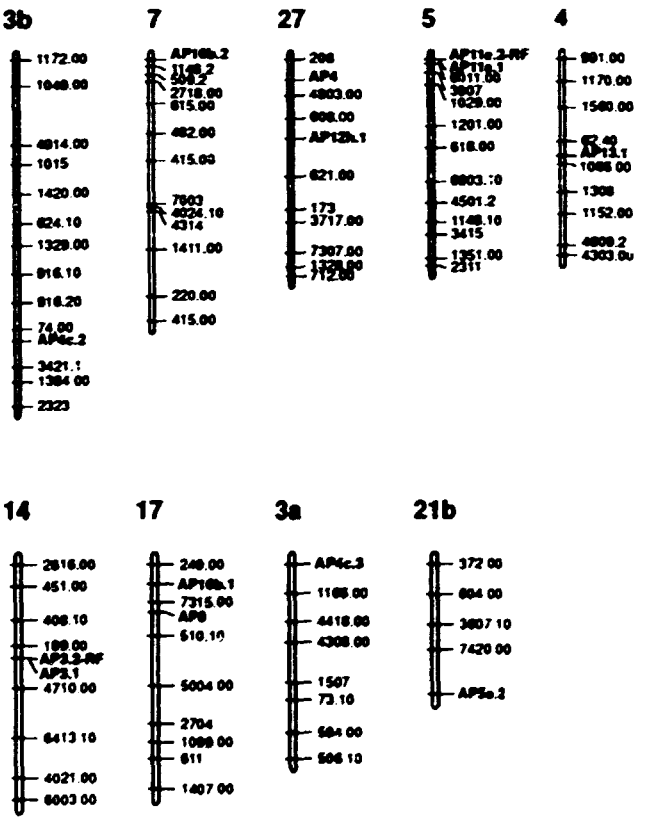

Figure 4. Genetic map of polymorphic loci detected by amplification. Amplification polymorphisms (bold type) and RFLPs identified using probes derived from amplified polymorphic DNA segments (bold type, suffix '-RF') were mapped in the soybean genome on the indicated linkage groups relative to classical RFLP markers (plain type) as described in Results. For each marker, the probability of the indicated map position is as follows: AP3.1 is $10^{10.1}$, AP3.2 RF, $10^{12.6}$, AP4, $10^{6.8}$, AP4c 2, $10^{11.1}$, AP4c 3, $10^{6.3}$, AP5a 2, $10^{3.7}$, AP8, $10^{15.4}$, AP10b 1, $10^{20.4}$, AP10b 2, $10^{5.4}$, AP11a 1, $10^{16.8}$, AP11a 2 RF, $10^{16.8}$, AP12h, $10^{4.3}$, AP13, $10^{12.8}$. Primer sequences are AP3 (5' TCGTAGCCAA), AP4 (TCACGATGCA), AP4c (TCTCCGATGCA), AP5a (CTGTTGCTAC), AP8 (TGGTCACTGA), AP10b (GGAAGTAGTG), AP11a (ACCTCGAGCACTGT CT), AP12h (CGGCCCCTGT), AP13 (ATTGCGTCCA).

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High output genetic mapping of polyploids using PCR-generated markers

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Summary. The polymerase chain reaction (PCR) with arbitrarily selected primers has been established as an efficient method to generate fingerprints that are useful in genetic mapping and genomic fingerprinting. To further increase the productivity of mapping and fingerprinting efforts, we have altered existing protocols to include the use of the Stoffel fragment, which is derived from genetically engineered *Taq* polymerase. We also optimized the thermal profile of the reaction to increase the number of useful primers. In mapping of the genome of *Saccharum spontaneum* 'SES 208', a polyploid wild relative of sugarcane, these modifications allowed for an increase of 30% in the number of loci screened per primer, and an 80% increase in the number of polymorphisms per primer. Furthermore, the enzyme cost per reaction was decreased approximately 1.6-fold. Finally, there was an increase from about 70% to about 97% in the number of primers that were useful (i.e., gave a reproducible fingerprint) using our protocol. We have placed some of these markers into linkage groups.

Key words: AP-PCR – RAPD – Sugarcane – *Saccharum* – Stoffel fragment – Genetic mapping – MapMaker – Molecular markers

Introduction

The polymerase chain reaction (Saiki et al. 1985) has become an increasingly important tool in molecular biology (for a recent review see Erlich et al. 1991). The applications of the PCR to problems in molecular biology and genetics have increased dramatically over the last few years. One of these new applications combines the PCR with primers of arbitrary sequence to amplify a 'fingerprint' of different loci from any genome. This method was independently and simultaneously developed by Welsh and McClelland (1990) and Williams et al. (1990) and has been called AP-PCR or RAPDs, respectively. For any primer, the resulting pattern of amplified genomic fragments is highly reproducible and can be used as a 'fingerprint' for: (1) varietal identification and parentage determination (Welsh et al. 1991a, 1992); (2) genetic mapping, because they are inherited in a Mendelian manner (Williams et al. 1990; Welsh et al. 1991b), and (3) for generating phylogenetic trees, especially at the intraspecific level (Welsh et al. 1992).

The AP-PCR technique has allowed the rapid construction of genetic linkage maps. As an example of the high throughput of the method, Sederoff and co-workers recently constructed a 191-marker map in loblolly pine in just 2 months (Neale and Sederoff 1991). Similar work using RFLPs would have taken at least ten-fold longer to complete. In addition, many species of pine trees have very large genomes, thus it is difficult to load enough DNA on the gel to allow for the detection of single, or low-copy, sequences using Southern hybridization. So, the AP-PCR has extended the number of species that are amenable to genetic mapping with molecular markers, in addition to offering increased mapping output in every other species.

We are constructing a genetic map of the genome of a 'wild' form of sugarcane, *Saccharum spontaneum*, using AP-PCR. Because sugarcane is a complex polyploid plant with variable ploidy, the only markers

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that can be mapped are those present in one parent, absent in the other, and that segregate 1:1 in the progeny, such as in single-dose restriction fragments (Wu et al. 1992). Polymorphic fragments that have other segregation ratios cannot be mapped because there is no statistical method for determining their linkages. Therefore, high numbers of polymorphic fragments are required to saturate the map with those that segregate 1:1. Because of high output and the requirement for single-dose markers, AP-PCR is the method of choice for genetic mapping in polyploids such as sugarcane. However, it would be useful to optimize protocols to maximize the number of loci screened in each experiment, with the intent of increasing the number of polymorphisms detected with each primer.

Materials and methods

Plant materials

S. spontaneum 'SES 208' is a 2n = 64 form of this species. Fitch and Moore (1983) derived haploids from SES 208 by tissue culture of anthers. *S. spontaneum* 'ADP 85-0068' is one such a haploid that underwent spontaneous doubling of chromosomes during regeneration (Paul Moore, USDA-ARS Aeia, Hawaii, personal communication). A sexual cross was made between SES 208 and ADP 85-0068, from which over 100 progeny were derived at the Hawaiian Sugarcane Planters' Association (Aeia, Hawaii). This constitutes our mapping population. Total genomic DNAs from *S. spontaneum* SES 208, ADP 85-0068, and the derived progeny were extracted according to Honeycutt et al. (1992). DNA concentrations were determined by averaging three spectrophotometric readings at 260 nm. DNA concentrations were standardized at 50 ng μl^{-1} in H_2O and finally checked on an agarose gel, after which final corrections were made as necessary.

DNA amplification protocols

All amplifications were done in 30-µl reaction volumes in a System 9600 cycler (Perkin-Elmer). Amplifications using *Taq* polymerase contained 1 U *Taq* polymerase (AmpliTaq, Perkin-Elmer), 30 ng template DNA, 0.1 mM of each dNTP (Pharmacia), 2.0 mM $MgCl_2$, 50 mM KCl, and 10 mM Tris-HCl, pH 8.3. Amplifications using the AmpliTaq Stoffel fragment (Perkin-Elmer) contained 2 U Stoffel fragment, 30 ng template DNA, 0.1 mM of each dNTP, 4.0 mM $MgCl_2$, 10 mM KCl, and 10 mM Tris-HCl, pH 8.3. Amplifications using the *Pfu* polymerase (Stratagene Cloning Systems) contained 1 U polymerase in 20 mM Tris-HCl (pH 8.2), 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 0.1% Triton X-100, and 100 µg ml^{-1} bovine serum albumin. Oligonucleotide primers (10-mers, Operon Technologies; 12-mers synthesized by Genosys) were used at 0.22 µM in all reactions.

Unless otherwise noted, the temperature profile to which all reactions were submitted was: 94 °C 3 min, followed by 40 cycles of 94 °C 1 min, 35 °C 1 min, then increase to 72 °C with a ramp of 0.41 °C extension for 7 min. Amplification products were maintained at 12 °C until loaded onto a gel.

When amplification products were labelled for subsequent autoradiography, 1 µCi of α^{32}[P]dCTP (3,000 mCi mmol^{-1}, NEN) was included in each reaction. Unless otherwise noted, agarose gels were composed of 1.4% (full-length gels) or 2% (half-length) LE agarose (FMC) dissolved in 0.5x or 1x TBE (Maniatis et al. 1982) and were run in a model HRH gel box (International Biotechnologies Incorporated). Full-length agarose gels were run for 1,300 V × h; half-length gels were run for 550 V × h. Optimal resolution of products was obtained using voltage gradients of 5–8 V cm^{-1}. Denaturing polyacrylamide-gel electrophoresis was done in a sequencing apparatus (Hoefer Scientific Instruments) using gels composed of 4% polyacrylamide, 8 M urea in 1x TBE. Polyacrylamide gels were 0.35 mm thick and were run at a constant power (50 W) for 3.5–4 h, after which autoradiography was done for 12–24 h without the need for gel drying. For agarose gels, 15–20 µl of reaction products were loaded into each well. For polyacrylamide gels, 4 µl of reaction products were added to 14 µl of stop dye containing 50% formamide, the products were denatured at 85 °C for 5 min, immediately put on ice, then 2 µl were loaded into each well.

Automated reaction preparation

Besides manually preparing and loading amplification products, we also used a Tecan RSP 5032 (Tecan SLT) two-arm robot liquid handling station to prepare and load reactions. In this case, the robot first took 10 µl aliquots of template (30 ng total) from an array and loaded them into the System 9600 array. Then, it took 20 µl from a master reaction mix that was composed of the previously described components at 1.5x concentration. After thermal cycling was complete, the tubes were uncapped and the robot loaded the samples onto agarose gels. Programs were also written to allocate primers into tubes for screening against SES 208 and ADP 85-0068 DNAs. For primer-pair amplifications (Welsh and McClelland 1991), the robot assembled arrays containing equimolar concentrations of two primers, then two reaction mixes containing the appropriate template DNAs were added.

Genetic mapping of AP-PCR single-dose polymorphisms

To determine the linkage relationships of markers that were detected by our approach, we ran MapMaker (Lander et al. 1987) using a minimum L.O.D. score of 4.00 and a maximum θ value of 0.25.

Results and discussion

In a pilot study we compared the output of AP-PCR (Welsh and McClelland, 1990) and RAPD (Williams et al. 1990) protocols, as originally reported by the authors, except that the temperature profile to which the reactions were submitted was altered as described in Material and methods. Using the temperature profile and conditions reported by Williams et al. (1990), nearly one-half of the 10-mers we screened against the mapping parents failed to give products; similar results were observed by Klein-Lankhorst et al. (1991) in genetic mapping of tomato using AP-PCR. This may be caused by differences in the temperature profiles of different cyclers. We fixed primer length at ten bases and tested various modifications to the temperature profile using 20 different primers. Data for optimization of the temperature profile were acquired using recombinant AmpliTaq polymerase. Results using

native *Taq* polymerase were not as reliable, and we therefore discontinued its use. In summary, temperature profile optimization showed that: (1) annealing temperatures of 15 °C, 30 °C, 35 °C, and 40 °C did not cause significant changes in the fingerprint, in agreement with Welsh and McClelland (1990), although some fragments were lost or gained at the temperature extremes; (2) there was a major effect of ramp time from the annealing step to the extension step, in agreement with Klein-Lankhorst et al. (1991), i.e., the longer it takes for the temperature to change from 35 °C to 72 °C, the more primers give reproducible, robust fingerprints; (3) extension times longer than 90 s did not significantly improve resulting fingerprints; (4) 40 cycles were sufficient for good results in 30 µl reactions; and (5) the fingerprints were extremely reliable (we have repeated some primers as many as ten times on two genotypes of sugarcane without observing significant differences).

Primer length and concentration are the most striking differences between the original AP-PCR and RAPD protocols. We fixed primer length at 12 and 20 bases and studied the effect of a 50-fold greater primer concentration in the initial steps of AP-PCR (10 µM) when compared to the RAPD (0.2 µM) protocol. Representative results of a primer titration for 12-mers are shown in Fig. 1. Decreasing the primer concentration caused a decrease in the number of products observed, and the fragments lost as primer concentration decreases were mostly in the lower molecular weight range. This has also been observed with longer primers (John Welsh, CIBR, personal communication; Sobral, unpublished data). In addition, fingerprints produced at lower primer concentrations were more erratic, in that more failed lanes were observed. In general, when using *Taq* or *Pfu* polymerases, primer concentrations of 0.10 µM caused significant failures, and some primers did not work reliably at this concentration.

More amplification fragments might be expected to yield more polymorphic fragments, if the ratio between total number of products and number of polymorphic products is constant for a given pair of DNA templates. Therefore, we might expect that having more fragments would increase the output of polymorphisms in mapping experiments, or the number of informative characters for phylogenetic experiments. Table 1, which summarizes the results of the pilot study, shows that this was not the case. Although more fragments were observed at higher primer concentrations, the number of polymorphisms per primer was approximately the same for both protocols despite the much larger number of fragments observed using higher primer concentrations and autoradiography.

We do not understand why the extra fragments generated using higher primer concentration, as in the original AP-PCR protocol, do not yield higher numbers of polymorphisms per primer. This result suggests that these fragments are products that are not sensitive to sequence variation in the template genome, which is rather bewildering. If these additional fragments were strictly a product of sequence variation of the genome being surveyed, then it would be expected that the number of polymorphisms per primer would be directly related to the total number of fragments amplified. Some of the additional fragments observed in the original AP-PCR setup are a direct consequence

Fig. 1. Effect of primer concentration on the AP-PCR fingerprint. A 12-mer was used at two different concentrations on three different concentrations of template DNA (*S. spontaneum* 'ADP85-0068'): 1, 3, and 5 ng µl^{-1}. Titration of primer to 0.2 µM caused all but the most intense amplification products to disappear (data not shown). PCR conditions are described in Materials and methods

Table 1. Comparative output of AP-PCR and RAPD protocols

Item	AP-PCR	RAPD
Number of primers tested	18	41
Number of loci screened	482	355
Number of informative primers	12	30
Number of polymorphisms	31	67
Single-dose polymorphisms	16[a]	15[b]
Average polymorphisms per primer	2.58	2.23
Average single-dose polymorphisms per primer	1.33	1.88

[a] The number of single-dose polymorphisms was derived from the 12 informative primers, each tested on 35 progeny from the mapping population

[b] The number of single-dose polymorphisms was derived from the eight most informative primers, each tested on 22 progeny from the mapping population

of the higher resolution and sensitivity of the gel and detection systems, but other fragments are clearly a product of the higher primer concentrations (Fig. 1). It is possible that some of these smaller products may be caused by interactions that do not reflect the level of polymorphism of the genome.

What was desired, then, was a protocol that would increase the total number of loci screened and the number of polymorphisms per primer. To increase the number of polymorphisms per primer, we tested alternate thermostable polymerases using the optimized temperature profile. We also tested pairs of thermostable polymerases in the same reaction. The Stoffel fragment is a 61 kDa modified form of recombinant AmpliTaq polymerase from which the 289 N-terminal amino acids have been deleted such that it lacks 5′–3′ exonuclease activity. The manufacturer claims that the Stoffel fragment is two-fold more thermostable than AmpliTaq and that it displays optimal activity over a broader range of Mg^{++} concentrations. *Pfu* polymerase is a thermostable polymerase from *Pyrococcus furiosis* that has 5′–3′ DNA polymerase activity as well as 3′–5′ exonuclease activity.

Reaction products obtained using the Stoffel fragment ranged from 0.1 to 1.5 kb in size, whereas the AmpliTaq and *Pfu* products generally ranged from 0.5 to 2.5 kb. This reduction in average size of products obtained with the Stoffel fragment might be expected because of the lower processivity of the enzyme (Erlich et al. 1991). In addition, the number of useful primers increased with the Stoffel fragment, in relation to both AmpliTaq and *Pfu*. We screened a total of 40 primers using AmpliTaq, 96 primers using *Pfu*, and 144 primers using the Stoffel fragment. Twelve of forty primers (30%) gave no products with AmpliTaq, 33 of 96 (34%) failed with *Pfu*, and 4 of 144 (2.8%) failed with the Stoffel fragment. DNA fragments amplified by the Stoffel fragment, being smaller, are more suited to being resolved on high-resolution polyacrylamide gels (with visualization using autoradiography or silver staining) than are AmpliTaq or *Pfu* products.

Because the products from Stoffel reactions and AmpliTaq or *Pfu* reactions were of different sizes, we tried using both Stoffel and *Pfu* or AmpliTaq in the same reaction to see whether all or most of the fragments amplified by each enzyme individually could be obtained from one reaction. Typical results for three primers are displayed in Fig. 2. Briefly, the use of Stoffel in conjunction with *Pfu* or AmpliTaq did not allow the amplification of all the products that each enzyme yields when used alone. We tried two different amounts of enzyme per reaction, though we kept the Stoffel: *Pfu* ratio constant at 2:1. The absolute amounts of enzyme influenced the resultant fingerprint: some polymorphic fragments were lost, others were gained, though the overall pattern was similar. The buffer used also affected the results. We used the Stoffel + *Pfu* combination in Stoffel buffer as well as in *Pfu* buffer, and some differences were observed. Given the results using combinations of thermostable polymerases, we do not think that mixing polymerases is a useful strategy.

We chose the ten primers that gave the most polymorphisms with AmpliTaq and the 20 primers that gave most polymorphisms with the Stoffel fragment and used them against the mapping population. With the Stoffel fragment, the average number of loci screened per primer increased by 30%, and, even more surprisingly, the average number of polymorphisms per primer increased by 80% (Table 2) relative to AmpliTaq. Note that four of the primers used to compile the data in Table 2 have been tested with both enzymes. Four primers, an average of 11 loci were screened and an average of 2.3 polymorphisms were observed if AmpliTaq was used, while averages of 15.8 loci screened and 3.8 polymorphic fragments were obtained using the Stoffel fragment in place of AmpliTaq. Figure 3 shows a comparison of the finger-

Table 2. Comparative mapping output using AmpliTaq and Stoffel fragment

Item	AmpliTaq	Stoffel
Number of primers tested	7	14
Number of loci screened	80	207
Number of polymorphisms observed[a]	14	57
Average number of loci per primer	11.4	14.8
Average number of polymorphisms per primer	2.0	3.6

[a] Polymorphisms were identified by comparing the fingerprints of SES 208 with those of ADP 85-0068, the other mapping parent

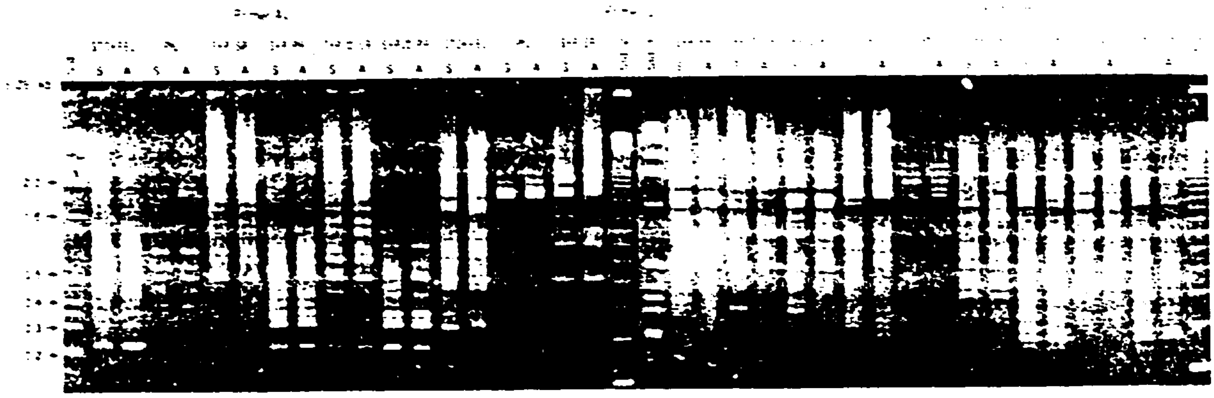

Fig. 2. Effect of different polymerases and buffers on AP-PCR fingerprints. Three 10-mers [illegible] and two template DNAs (mapping parents SES 208 and ADP85-0068) were used with either the Stoffel fragment alone [illegible] [illegible] DNA [illegible] BRL [illegible] DNA. Thermal cycling parameters and other information [illegible] Materials and methods

prints produced by AmpliTaq, *Pfu* and the Stoffel fragment using one representative primer. The majority of the products migrated to different positions on the gel, suggesting they represent different loci that are selected for amplification by the different enzymes. This has been a general observation when we have used the same primer with different polymerases. However, one amplified polymorphic DNA fragment that migrated to the same position on the gel when AmpliTaq and the Stoffel fragment were used. These polymorphisms mapped very close to each other (Fig. 4) and the slight discrepancy in their map positions is most likely due to differences in failed lanes.

To show that the single-dose AP-PCR polymorphisms could be used to construct a genetic linkage map, we selected some of the more informative primers and used them on the entire mapping population. In each PCR run, there were four repetitions each of the two parents, SES 208 and ADP85-0068, at template concentrations that covered a five-fold range. We only scored the progeny for polymorphisms that were reliably observed in all four repetitions. We discarded the 'sporadic' bands that were occassionally observed in some of the repetitions because these were usually influenced by template concentration. This approach allows the confidence level to be increased, especially because the progeny are only scored once for each polymorphism in mapping situations. The segregation data was used to run MapMaker and the resulting linkage groups are shown in Fig. 4. Approximately 70% of all polymorphisms present in SES 208 yet absent in ADP85-0068 were single-dose fragments. This number agrees with the ratio obtained using the RFLP approach in this same population (Burnquist 1991). However, RFLP single-dose fragments require 300-fold more DNA per lane, plus a 7–10 day exposure of autoradiograms, in addition to the normally long time spent on gel preparation, blotting, etc. (Burnquist 1991). Linkage was also detected between single-dose RFLPs (Burnquist 1991) and AP-PCR fragments. A high density AP-PCR map of this cross will be published elsewhere.

The reason for increased mapping output using the Stoffel fragment is unknown. However, Erlich et al. (1991) pointed out that genetically engineered variants of *Taq* polymerase might reveal properties that would be valuable to specific applications. It may be that the Stoffel fragment is more sensitive to mismatches between primer and template than are AmpliTaq or *Pfu* polymerases, although it would be expected that fewer fragments would be amplified if this were true. There are differences in Mg^{++} concentration of Stoffel reactions relative to AmpliTaq reactions, but we have also done Stoffel reactions in AmpliTaq and *Pfu* buffers, with lower Mg^{++} levels, and higher output with Stoffel was still observed.

Not only is the output using the Stoffel fragment and an optimized temperature profile nearly two-fold higher than that of reported protocols (Welsh and McClelland 1990; Williams et al. 1990), but the cost of data acquisition is significantly reduced because 1 U of AmpliTaq currently costs $0.62, whereas 2 U of Stoffel cost $0.39 (Perkin-Elmer catalog). Further savings can be obtained by reducing the volume of the reaction. Successful reactions have been done in 15-µl volumes (Ron Sederoff, North Carolina State, personal communication). Such a reduction in volume

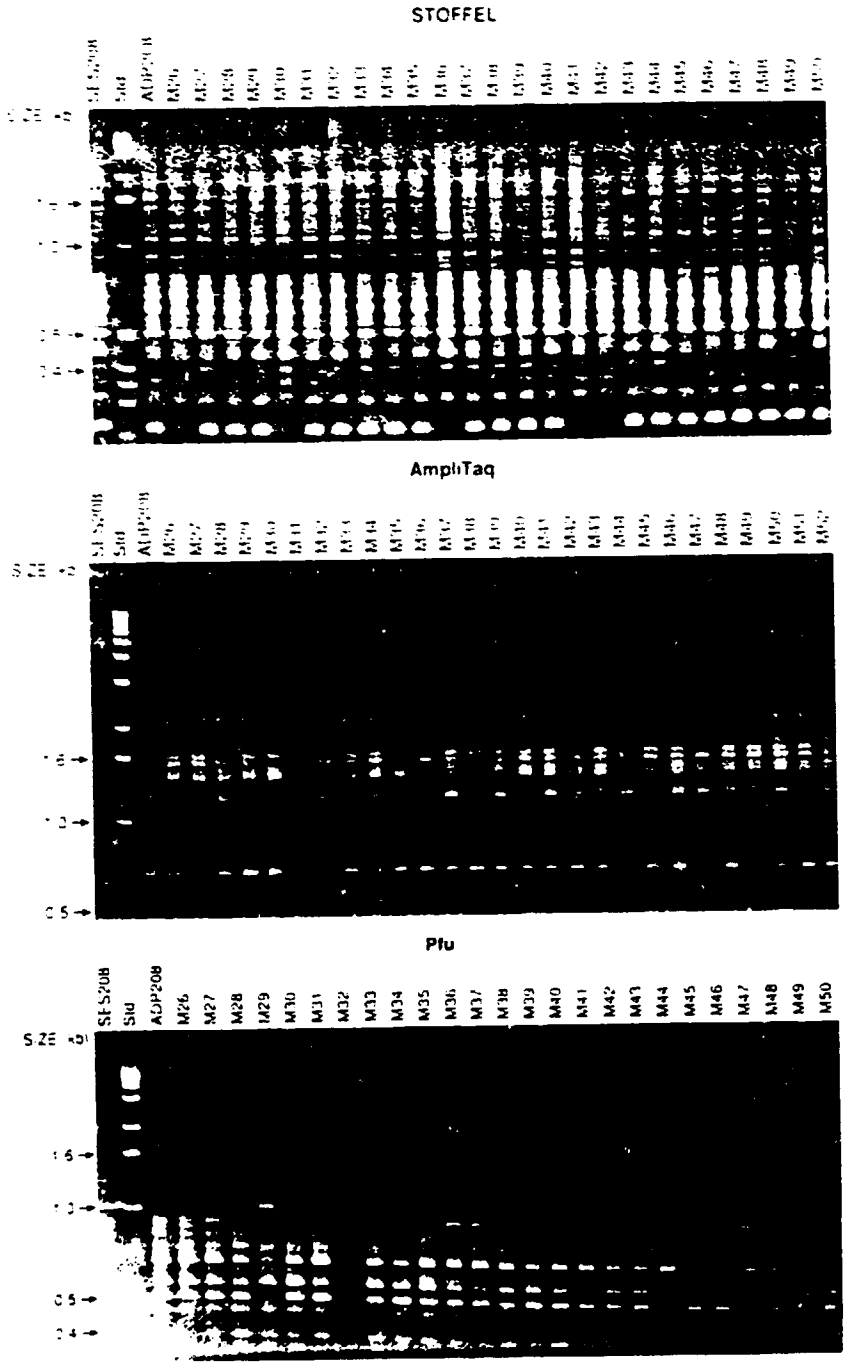

Fig. 3. Comparison of Stoffel, AmpliTaq, and *Pfu* polymerases using one 10-mer. Using the optimized thermal cycling profile and reaction conditions described in the text, primer A5 was used with all three polymerases and the entire mapping progeny; one of the four gel panels is shown here with the polymorphisms indicated by white asterisks to the right of the SFS 208 lane. In this case, the Stoffel fragment allowed the scoring of four polymorphisms, and AmpliTaq and *Pfu* each revealed one polymorphism. None of these polymorphisms have the same map location (Al-Janabi et al., submitted).

would lower the polymerase cost per reaction to approximately $0.17. In addition, the next generation of liquid handling robots will be capable of accurately handling even smaller volumes (David Juranas, Tecan SLT, personal communication).

Although we have increased the output of polymorphisms significantly, the bottleneck is rapidly becoming data acquisition. If data acquisition becomes automated, then using our protocol and a robot it would be possible to make a 200-marker genetic map for a diploid organism that had a similar level of polymorphism with less than 60 useful primers, without even using primers in pairwise combinations. In sugarcane, these 60 primers could be picked from screening

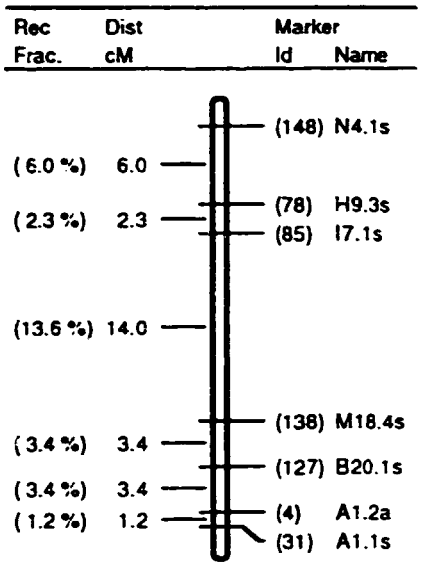

Fig. 4. One linkage group from a map being generated (Al-Janabi et al., in preparation) using AP-PCR and RFLP single-dose polymorphisms. This linkage group is composed exclusively of AP-PCR polymorphisms and includes the two similar sized polymorphisms that were produced by AmpliTaq and Stoffel fragment, *A1.2a and A1.1s*, respectively

approximately 100–180 primers in less than three full runs of the robot (much less than 1 day's work). Note that in our case with sugarcane, we can only map polymorphisms derived from one of the parents (SES 208). Furthermore, if a robot were to work at the rate of five runs/day (less than 5 h to setup reactions and load gels), then the primary data would be ready in 12 days, even with our output. Such potential productivity is especially important to those wishing to apply methodologies that have been developed to study marker-trait associations, such as bulked segregant analysis (Michelmore et al. 1991) and the use of near-isogenic lines (Martin et al. 1991; Yu et al. 1991), in combination with AP-PCR technology. Hundreds of primers, or pairwise combinations of primers, would allow the screening of thousands of loci in a large number of individuals in very few days. Marker-trait associations could be established with data from more individuals than before because of the increased output and lower cost per rection.

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Genetics, Plants, and the Polymerase Chain Reaction

Bruno W.S. Sobral and Rhonda J. Honeycutt

INTRODUCTION

The polymerase chain reaction (PCR) has given plant geneticists, ecologists, evolutionary, and population biologists a powerful new tool for studying their favorite organisms. In this chapter, we will use specific PCR to mean a standard, two-primer amplification that has as a target a specific genomic region, or gene, and therefore requires specific primers to be designed based on knowledge of DNA sequence. We differentiate this from PCR that uses primers of arbitrary sequence to specifically amplify a set of arbitrary loci in any genome, without the requirement for prior sequence knowledge. This is usually referred to as arbitrarily primed PCR or random amplified polymorphic DNA (RAPD) markers; herein, we will use the term arbitrarily primed PCR.

The first part of this chapter will discuss the applications that specific PCR has had in the plant sciences. The second part will discuss applications of arbitrarily primed PCR. In both sections we will attempt to exemplify existing applications and identify potential areas of improvement and research. We also note that, although a fairly recent introduction, the amount of published work using PCR in its various forms has exploded and therefore we will obviously be required to omit a variety of good work from various authors, because of space constraints. Our tendency will be to focus on areas of research that are similar to our own. Finally, we shall end by summarizing and commenting on future directions.

SPECIFIC PCR

Applications of specific PCR (Saiki *et al.*, 1985) to plant sciences have been of two major types: (i) to amplify and directly sequence or otherwise characterize specific DNA sequences for phylogenetic or parentage analyses, strain or cultivar identification; and (ii) to identify pathogens or soil microbes in mixtures of complex biological samples with minimal purification.

Phylogenetic analysis of a variety of loci has become easier because of PCR. A general strategy that can be used is to apply a set of nested primers to amplify then sequence the region of interest directly, using *Taq* polymerase (Ruanto and Kidd, 1991). Usually the primers flank a hypervariable region and hybridize to conserved flanking regions such that comparisons can be made against a wide variation of genotypes. Introns are particularly good candidates to be amplified for this approach because they generally evolve more rapidly than exons (Wolfe *et al.*, 1989) and tend to be short in plants (Hanley and Schuler, 1988; Hawkins, 1988). An internal pair of nested primers gives a secondary step of purification, during the sequencing reaction itself, and typically yield cleaner sequences. Longer sequences still require cloning before

sequencing. Direct sequencing of templates, without prior amplification, has been shown on chloroplast DNA (To *et al.*, 1992), suggesting that a variety of phylogenetic studies on chloroplast-encoded genes should be forthcoming.

The approach of amplifying and sequencing or digesting with restriction enzymes, not always using nested primers, has been successfully used on wild rice species (Barbier and Ishihama, 1990; Barbier *et al.*, 1991) and sugarcane (Sobral *et al.*, 1991; Al-Janabi and Sobral, unpublished data). Waugh *et al.* (1991) applied a similar strategy by using primers directed against the intergenic nucleotide sequences of the *U2snRNA* multigene family in potato, allowing identification of six new variant forms and determination of their genetic linkage. PCR amplification of the first intron in maize -tubulin has been used to estimate the number of different genes present in this multigene family, which was found to be higher than revealed by RFLP approaches (Montoliu *et al.*, 1989, 1992).

Another approach based on the high specificity of the PCR reaction is known as PCR amplification of specific alleles (PASA, Sarkar *et al.*, 1990; Sommer *et al.*, 1992), allele-specific PCR (ASPCR or ASP, Nichols *et al.*, 1989; Okayama *et al.*, 1989), and amplification-refractory mutation system (ARMS, Newton *et al.*, 1989). No matter what the acronym, the idea is to selectively amplify specific alleles by using primers that match the nucleotide sequence of one allele, but mismatch the sequence of a dissimilar allele. This method has been applied to analysis of the *waxy* locus in maize inbred lines (Shattuck-Eidens *et al.*, 1991). Development of allele-specific primers requires substantial time and effort, but once the primers have been developed, then large numbers of individuals can be quickly screened. Perhaps the PASA approach is a small glimpse of applications that will rely on specific PCR to identify superior genotypes in plant breeding. In this regard, a genetic linkage map of the mouse has been generated by Dietrich *et al.*, (1992) that allows the specific PCR typing of intraspecific cross progeny and contains 317 simple sequence length polymorphisms (SSLPs) based on primers that flank simple sequence repeats (SSRs, also known as microsatellites). SSRs are found in most eukaryotic genomes (Hamada *et al.*, 1982), suggesting that a similar approach could be taken in plant species. The main advantage of specific PCR polymorphisms is that they can be co-dominant (as SSLPs are) and, in some cases, multiallelic, thereby having the potential for more information per marker.

Because of phylogenetic conservation of some regulatory motifs across kingdoms, specific PCR approaches can also be used to determine whether specific types of regulators exist in other kingdoms, thereby allowing knowledge to be generated almost in parallel. For example, Singh *et al.* (1991) designed a pair of primers that hybridized to a cDNA clone of *Drosophila melanogaster* known to encode a modifier that suppresses variegation. Those primers amplified an 111-bp DNA fragment that was used as a heterologous probe to screen and clone full length murine cDNAs containing conserved chromo box motifs, now suggested to be a major regulatory motif (Singh *et al.*, 1991). Similar sequences were detected in plants (Singh *et al.*, 1991). These results should allow isolation and genetic studies on putative modifier genes in plant species where chromosomal imprinting has been described, and propel PCR applications into the study of epigenetic factors. Phylogenetic conservation of important motifs has also allowed specific PCR to aid in systematic studies of

plant retrotransposons, as well as permitting identification of elements in new species (Hirochika *et al.*, 1992). Perhaps more surprising, data accumulated from a variety of retrotransposons lead to the suggestion that horizontal transmission between different species has played a role in the evolution of these elements (Flavell and Smith, 1992; Flavell *et al.*, 1992). Homologues of the cdc2 protein kinase of *Saccharomyces cerevisiae*, which plays a central role in the regulation of cell division of eukaryotes (reviewed in Nurse, 1990), have been isolated from rice using PCR amplification of probes (Hashimoto *et al.*, 1992). PCR-based approaches can be used to study phylogeny of plant viral genomes as well. Asymmetric PCR amplification, followed by DNA sequencing has been used to study variability within the bipartite genome of bean golden mosaic geminivirus (Gilbertson *et al.*, 1991).

Pathogen identification is a major concern to those working with exchange and conservation of plant germplasm. A large number of plant pathogens can be found in a variety of plant tissues and locations; intracellularly, extracellularly, and superficially. In best-case scenarios, detection of plant pathogens has usually been at the level of antigen-antibody interactions, using antibodies tagged with some type of fluorescent or other type of label. More often, detection has been based on inoculation of susceptible (tester) genotypes and subsequent detection of specific symptoms, which is slow and laborious. Sophisticated approaches such as antibody detection can require relatively pure materials on which binding assays are performed. Such assays also require monoclonal or cross-adsorbed antibodies resulting in high cost and technical difficulty for many laboratories and quarantine facilities. Furthermore, detection of phloem pathogens, such as mycoplasmalike organisms (MLOs), has been a persistent problem because they resist laboratory culture, as do obligate parasites, such as some rusts and smuts.

Specific PCR approaches to pathogen identification in complex biological samples has been achieved by targeting conserved regions of their genomes, such as rDNA (Wilson *et al.*, 1989) and tDNA (McClelland *et al.*, 1992; Welsh and McClelland, 1992) genes. In general terms, the strategy involves aligning sequences from the organism under study and comparing those with homologous sequences from plants. The goal is to find regions that are specific to the pathogen in question (usually at the genus level) and yet not found in plants or other potential sources of contaminating DNA (such as human DNA from the operator or a variety of saprophytes that colonize plant surfaces or tissues) that may be in the PCR mixture. Variability of the amplified region is a plus, in that it allows strains or species to be separated, which in turn is useful for studies in epidemiology and population genetics. Primers are designed to hybridize and allow amplification *when and only when* genomic DNA from the pathogen is encountered. DNA sequence analysis of the amplified region, either using direct cycle-sequencing with *Taq* polymerase (circumventing cloning; Ruanto and Kidd, 1991) or traditional cloning and sequencing, allows identification of polymorphic restriction enzyme sites. Once a restriction map is made, then restriction enzyme analysis of the amplified region can be used to generate mapped restriction site polymorphism (MRSP) data for phylogenetic analysis (Ralph *et al.*, 1992). Using primers directed to 16*S* rDNA, Ahrens and Seemuller (1992) have designed a set of primers to detect MLOs in complex mixtures of field grown woody species. This required

as little as 170 pg of DNA from infected woody plants. A similar approach for detecting plant MLOs, targeting different regions, was reported by Deng and Hiruki (1991). These approaches have not only aided pathologists to quickly identify MLOs, they also are supplying information that is useful for systematic purposes. Viruses, both RNA and DNA, can also be quickly identified in complex mixtures using specific PCR or reverse-transcription PCR (RT-PCR). RT-PCR has been used to detect RNA viruses in apple, citrus, plum, peach, and grape (Yang *et al.*, 1992; Hadidi and Yang, 1990).

Identification of plant symbionts has also been a difficult area of research that has benefited by application of specific PCR. For example, identification of symbiotic nitrogen-fixing bacteria, such as *Rhizobium* or *Frankia*, from large numbers of plant-derived nodules has been a difficult task that has also relied mainly on antibodies. Recent work in *Frankia* has made use of specific primers that amplify a portion of the *nifH* gene, one of the structural genes for nitrogenase (Simonet *et al.*, 1990). Because nitrogenase is not present in plants, nodules can be crushed directly in microtiter plates and the crude extract subjected to PCR. Strain-specific primer pairs could be developed such that PCR could be used to study important questions of competition for nodule occupancy and survival in the soil. Similar work, using primers targeted against the T-DNA, has been reported for *Agrobacterium tumefaciens*, another difficult-to-detect plant pathogen (Dong *et al.*, 1992).

Following the fate of genetically engineered, introduced bacteria in complex environments, such as the soil, has also been an elusive goal that has become closer to reality by application of PCR strategies. For example, Van Elsas *et al.* (1991) used specific primers against a non-selected segment of a patatin cDNA, introduced to *Pseudomonas florescens*, to follow the fate of the strain and its plasmid (RP4) in the soil. This represents a large improvement over previous methods of detection of engineered organisms, such as antibiotic resistance, particularly because transfer of genes to non-culturable soil microbes can also be determined. RP4 is a broad host range, self-transmissible plasmid, and its lateral transfer is expected, even though the range of transfer may not be known. It would be interesting to insert similar genes into chromosomal loci to determine the amount of lateral transfer of chromosomal markers within a species or genus, as little is known about the population structure in most bacteria.

Because PCR is a highly automatable technique (Sobral and Honeycutt, 1993; Nelson *et al.*, 1992; Dietrich *et al.*, 1992), and because scoring of specific PCR amplifications can be of the DNA present or DNA absent type, we expect it will soon be possible to quickly type very large numbers (thousands) of nodules or soil samples for the presence of specific strains of symbiotic bacteria or engineered, introduced bacteria. This type of high output will require development of non-electrophoretic detection of amplified products, such as EtBr staining, followed by direct data acquisition via computer reading of stained microtiter plates. Quantitative methods for typing via PCR would be important for such applications.

Fungi are major pathogens of crops. Cereal rusts are among the major biotic pathogens limiting cereal production. Classification of many obligate parasites has been based on genetics of the interaction between host and pathogen, meaning that many of the pathogens have been classified primarily

(if not only) by their avirulence gene content, as determined by inoculation on tester plants. Although such classifications have been useful for breeding, little is known about the genetic diversity, epidemiology, population structure, and phylogenetic relations of most phytopathologically important fungal species. This is mainly caused by the inability of avirulence gene phenotype to characterize the genetic variability within the pathogen, just as seen above for major epitopes against which antibodies are prepared. Approaches relying on protein differences have generally not yielded sufficiently discriminatory patterns to be a significant improvement, besides being costly, time-consuming, and technically difficult (Kim *et al.*, 1992). Specific PCR of an intergenic region between the 26*S* and 5*S* rDNA genes of *Puccinia graminis* (cereal stem rust) allowed development of a rapid method for identification of races of *P. graminis* and opened the door for the study of genetic relations within this important species of fungal pathogen (Kim *et al.*, 1992). Liu and Sinclair (1992) used a series of six primers directed against the nuclear rDNA loci of *Rhizoctonia solani*, a very important soil pathogen of many crop species, to investigate MSRPs within anastomosis group 2. Five groups were obtained, either through analysis of isozyme alleles or PCR-amplification followed by restriction enzyme digestion, demonstrating that genetically independent groups can be established and monitored using this approach.

ARBITRARILY PRIMED PCR

The discovery that the use of PCR with an arbitrarily selected primer to amplify a specific set of arbitrarily distributed loci in any genome laid the foundation for high output of genetic markers that can be used for a variety of purposes (Welsh and McClelland, 1990; Williams *et al.*, 1990). The impact of arbitrarily primed PCR on plant genetics has been great. Less than one full year after publication of the protocol (Welsh and McClelland, 1990; Williams *et al.*, 1990), there were more than 30 posters describing work in progress at the biennial meeting of the International Society for Plant Molecular Biology (Tucson AZ, October 1991); one year after that, the Plant Genome I conference (San Diego CA, November 1992) was filled almost exclusively with posters that reported progress on genetic and systematic studies of various plant species and their pathogens, mostly using arbitrarily primed PCR.

Perhaps the main reason for the immediate success of arbitrarily primed PCR among plant scientists was the need for a high output marker acquisition method that was also low technology and immediately accessible to a variety of research or end-user environments. Because arbitrarily primed PCR is easily done with small amounts of DNA and without the requirement for clone banks or other forms of molecular characterization of the species in question, many crop species that were orphans because they lacked sufficient research investments became amenable to genetic studies. In addition, arbitrarily primed PCR does not normally require radioactively-labeled nucleotides, a limiting factor in most non-first-world countries. Finally, because any PCR method is inherently automatable, there is the prospect for fulfilling the promise of routine use of molecular markers by breeding programs *in loco*, something that has not been possible with RFLP technology.

The main applications of arbitrarily primed PCR in plant sciences have been: (i) genetic mapping; (ii) systematic studies with various goals; and (iii) identification of controlling regions for traits of agronomic importance, though

this area is still embryonic. Development of plant genetic maps using arbitrarily primed PCR has progressed impressively. Some plant species that have been mapped by this approach include: *Arabidopsis thaliana* (Reiter *et al.*, 1992); *Saccharum spontaneum*, a wild relative of sugarcane (Al-Janabi *et al.*, 1992, 1993); species of pines (Carlson *et al.*, 1991; Chaparro *et al.*, 1992a); alfalfa (Echt *et al.*, 1992); soybeans (Williams *et al.*, 1990); peach (Chaparro *et al.*, 1992b); citrus (Cai *et al.*, 1992); flax (Gorman and Parojcic, 1992); grapes (Lodhi *et al.*, 1992); tomato (Klein-Lankhorst *et al.*, 1991); and *Eucalyptus* trees (Grattapagalia *et al.*, 1992; Grattapagalia and Sederoff, 1992).

Arbitrarily primed PCR polymorphisms presumably are based on mismatches in primer binding sites or insertion/deletion events, and therefore usually result in the presence or absence of an amplified product from a single locus (Welsh *et al.*, 1992; Williams *et al.*, 1990). This means the arbitrarily primed PCR markers are usually dominant because individuals containing two copies of an allele (homozygous with presence phenotype) cannot be distinguished from individuals with one copy of the allele (heterozygous with presence phenotype). Mapping with dominant markers usually means that information is less per marker in F_2 populations because dominant markers in repulsion provide little information for genetic distance estimation, although selection of markers in coupling phase or from a single parent can increase the per-gamete informativeness of the marker to levels similar to those of codominant markers (Tingey *et al.*, 1992).

Although the dominant nature of arbitrarily primed PCR polymorphisms can be seen as a hindrance to their informativeness for genetic mapping, there are specific situations in the plant sciences that cannot use but dominant markers. In particular, genetic mapping of polyploid species and tree species that have high amounts of DNA and long generation times have been brought out of a near standstill by application of arbitrarily primed PCR technology. In polyploids of unknown genomic constitution, Al-Janabi *et al.* (1992, 1993) have adopted an approach based on selection of single-dose polymorphisms and subsequent determination of their linkages by analysis of what has been called the pseudo-testcross strategy by Grattapagalia and Sederoff (1992), which the latter authors have applied to mapping in tree species using existing crosses. This strategy has been proposed with various levels of detail by Bonierbale *et al.* (1988), Ritter *et al.* (1990), and Wu *et al.* (1992). In this approach, markers are selected from any cross between any two species from which an F_1 can be produced. Markers selected for mapping must fit two criteria: (i) they must be present in one parent and absent in the other and (ii) they must segregate 1:1 in the progeny. In the case of polyploid species, no matter what the genomic constitution (allopolyploid vs. autopolyploid) or ploidy level of the material, single-dose markers correspond to simplex alleles (autopolyploids) or heterozygous alleles in diploid loci (allopolyploids) (Wu *et al.*, 1992; Al-Janabi *et al.*, 1992, 1993). In tree species, crosses normally cannot be made and analyzed on short notice, so the method allows mapping of existing crosses. In addition, in pine species there is haploid, maternally-inherited tissue in the megagametophyte, meaning that all single-dose markers can be mapped and single-tree maps can be readily made (Chaparro *et al.*, 1992a). Not only are these maps readily made, they typically require approximately four to six person-months per 200 mapped markers (Reiter *et al.*, 1992; Al-Janabi *et al.*,

1993), which is significantly less than required to make similar RFLP maps. Of course, single-dose RFLP maps could be made in polyploid species as well, but single-copy RFLP probes tend to hybridize to many fragments in polyploids and the resulting complex fingerprint may cause difficulties in interpreting alternate alleles. In addition, in some tree species, DNA content is so high that single-copy Southern hybridization may be impractical, or at least very lengthy exposures are required. Therefore, one large impact of arbitrarily primed PCR has been to increase the species amenable to mapping activities. Furthermore, automation of PCR has progressed to the point that data generation for a 200 marker map can be done in one or two weeks by a robotic liquid handling station (Sobral and Honeycutt, 1993; Sobral *et al.*, 1993; Crawford *et al.*, 1992). Robotic liquid handling stations can be programmed to handle 384-well (24x16) microtiter plates, and should be able to handle even larger numbers of wells/plate. So, arbitrarily primed PCR also has increased the speed of map-making.

Fingerprinting of plant genomes using arbitrarily primed PCR polymorphisms has been done for a variety of systematic and population genetic studies. Although discussions abound as to the appropriate manner to analyze the data, this is due less to methodological constraints than to different perceptions of how systematics, population genetics, and phylogenetic inference are related and what are the appropriate ways to analyze data (for a brief review, see Moritz and Hillis, 1991). Once again, adjustments need to be made because of the dominant nature of the polymorphisms detected by arbitrarily primed PCR, which lowers their polymorphism information content (PIC, Botstein *et al.*, 1980) but does not invalidate phylogenetic analysis of appropriate biological situations. In particular, phylogenetic approaches can be taken if the genome in question fits the assumptions made by all methods of inference, that is, that organisms evolve mainly by drift and mutation under a bifurcating tree assumption (Swofford and Olsen, 1991). Most plant nuclear genomes are at least diploid and sexual, and presumably have reticulating trees that trace their evolutionary history, rather than bifurcating trees (Swofford and Olsen, 1991), so such analyses invalidate assumptions no matter what type of method is used to generate the data. If the biology of your organism fits the assumptions of the algorithms, then you can use PAUP (Swofford, 1991), with appropriate weighting of character state transformations (Albert *et al.*, 1992). Chloroplast, mitochondria, and some bacterial and fungal pathogens are generally believed to fit assumptions of bifurcating trees. If modest reticulation is expected, cladistic parsimony can be applied and resulting hypotheses can be tested within a framework of maximum likelihood (Felsentein, 1973; Lathrop, 1982; Thompson, 1973, 1975).

Even when phylogenetic analyses should not be conducted, fingerprinting data can be used for systematic or classification purposes (Honeycutt *et al.*, 1992), parentage determination (Welsh *et al.*, 1991), estimation of gene flow (Arnold *et al.*, 1991), and detection of genetic variation in natural populations (Chalmers *et al.*, 1992; Chapco *et al.*, 1992). These areas of research have not progressed greatly with development of molecular markers because thus far they have been either too costly and laborious to be used on large numbers of individuals (as is the case for RFLPs) or too limited in their genomic distribution and the level of diversity they can reveal (as is the case for

isozymes). Arbitrarily primed PCR has largely resolved these problems and we expect that large amounts of fingerprinting data from various plant species will be analyzed in the near future. These data will certainly have an impact on management, conservation, and improvement of plant genetic resources worldwide.

Analysis of arbitrarily primed PCR fingerprints for systematic or classification purposes can be done using similarity coefficients, as the general objective is to group similar germplasm. Choices of coefficients and their implications on subsequent analyses should be carefully considered (Jackson *et al.*, 1989; Swofford and Olsen, 1991; Weir, 1991), as should earlier choices regarding sampling (Baverstock and Moritz, 1991). For population studies, gene flow and mating system are known to be important determinants of genetic structure of plant populations and require the ability to detect heterozygotes (Clegg, 1980), which means that uses of arbitrarily primed PCR are limited. However, Chalmers *et al.* (1992) and Baird *et al.* (1992) have noted that arbitrarily primed PCR polymorphisms may be used to detect heterozygous individuals when a single primer generates at least one complementary polymorphism from each parent. In most studies of this type, each primer detects a variety of polymorphisms so this should not be difficult to achieve. Population-specific or species-specific markers can be generated in this way (Crowhurst *et al.*, 1991; Hadrys *et al.*, 1992; Sellstedt *et al.*, 1992; Smith *et al.*, 1992) and used to further characterize differences among populations or species, especially when genetic maps are available for the species being studied. For genealogical studies, polymorphisms can be used to infer parentage (Welsh *et al.*, 1991; Honeycutt *et al.*, 1992). Preliminary data for our work with elite maize inbred lines, using 15 arbitrarily selected primers on 22 genotypes, showed a high correlation between genetic distances measured by 99 mapped RFLP probes (Smith *et al.*, 1991) and those measured by the 15 primers (119 characters) (Figure 1). The RFLP probes have a distribution that allows coverage of most of the maize genome. In addition, good correlation was shown for pedigree coefficients and distances as measured by arbitrarily primed PCR (Figure 2), although the level of correlation was smaller than observed with the 99 mapped RFLP probes on a larger set of inbreds (Smith *et al.*, 1991). In maize inbreds, the incapacity to detect heterozygotes is not important, so the dominant nature of the polymorphisms is not relevant. In parentage analysis work, we have not observed non-parental bands (Reidy *et al.*, 1992). However, we note that we use a modified protocol of arbitrarily primed PCR (Sobral and Honeycutt, 1993), which produces a larger number of amplified products per primer, when compared to standard conditions (Welsh and McClelland, 1990; Williams *et al.*, 1990). Perhaps we have not experienced this problem because there may be a smaller context effect on the competition for primer binding when more amplified products are produced. For plant breeders, it is important to have information on genetic diversity because such data can be used to inform them of the degree of relatedness of the materials they work with, and therefore select which new crosses should be made and which materials need to be preserved in germplasm collections. This is clearly another important application that arbitrarily primed PCR has filled, especially in crops that have been classified exclusively by morphological or geographical attributes.

The other criticism frequently mentioned when arbitrarily primed PCR polymorphisms are used in a variety of studies is the potential co-migration of amplification products that are not from the same locus. On statistical grounds this should occur rarely, but gel resolution systems and the relatedness of the genomes in question also play a large role in how often this occurs. Our work in sugarcane and its relatives has suggested that higher resolution, polyacrylamide sequencing gels are superior to agarose gels for interspecific comparisons (Figure 3), whereas longer (20 cm usable running length) 2% agarose gels provided sufficient resolution for the aforementioned analysis of maize inbred lines (Figure 4). In any event, dubious polymorphisms can always be excised, re-amplified in the presence of a labeled nucleotide, and used in a Southern blot to show whether homology exists at the DNA sequence level (Peinado *et al.*, 1992).

The third major area of application of arbitrarily primed PCR has been the targeting of markers to specific genetic regions. This area of research has been substantially forwarded by Michelmore *et al.* (1991), who developed an elegant method of creating "*in vitro* near-isogenic lines (NILs)" by bulking segregants from a cross between parents that contrasted for a specific trait. The process is called bulked segregant analysis (BSA) and the idea is to select from an F_2 population individuals that represent the phenotypic extremes for the trait in question and separately pool their genomic DNAs in equimolar amounts. The assumption is that the ends of the phenotypic distribution represent individuals that have opposing homozygote alleles ("good" vs. "bad") for the trait in question. The number of individuals to be bulked can be as few as three or four and there is no need to construct near-isogenic lines, a procedure which is at best time-consuming, and virtually impossible for some species. Of course, use of traditional NILs and arbitrarily primed PCR can be done when such genotypes exist (Paran *et al.*, 1991; Martin *et al.*, 1991). BSA also can be used to target multiple loci of highly heritable quantitative traits that are controlled by few loci of large phenotypic effect (Michelmore *et al.*, 1992). Not only does BSA allow for very quick screening and enrichment for markers linked to specific traits, but the procedure also generates a genetic map for the region(s) under study (Michelmore *et al.*, 1991). This regional map can be refined by selection of appropriate progeny for further bulking and analysis as well as individual progeny testing. In addition, these regional maps can be integrated into existing genetic maps for the species being studied by using bulking to make specific, regional maps, and then cross-mapping those regions in the standard mapping population, as has been done in lettuce for disease resistance loci (Michelmore *et al.*, 1992). Cross-mapping the region of interest onto the general map is particularly important because the final product is a single, comprehensive genetic linkage map for any given species. Furthermore, regions on existing linkage maps that are poorly populated with markers can be mapped using bulks of informative individuals from existing mapping populations (Giovannoni *et al.*, 1991). Because of the elegant simplicity and wide applicability of BSA, we expect it to be used on a variety of important crop species.

SUMMARY AND FUTURE DIRECTIONS

In summary, there is little doubt that application of PCR in its various forms have caused a large increase in genetic knowledge of plant species and of the many species of other organisms important to plants. There is also little

doubt that further advances in methodology, particularly those that relate to increased speed and increased automation, will allow further leaps in genetic knowledge of plant genomes and their evolution. Of course, the ultimate DNA marker is DNA sequence information, and when it becomes sufficiently cheap and expedient to obtain and analyze large amounts of DNA sequence data, we would expect that other methods might be superseded, at least for well-funded species.

In the mean time, it seems that with small increments in data acquisition technologies, such as non-electrophoretic means of visualizing amplification results, and computer-assisted scoring of gel images, it may be possible to make genetic maps, screen thousands of genetic loci, or all mRNAs in a cell type (Welsh *et al.*, 1992b; Liang and Pardee, 1992) in a matter of days, rather than years. We expect this to directly benefit a large number of orphan crops that have not received much attention by funding agencies. It will also allow ecological and population genetics to address novel questions because of high marker output and the capacity to analyze large numbers of individuals through automation. Further improvements to arbitrarily primed PCR markers, such as their conversion to sequence characterized amplified regions (SCARs; Michelmore *et al.*, 1992), which are codominant and based on specific PCR reactions, as well as novel strategies that surely will be devised, may well realize the dream of applied marker-based breeding and construction of superior genotypes, and large studies in population genetics and germplasm conservation. In addition, we expect marker-assisted introgression, marker-aided selection and, eventually, map-based gene cloning to progress more quickly and be attempted on a larger number of plant species because of the lowered cost per data point and lower technological requirements of arbitrarily primed PCR in relation to other molecular markers. This will be especially true when the cost of thermostable polymerases reaches the levels of cloned restriction enzymes, as the major cost component of PCR-based analyses is the polymerase.

Finally, diagnostic techniques for biotrophic plant disease agents, as well as markers for the study of pathogens, should allow large gains in knowledge to be achieved by plant pathologists and entomologists. In particular, the ability to identify and characterize pathogens in complex biological mixtures, and without the need for a capacity to culture them in the laboratory has allowed previously impossible studies to be idealized and executed. Our general lack of understanding of fungal and bacterial pathogens most likely makes practical, energy-efficient control of these pathogens difficult if not impossible. As application of the various PCR-based strategies reaches these organisms, we may learn enough about their lives to control them more rationally in the future. Perhaps the most interesting results of the application of PCR to the plant sciences are already in the making and will soon available from the ongoing population studies that will eventually tell us more about plants and their environment. For those, we need only to wait for their completion.

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FIGURE LEGENDS

Figure 1: Regression analysis of RFLP genetic distance x arbitrarily primed PCR genetic distance on 22 maize inbred lines (from Honeycutt *et al.*, 1992). Data were scored as presence or absence of a DNA fragment of specified size. RFLP data is based on hybridization with 99 mapped probes that are known to be distributed throughout the maize genome (these data kindly furnished by Stephen Smith, Pioneer Hi-Bred International, Iowa). Arbitrarily primed PCR data was generated using 15 arbitrarily selected 10-mers (Operon Technologies). Genetic distances were calculated using PAUP 3.0s (Swofford, 1991). Pairwise genetic distances were then plotted for all possible combinations of the 22 inbred lines and linear regression was performed with the resulting regression coefficients shown above the line.

Figure 2: Regression analysis of genetic distance calculated by arbitrarily primed PCR x pedigree coefficient for 22 maize inbred lines. Pedigree coefficients were determined by Smith *et al.* (1991).

Figure 3: Comparison of gel resolution systems in study of sugarcane and its relatives. Arbitrarily primed PCR products were generated from *Saccharum* species according to Sobral and Honeycutt (1993), using AmpliTaq Stoffel fragment (Perkin-Elmer), and resolved on a 4% polyacrylamide denaturing sequencing gel (upper panel) run at 50 mA for 4 h or on a 2% agarose gel (lower panel) run for 1,100 V h. Amplifications were done in 30-µl reaction volumes in the presence of alpha^{32}[P] dCTP (Welsh and McClelland, 1990). For polyacrylamide gels, 4 µl of sample were taken and mixed with 14 µl of denaturing dye, the mixture was incubated at 85°C/5 min, put immediately on ice, after which 4 µl were loaded onto the gel. For agarose gels, 20 µl of the reaction was loaded directly. Saccharum DNAs were used at three template concentrations (0.3, 1, and 1.7 ng µl^{-1}, respectively), to detect potential "sporadic" fragments that are template-concentration-dependent. Molecular weight markers were: M1=pBR322 digested with *Msp*I; M2=BRL 123 bp ladder; M3=BRL 1 kb ladder. Arrows indicate the position of a fragment that potentially would be mis-scored on the agarose gel, especially if it were shorter than 20 cm.

Figure 4: Twenty-cm-long 2% agarose gel (Stratagene Cloning Systems -test box) used to resolve arbitrarily primed PCR products in maize inbred line study (Honeycutt *et al.*, 1992). Electrophoresis was done at 5 V cm^{-1} for a total of 1,100 V h. Data shown is for one 10-mer primer (Operon Technologies). Maize DNAs were analyzed using two template concentrations (1 ng µl^{-1} and 3 ng µl^{-1}, loaded sequentially for each genotype) to allow detection of rare, "sporadic", template-concentration-dependent faint products, which were not scored. Amplifications were done in 30-µl reaction volumes in a PTC-100 thermocycler (MJ Research) using a 96-well microtiter plate heating block. Cycling parameters were: 94°C/3 min, followed by 30 cycles of 94°C/1 min, 35°C/1 min, increase to 72°C at 1°C/2s, 72°C/2 min; cycling was completed by a single 72°C/7 min, and reaction products were maintained at 12°C until loading.

AmpliTaq Stoffel fragment (Perkin-Elmer) was used as the thermostable polymerase (Sobral and Honeycutt, 1993).

Comparison of arbitrarily primed PCR and RFLP distances in 22 maize inbred lines

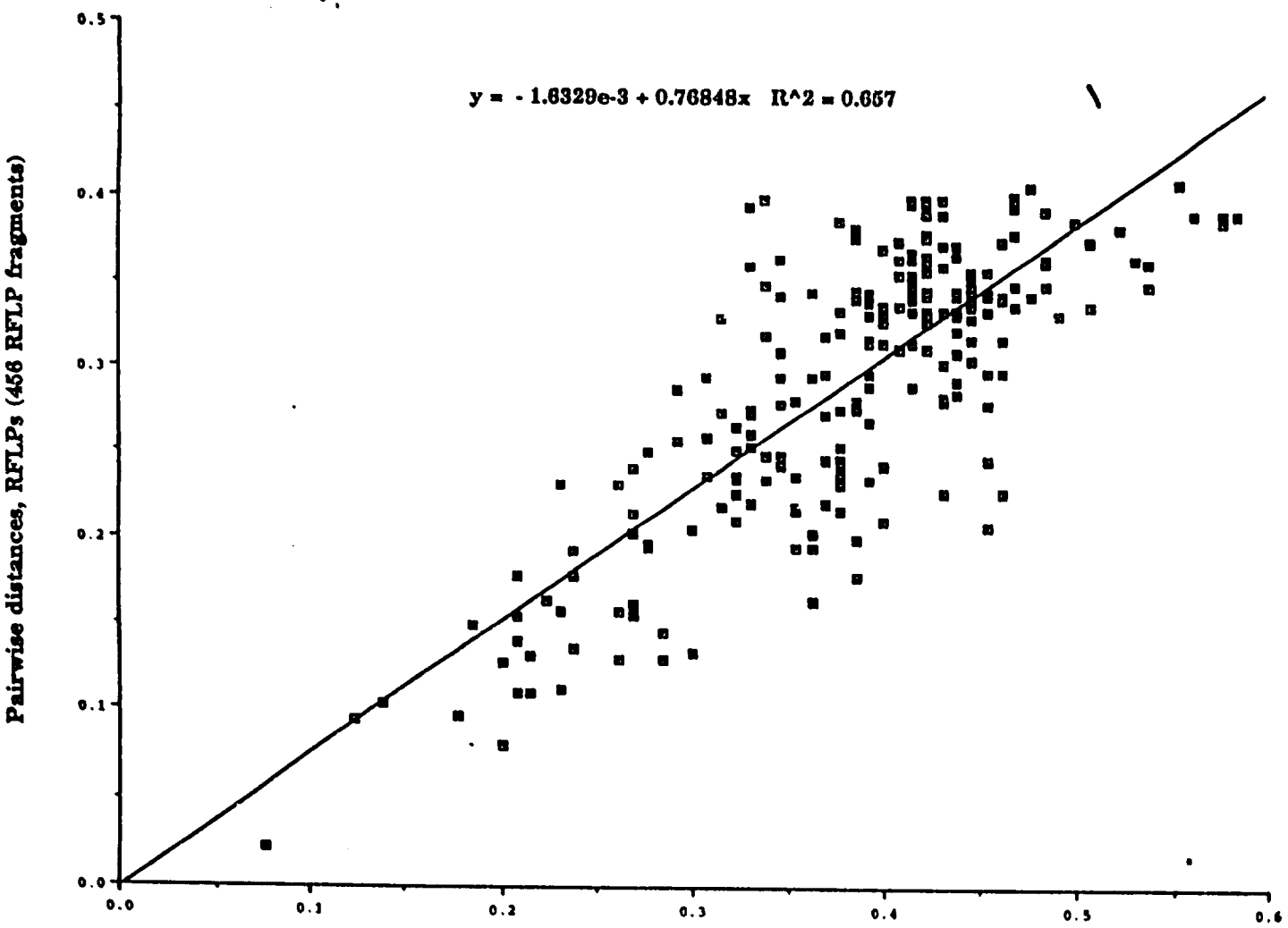


Fig 1

Comparison of arbitrarily primed PCR and pedigree distances in 22 maize inbred lines

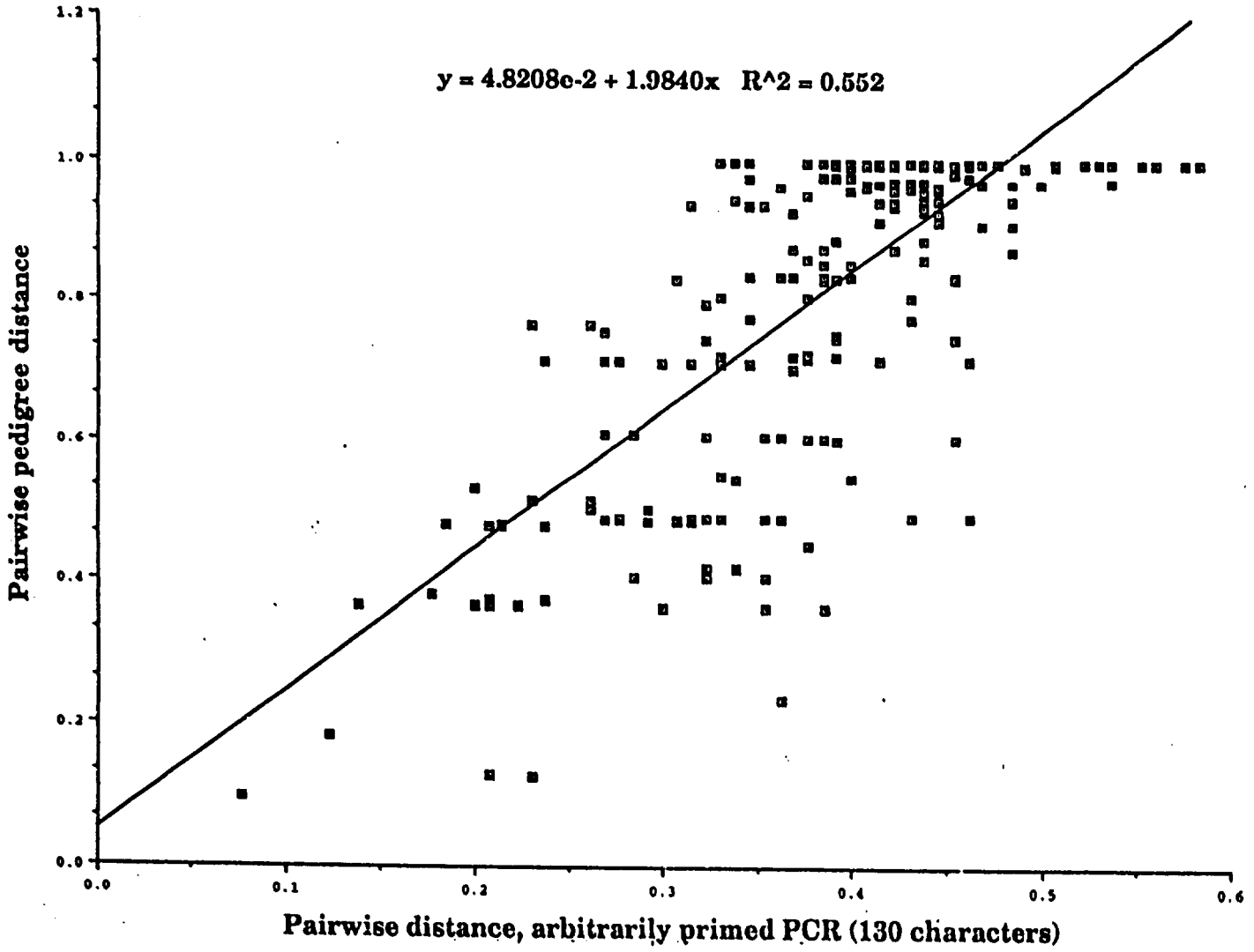
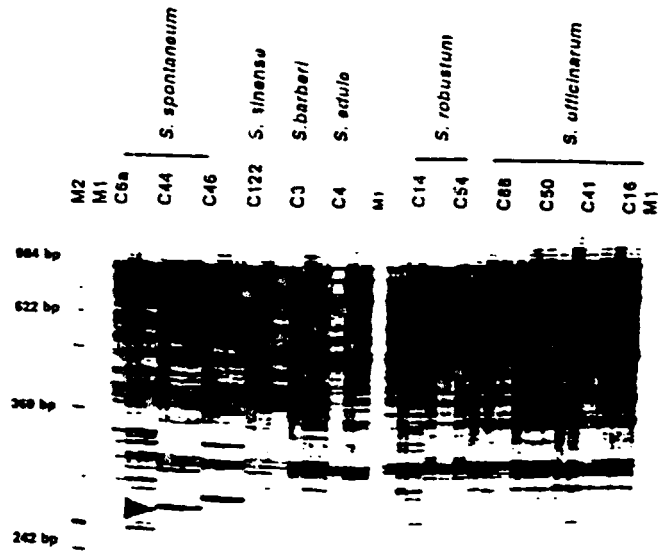
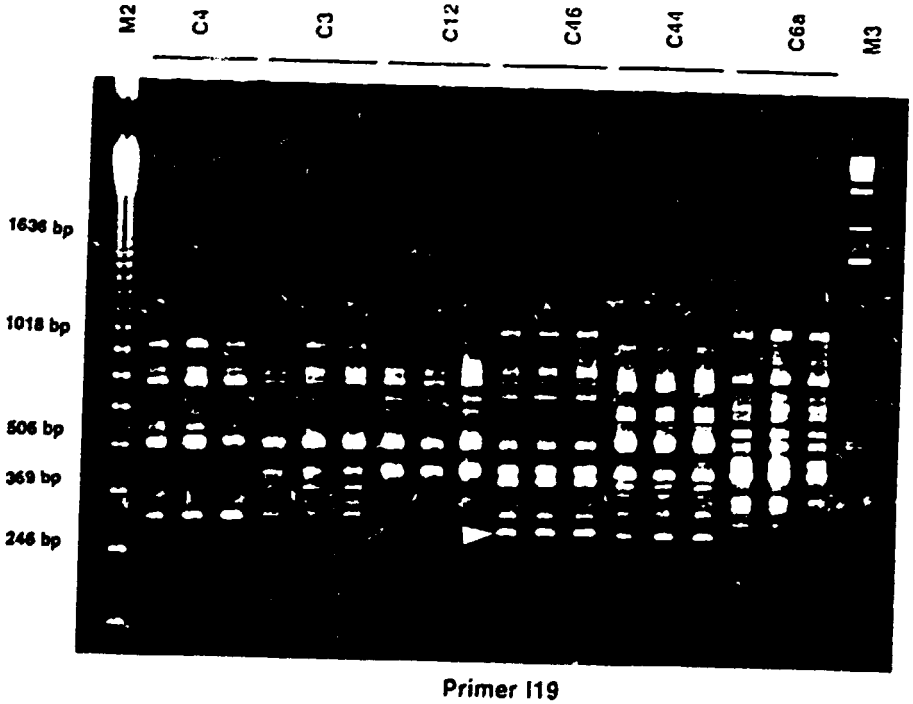


FIG 2

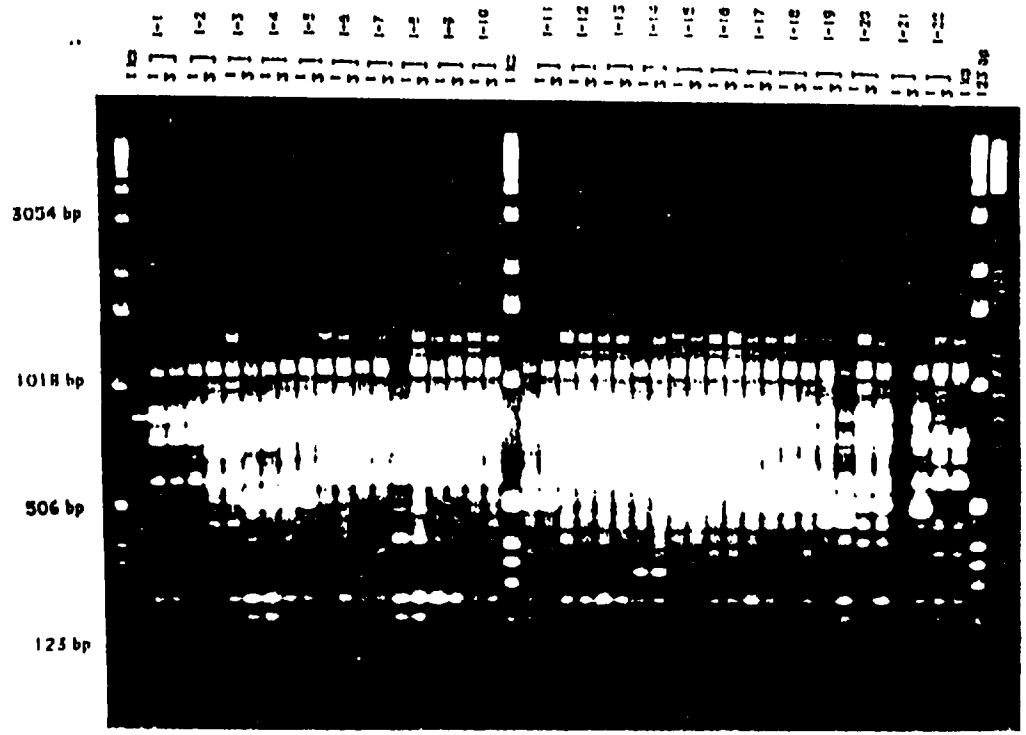


Primer I19



Primer I19

Fig3



Primer B11

CAMBIA Literature Search Service

A search of journal articles listed on BIOSIS CD ROMS dated January 1989 to June 1993.

Search critera were **RAPD** and (**DNA** or **PCR**).

This file contains 69 records extracted from BIOSIS Jan 1989 to June 1993 and 10 records extracted from BA/RRM on CD 1/93 - 3/93 (8 CDs).

The following search has been extracted from BA/RRM on CD 1/93 - 3/93

TI: RAPD markers for population genetic studies of Pyrenophora teres.
AU: PEEVER-T-L; LEE-K-C; MILGROOM-M-G
CS: Dep. Plant Pathol., Cornell Univ., Ithaca, N.Y. 14853, USA
SO: CANADIAN JOURNAL OF PLANT PATHOLOGY 14(3): 246-247
PY: 1992
LA: English

TI: Studies of oviposition behavior of Aedes aegypti using RAPD-PCR markers.
AU: APOSTOL-B-A; REITER-P; BLACK-W-C; MILLER-B-R; BEATY-B-J
CS: Arthopod Borne Infectious Disease Lab., Colo. State Univ., Fort Collins, Colo
SO: AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE 47(4 SUPPL.): 253
PY: 1992
LA: English

TI: New DNA probe development protocol: RAPD-PCR:PC: Application to development of species-specific DNA probes for characterization of sand flies.
AU: STITELER-J-M; DIAS-E-S; PERKINS-P-V
CS: Dep. Entomol., Walter Reed Army Inst. Res., Washington, D.C., USA
SO: AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE 47(4 SUPPL.): 185-186
PY: 1992
LA: English

TI: Sand fly DNA probes: RAPD-PCR:PC development: Requirement for multicopy genomic sequence targets.
AU: DIAS-E-S; STITELER-J-M; LAWYER-P-G; PERKINS-P-V
CS: Res. Cent. Rene Rachou-FIOCRUZ, Belo Horizonte, Brazil
SO: AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE 47(4 SUPPL.): 185
PY: 1992
LA: English

TI: Parentage analysis using RAPD PCR.
AU: SCOTT-M-P; HAYMES-K-M; WILLIAMS-S-M
CS: Dep. Zool., Univ. New Hampshire, Durham, N.H. 03824
SO: NUCLEIC ACIDS RESEARCH 20(20): 5493
PY: 1992
LA: English

TI: Genetic variation in the beach clam Donax revealed by the RAPD amplification of genomic DNA.
AU: ADAMKEWICZ-L
CS: Dep. Biol., George Mason Univ., Fairfax, Va. 22030, USA
SO: VIRGINIA JOURNAL OF SCIENCE 43(2): 227
PY: 1992
LA: English

TI: Barley mildew in Europe: Population structure based on virulence and RAPD variation.
AU: MCDERMOTT-J-M; MULLER-K; WOLFE-M-S
CS: Phytopathology Group, Swiss Federal Inst. Technology, CH-8092 Zurich, Switz
SO: PHYTOPATHOLOGY 82(10): 1167
PY: 1992
LA: English

TI: RAPD analysis of Colletotrichum gloeosporioides.
AU: CORRELL-J-C; RHOADS-D-D; GUERBER-J-C
CS: Dep. of Plant Pathol., Univ. of Arkansas, Fayetteville, Ariz. 72701
SO: PHYTOPATHOLOGY 82(10): 1142
PY: 1992
LA: English

TI: Differentiation of the angular leafspot fungus with RAPD markers: Evidence for coevolution with the common bean.
AU: GUZMAN-P; MANDALA-D; NODARI-R; MSUKU-W-A-B; MKANDAWIRE-A-B-C; GEPTS-P; TEMPLE-S; GILBERTSON-R-L
CS: Dep. Plant Pathology, University California, Davis, Calif. 95616
SO: PHYTOPATHOLOGY 82(10): 1124
PY: 1992
LA: English

TI: DNA fingerprinting and RAPD analysis of population diversity of Colletotrichum orbiculare.
AU: CORRELL-J-C; RHOADS-D-D; GUERBER-J-C
CS: Dep. Plant Pathology, University Arkansas, Fayetteville, Arkansas 72701
SO: PHYTOPATHOLOGY 82(10): 1124
PY: 1992
LA: English

TI: Selection of a genetic variant within Colletotrichum gloeosporioides isolates pathogenic on mango by passaging through wounded tomato fruits.
AU: ALAHAKOON-P-W; SREENIVASAPRASAD-S; BROWN-A-E; MILLS-P-R
CS: Dep. Applied Plant Science, Queen's University Belfast, Newforge Lane, Belfast BT9 5PX, UK
SO: PHYSIOLOGICAL AND MOLECULAR PLANT PATHOLOGY 41(4): 227-240
PY: 1992
LA: English
AB: A genetic variant virulent on tomato was selected by passaging field isolates of Colletotrichum gloeosporioides, obtained from mango, at least twice through wounded tomato (Lycopersicon esculentum) fruits. The field isolates and isolates virulent on tomato showed ribosomal **DNA** restriction fragment length polymorphisms (RFLP). They also had different random amplified polymorphic **DNA (RAPD)** banding patterns. The mango (Mangifera) field isolates produced conidia of sizes ranging from 12 to 22 mu-m and approx. 5% of these conidia were multinucleate. Cultures of the genetic variant produced uniformly-sized conidia (approx. 14 mu-m) which were uninucleate. The genetic variant was isolated in culture, only with difficulty, as a single conidium (L-2t-3) from a culture (L-2) which, in turn, was grown from a single approx. 20 mu-m conidium obtained from one of the mango field isolates (M11). It is suggested that two genetically different nuclei residing within individual multinucleate conidia gave rise to the observed variation. The importance of heterokaryosis in relation to the considerable genetic variation and apparent adaptability seen in C. gleosporioides is discussed.

TI: Species and strain differentiation of Eimeria spp. of the domestic fowl using DNA polymorphisms amplified by arbitrary primers.
AU: PROCUNIER-J-D; FERNANDO-M-A; BARTA-J-R
CS: Dep. Pathol., Univ. Guelph, Guelph, ON N1G 2W1, Can
SO: PARASITOLOGY RESEARCH 79(2): 98-102
PY: 1993
LA: English
AB: Eimeria spp. from the domestic fowl were examined for genetic relatedness by the random amplified polymorphic **DNA (RAPD)** assay. Nine different oilgonucleotide decamers with arbitrary **DNA** sequences were tested as primers to amplify **DNA** from six Eimeria species infecting chickens. Two strains each of E. acervulina and E. tenella were used. Depending on the species/strain-primer combination, between 1 and 12 **DNA** segments ranging in size from 0.16 to 4.95 kb were amplified. The two strains of E. acervulina showed minor and major differences in their amplified **DNA** patterns, giving a similarity coefficient of 61%. The two strains of E. tenella seemed to be more closely related, yielding a similarity coefficient of 98%. The differences observed between species were greater than those found between strains with every primer used, indicating that the RADP assay could be a useful tool for the study of relationships among these coccidia. The results obtained in this study also indicate the presence of unique, species-specific, amplified **DNA** segments that could be exploited to identify Eimeria species of the chicken.

TI: Inheritance of random amplified polymorphic DNA markers in an interspecific cross in the genus Stylosanthes.
AU: KAZAN-K; MANNERS-J-M; CAMERON-D-F
CS: Commonwealth Sci. and Industrial Res. Organization, Div. Tropical Crops and Pastures, Cunningham Lab., 306 Carmody Rd., St. Lucia, Queensland 4067, Australia
SO: GENOME 36(1): 50-56
PY: 1993
LA: English
AB: The inheritance of random amplified polymorphic **DNA (RAPD)** markers generated via the polymerase chain reaction amplification of genomic **DNA** sequences in an F-2 family of an interspecific cross between Stylosanthes hamata and S. scabra was investigated. An initial comparison between the parentasl species, S. hamata cv. Verano and S. scabra cv. Fitzroy, demonstrated that 34% of detected **RAPD** bands were polymorphic. Of 90 primers tested, 35 showed relatively simple and reliably scorable polymorphisms and were used for segregation analysis. Sixty F-2 individuals were scored for the segregation of 73 **RAPD** markers and 55 of these markers fit a 3:1 ratio. Segregation of eight other **RAPD** markers deviated significantly from a 3:1 ratio. There was no bias in the inheritance of **RAPD** markers regarding parental origin of the segregating **RAPD** markers. Linkage analysis revealed 10 linkage groups containing a total of 44 **RAPD** loc. Another 10 **RAPD** markers (7 of maternal origin) that were polymorphic between the parents did not segregate in the F-2 population. One of the maternally inherited **RAPD** bands hybridized to chloroplast **DNA**. Analysis of **RAPD** loci by **DNA** hybridization indicated that mainly repeated sequences were amplified. These data indicate that **RAPDs** are useful genetic markers in stylosanthes spp. and they may be suitable for genetic mapping.

TI: Genetic relationships and variation in the Stylosanthes guianensis species complex assessed by random amplified polymorphic DNA.
AU: KAZAN-K; MANNERS-J-M; CAMERON-D-F
CS: Commonweath Scientific and Industrial Res. Organization, Div. Tropical Crops and Pastures, Cunningham Lab., 306 Carmody Rd., St. Lucia, Queensland 4067, Australia
SO: GENOME 36(1): 43-49
PY: 1993
LA: English
AB: Genetic variation in the five taxonomic groups of the Stylosanthes guianensis (Aubl.) Sw. complex was investigated using random amplified polymorphic **DNA** markers (**RAPDs**). **DNA** samples from four plants of each of 45 accessisons within the S. guianensis species complex were analyzed using 20 oligonucleotides of random sequences. Little variation was found within each of the 18 accessions (1-7% of total **RAPD** bands in pairwise comparisons) and none within each of the other 27 accessions. However, higher levels of polymorphisms were observed both within (index of genetic distance = 1 -F 0.16-0.248) and between (1-F = 0.254-0.408) the five taxa. This level of differentiation at the **DNA** level supported an earlier classification of the taxa as distinct species. A phenogram based on band sharing was constructed to show genetic relationship among taxa studied. This phenogram corroborated the description of relationship based on morphological-agronomic characteristics, seed protein patterns, rhizobial affinities, crossability, and pollen stainability of the hybrids. In this phenogram, the most similar species were Stylosanthes grandiflora and S. hippocampoides (1 - F = 0.264), with S. acuminata also showing closest similarity to these two species (1 = F = 0.277 and 0.283, respectively). S. gracilis accessions showed the closest similarity (1 - F = 0.296) to S. guianesis ssp. guianensis accessions. Lowest similarity values (1 - F = 0.335-0.411) were found between these two species and S. grandiflora, S. acuminata, and S. hippocampoides.

TI: Examination of parameters affecting polymerase chain reaction in studying RAPD.
AU: YOON-C-S
CS: Dep. Plant Pathology, Univ. Ill., Urbana, IL 61801, USA
SO: KOREAN JOURNAL OF MYCOLOGY 20(4): 315-323
PY: 1992
LA: English
AB: The effects of several parameters on **PCR** amplification in using **RAPD** were studied (using Hypoxylon truncatum). The results of this study suggest that approximately 15 ng of genomic **DNA** in 20 mu-l of reaction mixture results in discrete and reproducible **PCR** products. In addition, the results indicate that concentration or amounts of reaction components studied are highly inter-dependent in their effects, and RNA can interfere severely with **PCR** amplification. Suitable concentrations or amounts of reaction components were found to be 30 ng of 10-mer primer, 200 mu-M of dNTP, 0.001% gelatin 1.5 mM MgCl-2, 10 mM Tris-Cl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 2 units of Taq **DNA** polymerase, and 15 ng of RNase-treated genomic **DNA** in 25 mu-l of reaction mixture.

TI: DNA sequence variation and interrelationships among Colletotrichum species causing strawberry anthracnose.
AU: SREENIVASAPRASAD-S; BROWN-A-E; MILLS-P-R
CS: Dep. Applied Plant Science, Queen's University Belfast, Newforge Lane, Belfast BT9 5PX, UK
SO: PHYSIOLOGICAL AND MOLECULAR PLANT PATHOLOGY 41(4): 265-281
PY: 1992
LA: English
AB: Restriction fragment length polymorphisms (RFLPs) of the ribosomal **DNA** (r**DNA**) and mitochondrial **DNA** (mt**DNA**) of isolates of the strawberry (Fragaria ananassa) anthracnose pathogens Collectrichum acutatum, C. fragariae and C. gloeosporioides were analysed using r**DNA** from Saccharomyces carlsbergensis and mt**DNA** extracted from C. acutatum, C. fragariae and C. gloeosporioides as probes. These analyses revealed considerable heterogeneity within C. acutatum from divese hosts. The European strawberry isolates formed a discrete group while the American strawberry isolates fell into a broad group which included isolates from other hosts. No polymorphisms in either r**DNA** or mt**DNA** were observed among C gloeosporioides isolates from strawberry. C. fragariae isolates divided into groups with distinct r**DNA** and mt**DNA** patterns. Randoms amplified polymorphic **DNA** (**RAPD**) analysis grouped isolates in a similar manner to the mt**DNA** RFLP analysis. From sequencing data, the internally transcribed spacer (ITS) 1 region of the r**DNA** repeat unit of C. gloeosporioides, C. fragariae and C. acutatum was shown to be 171, 171 or 172 and 180 or 181 bases, respectively. C. gloeosporioides and C. fragariae differed from one another by only three to seven bases compared with C. acutatum which differed from C. gloeosporioides and C. fragariae by approx. 36-37 bases. ITS 1 data for C. acutatum generally support RFLP and **RAPD** taxonomies; differences of eight-eleven bases between European strawberry isolates and all other isolates studied being the greatest.

TI: Towards an integrated linkage map of common bean: 2. Development of an RFLP-based linkage map.
AU: NODARI-R-O; TSAI-S-M; GILBERTSON-R-L; GEPTS-P
CS: Dep. Agron. Range Sci., Univ. Calif., Davis, CA 95616-8515, USA
SO: THEORETICAL AND APPLIED GENETICS 85(5): 513-520
PY: 1993
LA: English
AB: A restriction fragment length polymorphism (RFLP)-based linkage map for common bean (Phaseolus vulgaris L.) covering 827 centiMorgans (cM) was developed based on a F-2 mapping population derived from a cross between BAT93 and Jalo EEP558. The parental genotypes were chosen because they exhibited differences in evolutionary origin, allozymes, phaseolin type, and for several agronomic traits. The segregation of 152 markers was analyzed, including 115 RFLP loci, 7 isozyme loci, 8 random amplified polymorphic **DNA** (**RAPD**) marker loci, and 19 loci corresponding to 15 clones of known genes, 1 virus resistance gene, 1 flower color gene, and 1 seed color pattern gene. Using MAPMAKER and LINKAGE-1, we were able to assign 143 markers to 15 linkage groups, whereas 9 markers remained unassigned. The average interval between markers was 6.5 cM; only one interval was larger than 30 cM. A small fraction (9%) of the markers deviated significantly from the expected Mendelian ratios (1:2:1 or 3:1) and mapped into four clusters. Probes of known genes belonged to three categories:

seed proteins, pathogen response genes, and Rhizobium response genes. Within each category, sequences homologous to the various probes were unlinked. The I gene for bean common mosaic virus resistance is the first disease resistance gene to be located on the common bean genetic linkage map.

TI: Genetic characterization of six parasitic protozoa: Parity between random-primer DNA typing and multilocus enzyme electrophoresis.
AU: TIBAYRENC-M; NEUBAUER-K; BARNABE-C; GUERRINI-F; SKARECKY-D; AYALA-F-J
CS: Dep. Ecol. Evolutionary Biol., Univ. California, Irvine, CA 92917, USA
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 90(4): 1335-1339
PY: 1993
LA: English
AB: We have assayed genetic polymorphisms in several species of parasitic protozoa by means of random amplified polymorphic **DNA (RAPD)**. One goal was to ascertain the suitability of **RAPD** markers for investigating genetic and evolutionary problems, particularly in organisms, such as the parasitic protozoa, unsuitable for traditional methods of genetic analysis. Another goal was to test certain hypotheses concerning Trypanosoma cruzi, and other protozoa, that have been established by multilocus enzyme electrophoresis. The **RAPD** results corroborate the hypothesis that the population structure of T. cruzi is clonal and yield a phylogeny of the clonal lineages in agreement with the one obtained by enzyme electrophoresis. This parity between the two sets of results confirms that **RAPD** markers are reliable genetic markers. The **RAPD** markers are also suitable for reconstructing species phylogenies and as diagnostic characters of species and subspecific lineages. The number of **DNA** polymorphisms that can be detected by the **RAPD** method seems virtually unlimited, since the number of primers can be increased effectively at will. The **RAPD** method is well suited for investigating genetic and evolutionary questions in certain organisms, because it is cost effective and demands no previous genetic knowledge about the organism.

TI: Artifactual variation in randomly amplified polymorphic DNA banding patterns.
AU: ELLSWORTH-D-L; RITTENHOUSE-K-D; HONEYCUTT-R-L
CS: Dep. Wildlife, Fisheries Sciences, Texas A and M Univ., College Station, TX 77843, USA
SO: BIOTECHNIQUES 14(2): 214-217
PY: 1993
LA: English
AB: Randomly amplified polymorphic **DNA (RAPD)** and arbitrarily primed **PCR** (AP-**PCR**) represent novel **DNA** polymorphism assays that involve the amplification of random **DNA** segments using **PCR** and oligonucleotide primers of arbitrary sequence. Products defining the polymorphisms exhibit Mendelian inheritance and thus possess tremendous potential utility as genetic markers in a diverse array of scientific disciplines. Amplification profiles for specific oligonucleotide primers are highly dependent on the specific conditions of the reaction; banding patterns may thus vary extensively because of inconsistencies in a number of reaction parameters. Artifactual variation represents a potential problem in surveys of genetic variation in natural populations and must be discriminated from true polymorphism for the applications of **RAPD** to be both accurate and reliable.

TI: Pedigree assessment using RAPD-DGGE in cereal crop species.
AU: DWEIKAT-I; MACKENZIE-S; LEVY-M; OHM-H
CS: Dep. Agron., Purdue Univ., West Lafayette, IN 47907, USA
SO: THEORETICAL AND APPLIED GENETICS 85(5): 497-505
PY: 1993
LA: English
AB: The introduction of molecular biology methodologies to plant improvement programs offers an invaluable opportunity for extensive germplasm characterization. However, the detection of adequate **DNA** polymorphism in self-pollinating species remains on obstacle. We have optimized a denaturing-gradient-gel-electrophoresis (DGGE) system which, when used in combination with random amplified polymorphic **DNA (RAPD)** analysis, greatly facilitates the detection of reproducible **DNA** polymorphism among closely related plant lines. We have used this approach to estimate pedigree relationships among a spectrum of plant materials in wheat, barley and oat. (Triticum aestivum, Hordeum vulgare, Avena sativa). Based on analysis with one or two primers, we were able to distinguish soft from hard winter wheat, and 2-rowed from 6-rowed barley. Further analysis with additional primers allowed resolution of polymorphisms even among closely related lines in highly selected populations. We placed 17 cultivars of oat into two distinct clusters that differed significantly from previous oat pedigree assessments. We believe that DGGE-**RAPD** is a superior method for detecting **DNA** polymorphism when compared to RFLP, agarose-**RAPD**, or polyacrylamide-**RAPD** methods.

TI: Genomic fingerprinting of Haemophilus somnus isolates by using a random-amplified polymorphic DNA assay.
AU: MYERS-L-E; SILVA-S-V-P-S; PROCUNIER-J-D; LITTLE-P-B
CS: Dep. Pathology, Univ. Guelph, Guelph, Ontario N1G 2W1, Can
SO: JOURNAL OF CLINICAL MICROBIOLOGY 31(3): 512-517
PY: 1993
LA: English
AB: The random-amplified polymorphic **DNA (RAPD)** assay was used to generate **DNA** fingerprints for 16 isolates of "Haemophilus somnus," and one isolate each of "Haemophilus agni," "Histophilus ovis," "Actinobacillus seminis," Pasteurella haemolytica, and Escherichia coli. The **RAPD** assay differentiated among "H. somnus" isolates, which shared similarity coefficients of 0.46 to 1.00 on the basis of pairwise comparisons of **RAPD** markers produced with nine random decamer primers. Three virulent encephalitic "H. somnus" isolates exhibited identical banding patterns, suggesting a common clonal ancestry. The **RAPD** assay clearly distinguished between the "H. somnus"-"H. agni"-"H. ovis" group and the other bacterial species tested. The results of the present study suggest that **DNA** fingerprinting of "H. somnus" isolates by the **RAPD** assay could be valuable in revealing subspecific divisions within this largely unexplored species.

TI: Trypanosoma congolense: Molecular characterization of a new genotype from Tsavo, Kenya.
AU: MAJIWA-P-A-O; MAINA-M; WAITUMBI-J-N; MIHOK-S; ZWEYGARTH-E
CS: Int. Lab. Res. Anim. Dis., P.O. Box 30709, Nairobi, Kenya
SO: PARASITOLOGY 106(2): 151-162
PY: 1993
LA: English
AB: Trypanosoma (Nannomonas) congolense comprises morphologically identical but genetically heterogeneous parasites infective to livestock and other mammalian hosts; three different genotypes of this parasite have been described previously. Restriction enzyme fragment length polymorphisms (RFLPs) in both kinetoplast **DNA** minicircle and nuclear **DNA** sequences, and randomly amplified polymorphic deoxyribonucleic acid (**RAPD**) patterns have been used here to demonstrate the existence of another type of T. (N.) congolense that is genotypically distinct from those that have so far been characterized at the molecular level. A highly repetitive, tandemly arranged **DNA** sequence and oligonucleotide primers, for use in polymerase chain reaction (**PCR**) amplification are described, which can be used for specific identification of the trypanosome and its distinction from others within the Nannomonas subgenus.

TI: Genetic variation in monoploids of diploid potatoes and detection of clone-specific random amplified polymorphism DNA markers.
AU: SINGSIT-C; OZIAS-AKINS-P
CS: Dep. Hortic., Univ. Ga., Coastal Plain Exp. Stn., Tifton, GA 31793-0748, USA
SO: PLANT CELL REPORTS 12(3): 144-148
PY: 1993
LA: English
AB: Randomly amplified polymorphic **DNA** (**RAPD**) markers has been used to study the genetic variation among androgenic monoploids of diploid Solanum species. Cluster analysis of pairwise genetic distances was used to construct a genetic relationship among anther donor and anther-derived potato (S. tuberosum) plants. The clustering based on Rogers' distances resembled classifications based on parental origins and hybrid combinations. Six of the 32 **RAPD** primers used resulted in the selective amplifications of **DNA** fragments which were polymorphic between the two S. phureja parental clones, 1.22 and A95. It should be possible to construct a genetic linkage map, without making crosses, using monoploids derived from a single heterozygous dipliod clone and **RAPD** markers.

TI: Genetic and physical analysis of the rice bacterial blight disease resistance locus, Xa21.
AU: RONALD-P-C; ALBANO-B; TABIEN-R; ABENES-L; WU-K-S; MCCOUCH-S, TANKSLEY-S-D
CS: Dep. Plant Pathol., Univ. California Davis, Davis, CA 95165, USA
SO: MOLECULAR & GENERAL GENETICS 236(1): 113-120
PY: 1992
LA: English
AB: Nearly isogenic lines (NILs) of rice (Oryza sativa) differing at a locus conferring resistance to the pathogen Xanthomonas oryzae pv. oryzae were survived with 123 **DNA** markers and 985 random primers using restriction fragment length polymorphisms (RFLP) and random amplified polymorphic **DNA** (**RAPD**) and random amplified polymorphic **DNA** (**RAPD**) analysis. One chromosome 11 marker (RG103) detected polymorphism between the NILs that consegregated with Xa21. All other chromosome 11 **DNA** markers tested were monomorphic between the NILs, localizing the Xa21 introgressed region to an 8.3 cM interval on chromosome 11. Furthermore, we identified two polymerase chain reaction (**PCR**) products (**RAPD2148** and **RAPD818**) that detected polymorphisms between the NILs. Genomic sequences hybridizing with **RAPD818**. **RAPD248** and RG103 were duplicated specifically in the Xa21 NIL. All three markers cosegregated with the reistance locus, Xa11, in a F-2 population of 386 progeny. Based on the frequency with which we recovered polymorphic Xa21-linked markers, we estimated the physical size of the introgressed region to be approximately 800 kb. This estimation was supported by physical mapping (using pulsed field gel electrophoresis) of the sequences hybridizing with the three Xa21-linked **DNA** markers. The results showed that the three Xa21-linked markers are physically close to each other, with one copy of the **RAPD818** sequences located within 6 kb of **RAPD248** and the other copy within 270 kb of RG103. None of the enzymes tested generated a **DNA** fragment that hybridized with all three of the markers indicating that the introgressed region containing the reistance locus Xa21 is probably larger than 270 kb.

TI: Use of RAPD markers to document the origin of the intergeneric hybrid X Margyracaena skottsbergii (Rosaceae) on the Juan Fernandez Islands.
AU: CRAWFORD-D-J; BRAUNER-S; COSNER-M-B; STUESSY-T-F
CS: Dep. Plant Biol., the Ohio State Univ., Columbus, OH 43210, USA
SO: AMERICAN JOURNAL OF BOTANY 80(1): 89-92
PY: 1993
LA: English
AB: Bands of random amplified polymorphic **DNA** (**RAPDs**) were used as markers to test the hypothesis that the species times Margyracaena skottsbergii which is endemic to the island of Masatierra in the Juan Fernandez archipelago, represents an intergeneric hybrid between Acaena argentea and Margyricarpus digynus. Thirteen 10-me primers produced 18 consistent species-species bands for A. argentea and 27 for M. digynus, with all 45 bands present in the presumed hybrid times Margyracaena. A second species of Acaena on Masatierra, A. ovalifolia, has 23 unique amplified bands in all plants examined, and it shares none of these bands with times Margyracaena. The data from **RAPDs** are concordant with morphology in implicating Acaena argentea rather than A. ovalfolia as one parent of times Margyracaena. **RAPDs** can provide numerous genetic markers while requiring minimal quantities of **DNA**, thereby making them attractive for the study of hybridization, particularly in rare plants where **DNA** amounts may be limiting.

TI: Fast DNA isolation from Histoplasma capsulatum: Methodology for arbitrary primer polymerase chain reaction-based epidemiological and clinical studies.
AU: WOODS-J-P; KERSULYTE-D; GOLDMAN-W-E; BERG-D-E
CS: Dep. Genet., Box 8230, Washington Univ. Med. Sch., St. Louis, MO 63110
SO: JOURNAL OF CLINICAL MICROBIOLOGY 31(2): 463-464
PY: 1993
LA: English
AB: The arbitrary primer polymerase chain reaction (also called random amplified polymorphic **DNA**, or **RAPD**) is a **DNA** fingerprinting method that provides an efficient, sensitive way of discriminating between independent isolates of Histoplasma capsulatum, but its widespread application has been hampered by the arduous 2-day procedure traditionally used to extract **DNA** from H. capsulatum. We present here a quick (approx 2-h) extraction method and show that the resultant **DNA** is suitable for sensitive and reproducible identification of individual strains of this pathogenic fungus.

TI: Use of RAPD markers in potato genetics: Segregations in diploid and tetraploid families.
AU: QUIROS-C-F; CEADA-A; GEORGESCU-A; HU-J
CS: Dep. Veg. Crops, Univ. Calif., Davis, CA 95616
SO: AMERICAN POTATO JOURNAL 70(1): 35-42
PY: 1993
LA: English
AB: The objective of the present study was to evaluate random amplified polymorphic **DNA** (**RAPD**) as a source of markers for use in investigations in potato genetics. Segregation of 18 loci in diploid Solanum goniocalyx times S. phureja and 12 loci in tetraploid S. tuberosum ssp. tuberosum times S. tuberosum ssp. andigena families fitted Mendelian and tetrasomic ratios, respectively. Eight loci in the diploid progeny were arranged in three linkage groups. Segregations of these markers fitting expected fitting expected ratios indicate that they can be effectively used in potato genetics, breeding and evolution.

TI: Use of RAPD markers to screen somatic hybrids between Solanum tuberosum and Solanum brevidens.
AU: XU-Y-S; CLARK-M-S; PEHU-E
CS: Dep. Crop Production, Univ. Helsinki, SF-00710 Helsinki, Finl
SO: PLANT CELL REPORTS 12(2): 107-109
PY: 1993
LA: English
AB: The identification of somatic hybrids between Solanum tuberosum and S. brevidens can be carried out using polymerase chain reaction (**PCR**) and arbitrary 10-mer primers to generate random amplified polymorphic **DNA** (**RAPD**) markers. Five commercial primers have been tested. Each primer directed the amplification of a genome-specific "fingerprint" for the fusion parents and S. brevidens. The size of the amplified **DNA** fragments ranged from 100 to 1800 base pairs. The somatic hybrids showed a combination of the parental banding profiles with four of the five primers surveyed, whereas regenerants from one of the parents had the same or a similar banding pattern to that of the parent. Thus **RAPD** markers provide a quick, simple and preliminary screening method for putative somatic hybrids.

TI: Analysis of genetic stability of plants regenerated from suspension cultures and protoplasts of meadow fescue (Festuca pratensis Huds.).
AU: VALLES-M-P; WANG-Z-Y; MONTAVON-P; POTRYKUS-I; SPANGENBERG-G
CS: Inst. Plant Sci., Swiss Federal Inst. Technol., CH-8092 Zurich, Switz
SO: PLANT CELL REPORTS 12(2): 101-106
PY: 1993
LA: English
AB: A cytological and molecular analysis was performed to assess the genetic uniformity and true-to-type character of plants regenerated from 20 week-old embryogenic suspension cultures of meadow fescue (Festuca pratensis Huds.), and compared to protoplast-derived plants obtained from the same cell suspension. Cytological variation was not observed in a representative sample of plants regenerated directly from the embryogenic suspensions and from protoplasts isolated therefrom. Similarly, no restriction fragment length polymorphisms (RFLPs) were detected in the mitochondrial, plastid and nuclear genomes in the plants analyzed. Randomly amplified polymorphic **DNA** markers (**RAPDs**) have been used to characterise molecularly a set of mature meadow fescue plants regenerated from these in vitro cultures. **RAPD** markers using 18 different short oligonucleotide primers of arbitrary nucleotide sequence in combination with polymerase chain reaction (**PCR**) allowed the detection of pre-existing polymorphisms in the donor genotypes, but failed to reveal newly generated variation in the protoplast-derived plants compared to their equivalent suspension-culture regenerated materials. The genetic stability of meadow fescue plants regenerated from suspension cultures and protoplasts isolated therefrom and its implications on gene transfer technology for this species are discussed.

TI: Polymerase chain reaction approaches to Culicoides (Diptera: Ceratopogonidae) identification.
AU: RAICH-T-J; ARCHER-J-L; ROBERTSON-M-A; TABACHNICK-W-J; BEATY-B-J
CS: Dep. Microbiol., Colorado State University, Fort Collins, CO 80523, USA
SO: JOURNAL OF MEDICAL ENTOMOLOGY 30(1): 228-232
PY: 1993
LA: English
AB: Molecular genetic approaches such as polymerase chain amplification (**PCR**) of target genomic sequences are finding wide application in systematic and taxonomic studies of arthropods. **PCR**-based techniques that preclude the need for target **DNA** sequence information of the species of interest facilitate molecular taxonomic studies. Two such techniques, **tDNA-PCR** (**DNA** encoding tRNAs is the analyte) and **RAPD-PCR** (randomly amplified polymorphic **DNA**) were investigated for their ability to differentiate certain North American Culicoides spp. larvae and adults.

TI: The identification of Y chromosome-linked markers with random sequence oligonucleotide primers.
AU: WARDELL-B-B; SUDWEEKS-J-D; MEEKER-N-D; ESTES-S-S; WOODWARD-S-R; TEUSCHER-C
CS: Dep. Microbiol., Brigham Young Univ., 873 WIDB, Provo, Utah 84602, USA
SO: MAMMALIAN GENOME 4(2): 108-112
PY: 1993
LA: English
AB: The polymerase chain reaction (**PCR**)-based technique of random amplification of polymorphic **DNA** (**RAPD**) is extremely useful for developing **DNA**-based markers. We previously identified a linkage group of eight unmapped **RAPD** markers that distinguish C57BL/6J and DBA/2J mice (Mammalian Genome 3: Woodward et al., 73-78, 1992). In this study, we report that all eight markers are Y Chromosome (Chr)-linked. One additional Y-linked **RAPD** was discovered serendipitously during the screening of a C3H/HeJ times (C3H/HeJ times SJL/J)F-1 BC1 population. The segregation of all nine markers was analyzed with a panel of 14 independent inbred strains of male mice. The nine markers could be divided into three distinct groups: (1) DYByu2, DYByu5, DYByu6, and DYByu8 identify both the M.m. musculus and M.m domesticus type Y Chr; (2) DYByu1, DYByu3, DYByu4, and DY-Byu7 is specific for the M.m. domesticus type. The results clearly indicate that the **RAPD** technique can be used to identify Y Chr-linked, **DNA**-based markers in mammalian species.

TI: Characterization of a Red Jungle Fowl by White Leghorn backcross reference population for molecular mapping of the chicken genome.
AU: CRITTENDEN-L-B; PROVENCHER-L; SANTANGELO-L; LEVIN-I; ABPLANALP-H; BRILES-R-W; BRILES-W-E; DODGSON-J-B
CS: Dep. Microbiol., Giltner Hall, Mich. State Univ., East Lansing, MI 48824
SO: POULTRY SCIENCE 72(2): 334-348
PY: 1993
LA: English
AB: A reference population designed for molecular genetic mapping of the chicken genome was produced by backcrossing a partially inbred Red Jungle Fowl (JF) line to a highly inbred White Leghorn (WL) line. The parental lines were chosen to maximize the expected genetic polymorphisms between them. Two full-sib F-1 males, produced by crossing a JF male with a WL female, were each individually mated to about 10 WL females to produce 400 progeny. All the progeny were classified for segregation of three loci controlling color phenotype and six blood group loci, some of which have been mapped by classical methods. Segregation of these nine loci did not differ significantly from the expected 1:1 ratio with one exception. At least 20 mL of whole blood was stored from all the parents and progeny to provide **DNA** for molecular analysis. Screening of the parental lines and F-1 crosses by Southern blot with cloned genes and by the random amplified polymorphic **DNA** (**RAPD**) procedure revealed a large number of molecular markers that were parental line-specific. A preliminary analysis of 16 backcross progeny classified for polymorphisms at 2 color loci, 6 blood group loci, 16 loci detected by cloned chicken genes, and 4 loci detected by the **RAPD** method has been completed. Segregation at 27 out of 28 loci did not differ significantly from the expected 1:1 ratio, showing that two alternative alleles were detected at each locus. Five pairs of linked loci were detected (P ltoreq .01). Thus, this population is polymorphic and gives simple segregation for two types of molecular probes, providing a good resource for collaborative mapping of the chicken genome.

TI: Identification of four major Meloidogyne spp. by random amplified polymorphic DNA (RAPD-PCR).
AU: CENIS-J-L
CS: CRIA, La Alberca, Spain
SO: PHYTOPATHOLOGY 83(1): 76-78
PY: 1993
LA: English
AB: The random amplified polymorphic **DNA** (**RAPD**) assay is a variant of the polymerase chain reaction (**PCR**) in which a single primer of random sequence is used at a low-annealing temperature. Twenty-two primers were evaluated for their usefulness in identifying 18 populations of Meloidogyne incognita, M. arenaria, M. javanica, and M. hapla collected worldwide. Reactions with primer ORA-01 produced amplified **DNA** bands whose size allowed the separation of the four species. Reactions with another group of 11 primers made possible the separation of one, two, or three species. Numerous polymorphisms were found between one population of race A and one of race B of M. hapla as well as between populations of M. arenaria. In contrast, the four races of M. incognita were indistinguishable.

TI: The random amplification of polymorphic DNA allows the identification of strains and species of schistosome.
AU: NETO-E-D; SOUZA-C-P-D; ROLLISON-D; KATZ-N; PENA-S-D-J; SIMPSON-A-J-G
CS: Centro de Pesquisas 'Rene Rachou', Avenida Augusto de Lima, 1715, Belo horinzonte, 30.190, Minas Gerais, Brazil
SO: MOLECULAR AND BIOCHEMICAL PARASITOLOGY 57(1): 83-88
PY: 1993
LA: English
AB: The use of arbitrarily selected primers (10-24 nucleotides) and very low stringency annealing conditions (30 degree C followed by 40 degree C) for the polymerase chain reaction amplification of 1.0 ng of schistosome **DNA** resulted in relatively complex patterns of products. Amongst the primers tested some, for example 5'-TCGTAGCCAA, produced patterns that included bands that were polymorphic between strains of Schistosoma mansoni. Other primers, for example 5'-TCACGATGCA, produced apparently identical products using **DNA** from 5 S. mansoni strains but highly variable patterns when **DNA** from different schistosome species (e.g., S. haematobium, S. bovis, S. intercalatum, S. curassoni, S. mattheei, S. margrebowiei, and S. vodhaini) was used. The results indicate that the random amplification of polymorphic **DNA** (**RAPD**) may be an extremely useful approach to the identification of schistosome strains and species.

TI: Genetic fingerprinting in cauliflower by the RAPD method and determination of the level of inbreeding in a set of F-1 hybrid seeds.
AU: BOURY-S; LUTZ-I; GAVALDA-M-C; GUIDET-F; SCHLESSER-A
CS: Groupement d'interet Publique, Prince de Bretagne Biotechnologie, Penn Ar Prat, 29250 Saint-Pol-de-Leon, France

SO: AGRONOMIE (PARIS) 12(9): 669-681
PY: 1992
LA: French
AB: F-1 hybrids varieties of cauliflower (Brassica oleracea var botrytis), which produced homogeneous crops, are popular with growers. However, the production of these hybrid seeds is difficult and they may be contaminated by inbreds is seeds arising from self-fertilization of the maternal line. The level of inbred occurrence is therefore determined before marketing the hybrid to avoid problems with th crop. At the moment this is carried out by a phenotypical test. In order to save time and ensure non-ambiguous results, detection of the level of inbreds is proposed via a new molecular biology technique, the **RAPD** (random amplified polymorphic **DNA**) method, which is based on **PCR** (polymerase chain reaction) amplifications each carried out with a single primer, the short sequence of which has been randomly written. The goal is obtain a marker of the paternal line which can only be detected in hybrids. To adapt this method to our plant material, we tested and modified various **PCR** parameters. We were thus able to determine that the annealing temperature and the primer concentration act upon amplifications. The **RAPD** method therefore proved to be a reliable and fast tool for assessing the purity of cauliflower F-1 hybrid seed.

TI: PCR-based RFLP analysis of DNA sequence diversity in the gastric pathogen Helicobacter pylori.
AU: AKOPYANZ-N; BUKANOV-N-O; WESTBLOM-T-U; BERG-D-E
CS: Department Molecular Microbiology, Campus Box 8230, Washington University Medical School, St. Louis, MO 63110
SO: NUCLEIC ACIDS RESEARCH 20(23): 6221-6225
PY: 1992
LA: English
AB: **DNA** sequence diversity among 60 independent isolates of the gastric pathogen Helicobacter pylori was assessed by testing for restriction fragment length polymorphisms (RFLPs) in several **PCR**-amplified gene segments. 18 MboI and 27 HaeIII RFLPs were found in the 2.4 kb ureA-ureB (urease) segment from the 60 strains; this identified 44 separate groups, with each group containing one to four isolates. With one exception, each isolate not distinguished from the others by RFLPs in ureA-ureB was distinguished by MboI digestion of the neighboring 1.7 kb ureC-ureD segment. The 1.5 kb flaA (flagellin) gene, which is not close to ure gene cluster, was also highly polymorphic. In contrast, isolates from initial and followup biopsies yielded identical restriction patterns in each of the three cases tested. The potential of this method for detecting population heterogeneity was tested by mixing **DNAs** from different strains before amplification: the arrays of restriction fragments obtained indicated co-amplification from both genomes in each of the five pairwise combinations tested. These results show that H. pylori is a very diverse species, that indicate **PCR**-based RFLP tests are almost as sensitive as arbitrary primer **PCR** (**RAPD**) tests, and suggest that such RFLP tests will be useful for direct analysis of H. pylori in biopsy and gastric juice specimens.

TI: Detection and differentiation of Colletotrichum gloeosporioides isolates using PCR.
AU: MILLS-P-R; SREENIVASAPRASAD-S; BROWN-A-E
CS: Dep. Mycology, Plant Pathology, Queen's Univ. Belfast, Newforge Lane, Belfast BT9 5PX, UK
SO: FEMS (FEDERATION OF EUROPEAN MICROBIOLOGICAL SOCIETIES) MICROBIOLOGY LETTERS 98(1-3): 137-144
PY: 1992
LA: English
AB: An oligonucleotide primer (CgInt), synthesized from the variable internally transcribed spacer (ITS) 1 region of ribosomal **DNA** (r**DNA**) of Colletotrichum gloeosporioides was used for **PCR** with primer ITS4 (from a conserved sequence of the r**DNA**) to amplify a 450-bp-fragment from the 25 C. gloesporioides isolated tested. This specific fragment was amplified from as little as 10 fg of fungal **DNA**. A similar sized fragment was amplified from **DNA** extracted from C. gloeosporiodes-infected tomato tissue. **RAPD** analysis divided 39 C. gloeosporioides isolates into more than 12 groups linked to host source geographic origin. Based on the results obtained, the potential of **PCR** for detection and differentiation of C. gloeosporioides is discussed.

TI: Use of genetic polymorphisms detected by the random-amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) for differentiation and identification of Aedes aegypti subspecies and populations.
AU: BALLINGER-CRABTREE-M-E; BLACK-W-C-IV; MILLER-B-R
CS: Medical Entomology Ecology Branch, Division Vector-Borne Infectious Diseases, Cent. Dis. Control, PO Box 2087, Fort Collins, CO 80522
SO: AMERICAN JOURNAL OF TROPICAL MEDICINE AND HYGIENE 47(6): 893-901
PY: 1992
LA: English
AB: Amplification of random regions of genomic **DNA** using 10-base primers in the random-amplified polymorphic **DNA** polymerase chain reaction (**RAPD-PCR**) was used to differentiate and identify mosquito populations based on genetic variation. Genomic DN was extracted from individual mosquitoes from 11 geographic populations of Aedes aegypti and amplified in **PCR** reactions using single primers of arbitrary nucleotide sequence. Discriminant analysis of the population frequencies of **RAPD** fragments produced using three different primers allowed accurate discrimination between the geographic populations in 89% of individuals and between subspecies (Ae. aegypti aegypti versus Ae. Aegypti formosus) in 100% of mosquitoes tested. The gentic relatedness of the populations was estimated using three different statistical methods, and unknown populations were correctly classified in a blind test. These results indicate that the **RAPD-PCR** technique will be useful in studies of arthropod molecular taxonomy and in epidemilogic studies of the relatedness of geographic populations and vector movement.

TI: Ribosomal DNA and RAPD variation in the rare plant family Lactoridaceae.
AU: BRAUNER-S; CRAWFORD-D-J; STUESSY-T-F
CS: Dep. Biol., Ashland Univ., Ashland, Ohio 44805
SO: AMERICAN JOURNAL OF BOTANY 79(12): 1436-1439
PY: 1992
LA: English
AB: Lactoris fernandeziana, endemic to the island of Masatierra in the Juan Fernandez Archipelago, is the only living member of the primitive angiosperm family, Lactoridaceae. The species was surveyed for ribosomal **DNA** (r**DNA**) and **RAPD** (Random

amplified polymorphic **DNA**) variation. Previous analyses of allozymes had revealed no variation within the species. Variation was found for length in the intergenic spacer and for restriction sites in the 18S-25S genes of **rDNA**, and for the presence of amplified bands using 16 primers. Different **rDNA** repeat lengths and restriction site variants were detected within individuals as well as within and among populations. The level of variation in **RAPDs** is low relative to other Juan Fernandez endemic species surveyed, and nearly all variants were restricted to single populations. The **rDNA** length variants were distributed throughout the island, whereas the **rDNA** restriction site variants and **RAPD** markers indicated minor genetic differences among the populations.

TI: Characterization of genetic identities and relationships of Brassica oleracea L. via a random amplified polymorphic DNA assay.
AU: KRESOVICH-S; WILLIAMS-J-G-K; MCFERSON-J-R; ROUTMAN-E-J; SCHAAL-B-A
CS: US Dep. Agric., Agric. Res. Serv., Plant Genetic Resources Unit, Cornell Univ., Geneva, N.Y. 14456-0462
SO: THEORETICAL AND APPLIED GENETICS 85(2-3): 190-196
PY: 1992
LA: English
AB: Effective conservation and the use of plant genetic resources are essential for future agricultural progress. Critical to this conservation effort is the development of genetic markers which not only distinguish individuals and accessions but also reflect the inherent variation and genetic relationships among collection holdings. We have examined the applicability of the random amplified polymorphic **DNA (RAPD)** assay for quick, cost-effective, and reliable use in addressing these needs in relation to collection organization and management. Twenty-five decamer oligonucleotide primers were screened individually with a test array composed of individuals representing a range of genetic relationships in Brassica oleracea L. (vegetable and forage cole crops). Over 140 reproducible, polymorphic fragments were generated for study. Each individual of the test array exhibited a unique molecular genotype and composites specific for accessions and botanical varieties could be established. An analysis of similarity based on amplified **DNA** fragments reflected the known genetic relationships among the selected entries. These results demonstrated that **RAPD** markers can be of great value in gene bank management for purposes of identification, measurement of variation, and establishment of genetic similarity at the intraspecific level.

TI: Segregating random amplified polymorphic DNAs (RAPDs) in Betula alleghaniensis.
AU: ROY-A; FRASCARIA-N; MACKAY-J; BOUSQUET-J
CS: Centre de Recherche Biologie Forestiere, Faculte de Foresterie de Geomatique, Universite Laval, Saint-Foy, Quebec, Canada G1K 7P4
SO: THEORETICAL AND APPLIED GENETICS 85(2-3): 173-180
PY: 1992
LA: English
AB: Molecular markers are currently being developed for Betula alleghaniensis Britton using random amplified polymorphic **DNA (RAPD)**. Arbitrarily designed 11-mer primers were tested on three intraspecific controlled crosses for which more than 15 full-sibs were available. Using two of these primers, we were able to genetically characterize a total of nine polymorphic **RAPD** markers. Segregation of these markers was consistent with a biparental diploid mode of inheritance, and all appeared dominant. **RAPDs** were valuable in detecting contaminants and, therefore, in assessing the validity of controlled crosses. Limitations of the technique are discussed in relation to the determination of parental genotypes and construction of linkage maps for hardwood species.

TI: Genotypic identification and characterization of species and strains within the genus Candida by using random amplified polymorphic DNA.
AU: LEHMANN-P-F; LIN-D; LASKER-B-A
CS: Dep. Microbiol., Med. Coll. of Ohio, P.O. Box 10008, Toledo, Ohio 43699-0008
SO: JOURNAL OF CLINICAL MICROBIOLOGY 30(12): 3249-3254
PY: 1992
LA: English
AB: Random amplified polymorphic **DNA (RAPD)** was used to better characterize the genotypic relatedness among medically important Candida species. By using short oligomer primers (10-mers) with arbitrarily chosen sequences in the polymerase chain reaction, distinctive and reproducible sets of polymerase chain reaction products were observed for isolates of C. albicans, C. lusitaniae, C. tropicalis, and Torulopsis (Candida) glabrata. The **RAPD** analysis differentiated a physiologically homogeneous panel of C. parapsilosis into three distinct groups and showed genetic diversity within C. haemulonii. Intraspecices **DNA**-length polymorphisms were seen for **RAPD** profiles derived from different isolates of each species. Analysis of **RAPDs** from a panel of C. albicans, which included 16 laboratory derivatives of two reference strains, showed that the profiles of unrelated strains differed and that the derivatives of each reference strain were identifiable. Minor differences in the **RAPD** profiles, suggestive of mutations that had occurred during the long-term maintenance of the strains, were detected. Because of its ease and reliability, **RAPD** analysis should be useful in providing genotypic characters for taxonomic descriptions, for confirming the identities of stock isolates, for typing Candida species in epidemiologic investigations, and for use in the rapid identification of pathogenic fungi.

TI: Diversity among clinical isolates of Histoplasma capsulatum detected by polymerase chain reaction with arbitrary primers.
AU: KERSULYTE-D; WOODS-J-P; KEATH-E-J; GOLDMAN-W-E; BERG-D-E
CS: Dep. Mol. Microbiol., Washington University Med. Sch., St. Louis, Missouri 63110
SO: JOURNAL OF BACTERIOLOGY 174(22): 7075-7079
PY: 1992
LA: English
AB: Clinical isolates of the fungal respiratory and systemic pathogen Histoplasma capsulatum have been placed in several different classes of using genomic restriction fragment length polymorphisms (RFLPs), but in general have not been

distinguished further. We report here that a polymerase chain reaction (**PCR**)-based **DNA** fingerprinting method that has been termed arbitary primer or random amplified polymorphic **DNA** (**RAPD**) **PCR** can distinguish among isolates in a single RFLP class. In this method, arbitrarily chosen oligonucleotides are used to prime **DNA** synthesis from genomic sites that they fortuitously match, or almost match, to generate strain-specific arrays of **DNA** fragments. Each of 29 isolates of RFLP class 2, the group endemic in the American Midwest was distinguished by using just three arbitray primers. In contrast, laboratory-derived S and E colony morphology variants of two strains were not distinguished from their R parents by using 18 such primers. Thus, the clinical isolates of H. capsulatum are quite diverse, but their genomes remain stable during laboraory culture. These outcomes suggest new possibilities for epidemiological analysis and studies of fungal populations in infected hosts.

TI: Random amplified polymorphic DNA of mosquito species and populations (Diptera: Culicidae): Techniques, statistical analysis, and applications.
AU: KAMBHAMPATI-S; BLACK-W-C-IV; RAI-K-S
CS: Dep. Biological Sciences, Univ. Notre Dame, Notre Dame, IN 46556
SO: JOURNAL OF MEDICAL ENTOMOLOGY 29(6): 939-945
PY: 1992
LA: English
AB: A polymerase chain reaction (**PCR**)-based method is described for the identification and differentiation of mosquito species and populations. The method, described first by Williams et al. (1990), employs single 10 base-long primers of arbitrary **DNA** sequence and results in the amplification of random segments of **DNA** known as random amplified polymorphic **DNA** (**RAPD**). We wished to determine if **RAPD** of mosquito **DNA** could be used for the differentiation of species and populations, identification of unknown specimens, and the reconstruction of phylogeny. **RAPD** of mosquito **DNA** results in the amplification of a series of **DNA** fragments of varying length. Most amplified fragments are unique to an individual; however, our data indicated that in each of the five species of Aedes examined, some fragments are species-specific and are present in all individuals of that species. This enabled us to derive a diagnostic profile for each of the five species. A nearest-neighbor analysis of all the amplified **DNA** fragments discriminated among species on a multivariate basis. Several individuals of Aedes albopictus (Skuse), included in the analysis as "unknowns," were correctly identified as belonging to Ae. albopictus. UPGMA clustering of presence-absence data enabled the separation of different Aedes species as well as different populations of Ae. albopictus. The entomological applications of **RAPD** include the construction of diagnostic profiles for species identification and differentiation among conspecific populations. (Other species mentioned are A. katharinensis, A. riversi and A. polynesiensis.)

TI: Patterns of inheritance with RAPD molecular markers reveal novel types of polymorphism in the honey bee.
AU: HUNT-G-J; PAGE-R-E-JR
CS: Dep. Entomology, Univ. California, Davis, Calif. 95616
SO: THEORETICAL AND APPLIED GENETICS 85(1): 15-20
PY: 1992
LA: English
AB: The polymerase chain reaction (**PCR**) was used to generate random amplified polymorphic **DNA** (**RAPD**) from honey bee **DNA** samples in order to follow the patterns of inheritance of **RAPD** markers in a haplodiploid insect. The genomic **DNA** samples from two parental bees (Apis mellifera), haploid drone and a diploid queen, were screened for polymorphism with 68 different ten-nucleotide primers of random sequence. Parents were scored for the presence or absence of individual bands. An average of 6.3 bands and 1.3 polymorphisms for presence/absence were observed per primer between the parents. Thirteen of these primers were used to determine the inheritance of **RAPD** marker alleles in the resulting progeny and in haploid drones from a daughter queen. Four types of polymorphisms were observed. Polymorphisms for band presence/absence as well as for band brightness were inherited as dominant marker, meeting Mendelian expectations in haploid and diploid progeny. Polymorphisms for fragment-length were also observed. These segregated in a near 1:1 ratio in drone progeny. The last type of polymorphism was manifested as a diploid-specific band. Mixing of amplification products after **PCR** showed that the diploid-specific band was the result of heteroduplex formation from the **DNA** of alternate alleles in heterozygotes. In two of the four cases of heteroduplex formation, the alternative alleles were manifested as small fragment-length polymorphisms, resulting in co-dominant markers. This is the first demonstration that a proportion of **RAPD** markers are not inherited in a dominant fashion.

TI: Use of randomly amplified polymorphic DNA markers to distinguish isolates of Aspergillus fumigatus.
AU: AUFAUVRE-BROWN-A; COHEN-J; HOLDEN-D-W
CS: Dep. Infectious Diseases Bacteriology, Royal Postgradauate Medical School, Hammersmith Hospital, Du Cane Road, London W12 0NN, U.K
SO: JOURNAL OF CLINICAL MICROBIOLOGY 30(11): 2991-2993
PY: 1992
LA: English
AB: Forty-four oligonucleotide decamers were tested for their abilities to generate randomly amplified polymorphic **DNA** (**RAPD**) markers from genomic **DNAs** of three different isolates of Aspergillus fumigatus. Seven primers generated **RAPDs** that allowed the three isolates to be differentiated; one of the primers also yielded a unique **RAPD** pattern in each of an additional six fungal isolates, demonstrating the utility of this technique for distinguishing between A. fumigatus isolates.

TI: DNA diversity among clinical isolates of Helicobacter pylori detected by PCR-based RAPD fingerprinting.
AU: AKOPYANZ-N; BUKANOV-N-O; WESTBLOM-T-U; KRESOVICH-S; BERG-D-E
CS: Dep. Mol. Microbiol., Campus Box 8230, Washington Univ. Med. Sch., St. Louis, Mo. 63110
SO: NUCLEIC ACIDS RESEARCH 20(19): 5137-5142
PY: 1992
LA: English
AB: The **RAPD** (or AP-**PCR**) **DNA** fingerprinting method was used to distinguish among clinical isolates of Helicobacter pylori, a bacterium whose long term carriage is associated with gastritis, peptic ulcers and gastric carconomas. This method uses arbitrarily chosen oligonucleotides to prime **DNA** synthesis from genomic sites to which they are fortuitously matched, or almost matched. Most 10-nt primers with gtoreq 60% G + C yielded strain-specific arrays of up to 15 prominent fragments, as did most

longer (gtoreq 17-nt) primers, whereas most 10-nt primers with 50% G + C did not. Each of 64 independent H. pylori isolates, 60 of which were from patients in the same hospital, was distinguishable with a single **RAPD** primer, which suggests a high level of **DNA** sequence diversity within this species. In contrast, isolates from initial and followup biopsies were indistinguishable in each of three cases tested.

TI: Detection of genetic variation between and within populations of Gliricidia sepium and Gliricidia maculata using RAPD markers.
AU: CHALMERS-K-J; WAUGH-R; SPRENT-J-I; SIMONS-A-J; POWELL-W
CS: Scottish Crop Res. Inst., Invergowrie, Dundee DD2 5DA, Scotland
SO: HEREDITY 69(5): 465-472
PY: 1992
LA: English
AB: Gliricidia sepium and G. maculata are multi-purpose leguminous trees native to Central America and Mexico. Research programs have been initiated to define the native distribution of Gliricidia and sample the spectrum of genetic variation. To date, there has been little systematic assessment of genetic variability in multi-purpose tree species. Accurate estimates of diversity between- and within-populations are considered a prerequisite for the optimization of sampling and breeding strategies. We have used a **PCR**-based polymorphic assay procedure (**RAPDs**) to monitor genetic variability in Gliricidia. Extensive genetic variability was detected between species and the variability was partitioned into between- and within-population components. On average, most (60 per cent) of the variation occurs between G. sepium populations but oligonucleotide primers differed in their capacity to detect variability between an dwithin populations. Population-specific genetic markers were identified. **RAPDs** provide a cost-effective method for the precise and routine evaluation of variability and may be used to identify areas of maximum diversity. The approaches outlined have general applicability to a range of organisms and are discussed in relation to the exploitation of multipurpose tree species of the tropics.

TI: Sequence-tagged-site-facilitated PCR for barley genome mapping.
AU: TRAGOONRUNG-S; KANAZIN-V; HAYES-P-M; BLAKE-T-K
CS: Plant Soil Sci. Dep., Montana State University, Bozeman, MT 59717
SO: THEORETICAL AND APPLIED GENETICS 84(7-8): 1002-1008
PY: 1992
LA: English
AB: Speed, efficiency, and safety considerations have led many genome mapping projects to evaluate polymerase chain reaction (**PCR**) sequence amplification as an alternative to Southern blot analysis. However, the availability of informative primer sequences can be a limiting factor in **PCR**-based mapping. An alternative to random amplified polymorphism detection (**RAPD**) is the sequence-tagged-site (STS) approach. If informative primer sequences could be derived from known sequences, then current maps, which are based on both known function and anonymous clones, might be easily converted to maps utilizing **PCR** technology. In this paper, four pairs of primer sequences were obtained from published sequences, and four pairs were obtained by sequencing portions of **DNA** clones from genomic clones derived from a random genomic library used in the North American Barley Genome Mapping Project (NABGMP). These primers were used to screen for polymorphisms in the progeny of a winter times spring and a spring times spring barley cross. Two types of polymorphisms were distinguished using these primer sets: (1) insertion/deletion events that could be read directly from agarose gels, and (2) point mutation events. The latter were identified using polyacrylamide-gel electrophoresis of **PCR** products following digestion with restriction endonucleases (for-base cutters). To determine whether the **PCR**-based polymorphisms were allelic to polymorphisms identified by the clones from which the primer sequences derived, chromosomal assignments and (when possible) co-segregation analysis was performed.

TI: Use of RAPD markers to determine the genetic diversity of diploid, wheat genotypes.
AU: VIERLING-R-A; NGUYEN-H-T
CS: Genetics Lab., Indiana Crop Improvement, 3510 US 52 South, Lafayette, Indiana, 47905
SO: THEORETICAL AND APPLIED GENETICS 84(7-8): 835-838
PY: 1992
LA: English
AB: The genetic diversity of two diploid wheat species, Triticum monococcum and Triticum urartu (2n = 2x = 14), was assessed using random primers and the polymerase chain reaction (**PCR**). Electrophoretic analysis of the amplification products revealed a higher incidence of polymorphism in T. urartu than T. monococcum. Pair-wise comparisons of unique and shared polymorphic amplification products, were used to generate Jaccard's similarity coefficients. These were employed to construct phenograms using an unweighed pair-group method with arithmetical averages (UPGMA). The UP-GMA analysis indicated a higher similarity among T. monococcum with T. urartu. Analysis of **RAPD** data appears to be helpful in determining the genetic relationships among genotypes.

TI: Potential taxonomic use of random amplified polymorphic DNA (RAPD): A case study in Brassica.
AU: DEMEKE-T; ADAMS-R-P; CHIBBAR-R
CS: Agric. Can., Lethbridge Res. Stn., P.O. Box 3000 Main, Lethbridge, AB T1J4B1, Canada
SO: THEORETICAL AND APPLIED GENETICS 84(7-8): 990-994
PY: 1992
LA: English
AB: The potential use of **RAPDs** for taxonomic studies were investigated using Brassica, Sinapis and Raphanus taxa. Principal coordinate analysis of 284 **RAPD** bands revealed the classical U triangle relationship between diploid and amphidiploid Brassica taxa (B. napus, B. cannata, B. campestris, B. juncea, B. nigra, B. oleracea). Raphanus sativus and S. alba were distinct from the Brassica taxa. It appears that at least ten primers with approximately 100 total bands are needed to adequately portray these relationships. Cultivars of cabbage and cauliflower were separated by **RAPDs**. Analysis of **RAPDs** from individual plants of B. carinata cv. dodola resulted in 69 **RAPDs**, with 91.7% monomorphic and 8.3% polymorphic bands. **RAPDs** appear to be useful for taxonomic studies at levels ranging from populations to species and perhaps genera.

TI: Low levels of genetic diversity in red pine confirmed by random amplified polymorphic DNA markers.
AU: MOSSELER-A; EGGER-K-N; HUGHES-G-A
CS: Petawawa Natl. Foresty Inst., P.O. Box 2000, Chalk River, Ont. K0J 1J0, Can
SO: CANADIAN JOURNAL OF FOREST RESEARCH 22(9): 1332-1337
PY: 1992
LA: English
AB: Random amplified polymorphic **DNA** (**RAPD**) markers were used to characterize genetic variation in disjunct Newfoundland populations of red pine (Pinus resinosa Ait.) for comparison with individuals from throughout the mainland range of red pine. Red pine demonstrated a largely monomorphic profile for 69 arbitrary oligonucleotide primers. **DNA** samples from white spruce (Picea glauca (Moench) Voss) and black spruce (Picea mariana (Mill.) B.S.P.) that were screened together with red pine for 11 oligonucleotide primers showed abundant polymorphisms, confirming the genetic heterogeneity that characterizes these Boreal Zone spruces. Results with **RAPD** markers correspond with genetic diversity estimates using isozyme gene markers for both spruce species and red pine. **RAPD** markers provided further confirmation of low levels of genetic variation for a random sample of the red pine genome. A period of between 8000 and 10,000 years of isolation on the island of Newfoundland has resulted in very little detectable genetic differentiation of island populations from mainland populations, and the mainland populations have not recovered from losses of genetic diversity following a hypothesized genetic bottleneck that may have been experienced during glacial episodes of the Holocene. The low levels of genetic variation observed in red pine demonstrate the long time periods required for recovery following a loss of genetic divesity in long-lived, long-generation organisms like trees.

TI: Genetic and morphological comparisons of Glomerella (Colletotrichum) isolates from maize and from sorghum.
AU: VAILLANCOURT-L-J; HANAU-R-M
CS: Dep. Botany Plant Pathol., 1155 Lilly Hall, Purdue Univ., West Lafayette, Indiana 47907-1155
SO: EXPERIMENTAL MYCOLOGY 16(3): 219-229
PY: 1992
LA: English
AB: Various morphological and genetic characteristics were compared among six isolates of Colletotrichum from maize and six from sorghum. For the first time, a teleomorph was induced in sorghum isolates by pairing them on autoclaved sorghum leaves in a humidity chamber. The sorghum teleomorph was morphologically similar to Glomerella graminicola and Glomerella tucumanensis, the teleomorphs of Colletotrichum isolates from maize and from sugarcane, respectively. Mating tests demonstrated that Glomerella isolates from maize and sorghum were not interfertile. Several small but consistent differences in the morphologies of the isolates from maize and from sorghum were observed which agreed with earlier reports. **DNA** fingerprints detected as restriction fragment length polymorphisms of mitochondrial **DNA** and random polymorphic **DNA** (**RAPD**) produced from nuclear **DNA** by the polymerase chain reaction could be used to reliably and unambiguously distinguish members of the two groups of isolates. Results of a statistical analysis of similarity of the **RAPD** fingerprints suggested that maize and sorghum isolates of Colletotrichum are only about 45% similar (+- 10%) and represent two distinct and separate genetic lineages. We conclude that isolates of Colletotrichum from maize and sorghum are sibling species since they are morphologically very similar but reproductively completely isolated.

TI: Use of random amplified polymorphic DNAs (RAPDs) for identification of rice accessions.
AU: FUKUOKA-S; HOSAKA-K; KAMIJIMA-O
CS: Lab. Plant Breeding, Fac. Agric., Kobe Univ., 1-1 Rokko, Nada, Kobe 657, Jpn
SO: JAPANESE JOURNAL OF GENETICS 67(3): 243-252
PY: 1992
LA: English
AB: Randomm amplified polymorphic (**DNAs** (**RAPDs**) were identified in rice by amplification using single 10-mer primers of arbitrary sequence. The number of amplification products increased with increasing GC content of the primer in the range between 40% and 60% GC. Single-base substitutions of a primer altered amplification, providing new polymorphisms. The size of amplified **DNA** was mostly between 0.5kbp and 2.2kbp with the most common bands at 1.5bkp. Sixteen rice accessions were assayed with 28 primers which generated 116 polymorphic amplified **DNAs** or **RAPDs**. All accessions were uniquely distinguished by at least one **RAPD** and clustered into three distinct groups which corresponded to Japonica, Javanica and Indica. Japonica differed on average from Indica with 80.1 **RAPDs** and from Javanica with 30.8 **RAPDs**. It was, thus, demonstrated that **RAPDs** were useful polymorphisms in rice and superior to RFLPs for their technological simplicity. **RAPDs** would be good alternatives for the construction of a genetic map because of the higher frequencies of polymorphism detection.

TI: Random amplified polymorphic DNA analysis of Heterodera cruciferae and Heterodera schachtii populations.
AU: CASWELL-CHEN-E-P; WILLIAMSON-V-M; WU-F-F
CS: Dep. Nematol., Univ. Calif., Davis, Calif. 95616
SO: JOURNAL OF NEMATOLOGY 24(3): 343-351
PY: 1992
LA: English
AB: Heterodera schachtii and H. cruciferae are sympatric in California and frequently occur in the same field upon the same host. We have investigated the use of polymerase chain reaction (**PCR**) amplification of nematode **DNA** sequences to differentiate H. schachtii and H. cruciferae and to assess genetic variability within each species. Single, random oligodeoxyribonucleotide primers were used to generate **PCR**-amplified fragments, termed **RAPD** (random amplified polymorphic **DNA**) markers, from genomic **DNA** of each species. Each of 19 different random primers yielded from 2 to 12 fragments whose size ranged from 200 to 1,500 bp. Reproducible differences in fragment patterns allowed differentiation of the two species with each primer. Similarities and differences among six different geographic populations of H. schachtii were detected. The potential application of **RAPD** analysis to relationships among nematode populations was assessed through cluster analysis of these six different populations, with 78 scorable markers from 10 different random primers. **DNA** from single cysts was successfully amplified, and genetic variability was revealed within geographic populations. The use of **RAPD**

markers to assess genetic variability is a simple, reproducible technique that does not require radioisotopes. This powerful new technique can be used as a diagnostic tool and should have broad application in nematology.

TI: Mapping of molecular markers on Brassica B-genome chromosomes added to Brassica napus.
AU: STRUSS-D; QUIROS-C-F; ROEBBELEN-G
CS: Inst. Pflanzenbau Pflanzenzuechtung, Georg-August-Universitaet, Von-Siebold-Strasse 8, W-3400 Goettingen, Germany
SO: PLANT BREEDING 108(4): 320-323
PY: 1992
LA: English
AB: Using interspecific hybridization among various Brassica species, B-genome chromsomes from different sources of Brassica, i.e. B. nigra (BB, 2n = 18), B. carinata (BBCC, 2n = 34) and B. juncea (AABB, 2n = 36) were transferred into the Canadian variety 'Andor' of B. napus. Monosomic addition lines were selected (AACC + 1B, 2 n = 39) by cytological control. For characterization of the alien chromosomes, series of isozymes, RFLPs and **RAPD** markers were employed. This permitted the identification of a total of 39 lines representing seven of the eight B-genome chromosomes.

TI: DNA amplification polymorphisms of the cultivated mushroom Agaricus bisporus.
AU: KHUSH-R-S; BECKER-E; WACH-M
CS: Monterey Laboratories, P.O. Box 189, Watsonville, Calif. 95076
SO: APPLIED AND ENVIRONMENTAL MICROBIOLOGY 58(9): 2971-2977
PY: 1992
LA: English
AB: Single 10-bp primers were used to generate random amplified polymorphic **DNA** (**RAPD**) markers from commercial and wild strains of the cultivated mushroom Agaricus bisporus via the polymerase chain reaction. Of 20 primers tested, 19 amplified A. bisporus **DNA**, each producing 5 to 15 scorable markers ranging from 0.5 to 3.0 kbp. **RAPD** markers identified seven distinct genotypes among eight heterokaryotic strains; two of the commercial strains were shown to be related to each other through single-spore descent. Homokaryons recovered from protoplast regenerants of heterokaryotic strains carried a subset of the **RAPD** markers found in the heterokaryon, and both of the haploid nuclei from two heterokaryons were distinguishable. **RAPD** markers also served to verify the creation of a hybrid heterokaryon and to analyze meiotic progeny from this new strain: most of the basidiospores displayed **RAPD** fingerprints identical to that of the parental heterokaryon, although a few selected slow growers were homoallelic at a number of loci that were heteroallelic in the parent, suggesting that they represented rare homokaryotic basidiospores; crossover events between a **RAPD** marker locus and its respective centrometre appeared to be infrequent. These results demonstrate that **RAPD** markers provide an efficient alternative for strain fingerprinting and a versatile tool for genetic studies and manipulations of A. bisporus.

TI: Random amplified polymorphic DNA (RAPD) markers in sugar beet (Beta vulgaris L.): Mapping the genes for nematode resistance and hypocotyl colour.
AU: UPHOFF-H; WRICKE-G
CS: Inst. Angewandte Genetik, Univ. Hannover, Herrenhaeuser Str. 2, W-3000 Hannover 21, Ger
SO: PLANT BREEDING 109(2): 168-171
PY: 1992
LA: English
AB: The random amplified polymorphic **DNA** (**RAPD**) technique was adapted for segregation analysis in sugar beet. 83 I-O/I individuals were scored with a set of 20 arbitrary decamer primers. 4 preliminary linkage groups could be established, enclosing 9 **RAPD** markers, 2 isozyme loci, a gene for the hypocotyl colour and a gene for resistance to the root knot nematode (Heterodera schachtii Schm.).

TI: Detection of DNA sequence polymorphisms among wheat varieties.
AU: HE-S; OHM-H; MACKENZIE-S
CS: Dep. Agronomy, Purdue University, West Lafayette, Indiana 47907
SO: THEORETICAL AND APPLIED GENETICS 84(5-6): 573-578
PY: 1992
LA: English
AB: A **DNA** marker detection strategy that allows the rapid, efficient resolution of high levels of polymorphism among closely related lines of common wheat (Triticum aestivum) has been developed to circumvent the apparent lack of restriction fragment length polymorphism in many important self-pollinated crop species. The technique of randomly amplified polymorphic **DNA** (**RAPD**) was combined with a denaturing gradient gel electrophoresis system (DGGE) to explore **DNA** sequence polymorphisms among different genotypes of wheat. Of the 65 primer combinations used for the polymerase chain reaction (**PCR**) amplifications, over 38% of them produced readily detectable and reproducible **DNA** polymorphisms between a spring wheat line, SO852, and a winter wheat variety, 'Clark'. A high level of polymorphism was observed among a number of commercial varieties and breeding lines of wheat. This procedure was also used to detect polymorphisms in a recombinant inbred population to test the feasibility of its application in genome mapping. This **DNA** polymorphism detection system provides an opportunity for pedigree analysis and fingerprinting of developed wheat lines as well as construction of a high density genetic map of wheat. Without the need for 32P and sophisticated **DNA** extraction procedures, this approach should make it feasible to utilize marker-based selection in a plant breeding program.

TI: The use of random amplified polymorphic DNA markers in wheat.
AU: DEVOS-K-M; GALE-M-D
CS: Cambridge Lab., Colney Lane, Norwich NR4 7UJ, UK
SO: THEORETICAL AND APPLIED GENETICS 84(5-6): 567-572
PY: 1992
LA: English

AB: An evaluation was made of the use of random amplified polymorphic **DNA** (**RAPD**) as a genetic marker system in wheat. Reproducible amplification products were obtained from varietal, homozygous single chromosome recombinant line and wheat/alien addition line genomic **DNA** with selected primers and rigorously optimized reaction conditions. Factors influencing the **RAPD** patterns are **DNA** concentrations, Mg-2+ concentration, polymerase concentration and denaturing temperature. In wheat, the non-homoeologous, non-dose responsive and dominant behaviour of **RAPD** products devalues their use as genetic markers for the construction of linkage maps, and the high probability that the amplified fragments derive from repetitive **DNA** limits their use as a source of conventional RFLP probes. However, **RAPD** markers will most certainly find many applications in the analysis of genotypes where single chromosomes or chromosomes segments are to be manipulated.

TI: A feasibility study of the use of random amplified polymorphic DNA in the population genetics and systematics of grasshoppers.
AU: CHAPCO-W; ASHTON-N-W; MARTEL-R-K-B; ANTONISHYN-N; CROSBY-W-L
CS: Dep. Biol., University Regina, Regina, Sask., Canada S4S 0A2
SO: GENOME 35(4): 569-574
PY: 1992
LA: English
AB: Single, short primers of arbitrary nucleotide sequence were used in polymerse chain reactions to amplify regions of **DNA** isolated from several melanopline and oedipodine grasshoppers collected from local Saskatchewan populations. This represents one of the first applications of the method, called randomly amplified polymorphic **DNA** (or **RAPD**), to natural populations. Twenty-four different oligonucleotide primers, nine nucleotides in length, yielded clear and reproducible bands corresponding to amplified products and separable by agarose gel electrophoresis. On average, but 8.1 bands (range 0-17) were obtained per primer individual. The mean percent similarity between band profiles of conspecific individuals was 51.2% whereas the mean value for individuals representing different species or genera was 35.0%. Clearly greater numbers of insects and primers will be required to achieve a satisfactory level of phyolgenetic resolution. Given **RAPDs** technical advantages and ease execution, however, this should not be problematic to the molecular systematist.

TI: The use of RAPD markers for the detection of gene introgression in potato.
AU: WAUGH-R; BAIRD-E; POWELL-W
CS: Scottish Crop Res. Inst., Invergowrie, Dundee DD2 5DA, Scotland
SO: PLANT CELL REPORTS 11(9): 466-469
PY: 1992
LA: English
AB: Randomly Amplified Polymorphic **DNAs** were employed to demonstrate that potato dihaploids generated after interspecific pollination of a tetraploid Solanum tuberosum cultivar (Pentland Crown) by Solanum phureja dihaploid inducer clones could not be of parthenogenetic origin. of six different 10-mer oligonucleotides, four generated products from total potato dihaploid genomic **DNAs** which were not derived from the S. tuberosum parent. Gel electrophoresis and Southern analysis indicated that these amplified bands originated from S. phureja. The results are discussed in the contextt of recent cytological and molecular evidence which demonstrates that potato dihaploids are aneusomatic (Clulow et al. 1991) and emphasises this approach as a general methodology for the detection of alien gene introgression in both natural and cultivated plant populations.

TI: Electrophoretic karyotype analysis of the button mushroom, Agaricus bisporus.
AU: ROYER-J-C; HINTZ-W-E; KERRIGAN-R-W; HORGEN-P-A
CS: Centre Plant Biotechnology, University Toronto, Erindale Campus, Mississauga, Ont., Canada L5L 1C6
SO: GENOME 35(4): 694-698
PY: 1992
LA: English
AB: An efficient procedure is presented for the generation of protoplasts from shake flask cultures of the commercial mushroom Agaricus bisporus. Orthogonal field electrophoresis (OFAGE) of high molecular weight **DNA** from lysed protoplasts resolved the genome of A. bisporus into at least 10 bands, ranging in size from 1.2 to 4 Mb. The illustrated electrophoretic karyotypes of two homokaryons were highly polymorphic. A heterokaryon of the two homokaryons contained a mixture of the two electrophoretic patterns, though the ratio of nuclear types was not equal. A number of RFLP and **RAPD** markers and the r**DNA** repeat have been localized to specific chromosomes. Based on ethidium bromide staining intensity and autoradiography, we have estimated the chromosome number of A. bisporus to be 13.

TI: A comparative study of randomly amplified polymorphic DNA analysis and conventional phage typing for epidemiological studies of Listeria monocytogenes isolates.
AU: MAZURIER-S-I; AUDURIER-A; MARQUET-VAN-DER-MEE-N; NOTERMANS-S; WERNARS-K
CS: Lab. Water Food Microbiol., National Inst. Public Health Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, Netherlands
SO: RESEARCH IN MICROBIOLOGY 143(5): 507-512
PY: 1992
LA: English
AB: The analysis of **RAPD** profiles generated by **PCR** with a single 10-mer, HLWL74, was compared to bacteriophage susceptibility data for epidemiological typing of Listeria monocytogenes strains. A total of 104 L. monocytogenes strains was screened, all from serogroup 1 or serotype 4b. Of these, 53 had been isolated during 6 different listeriosis outbreaks. The remaining 51 strains were chosen randomly from our collection. A total of 38 **RAPD** types were observed, although each epidemic group of strains isolated during one of these outbreaks displayed a specific **RAPD** profile. For 98% of the strains isolated during outbreaks, the correlation between **RAPD** typing and phage typing was complete. Only one strain, typed as epidemic by phage typing, was clearly distinguishable from the others by **RAPD** analysis. Among the 51 strains not related to an outbreak, 12 was linked to epidemic groups by **RAPD** analysis. Two of these rearrangements were supported by phage typing. The remaining 10 strains could be excluded by phage typing from any of the epidemic groups studies. Considering all 104 isolates, the decision to relate a strain to a particular epidemic group or to exclude a strain from any epidemic group was

the same for 92 isolates, using either phage typing or **RAPD** analysis. The **RAPD** analysis, which is quick, simple and suited for automation, is proposed as an attractive alternative for phage typing in epidemiological studies of listeriosis.

TI: Typing of Listeria strains by random amplification of polymorphic DNA.
AU: MAZURIER-S-I; WERNARS-K
CS: Lab. Water Food Microbiol., National Inst. Public Health Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, Netherlands
SO: RESEARCH IN MICROBIOLOGY 143(5): 499-505
PY: 1992
LA: English
AB: The polymerase chain reaction (**PCR**) was used to obtain randomly amplified polymorphic **DNA** (**RAPD**) profiles for typing of Listeria strains. In this procedure, whole cells were incubated in the reaction mixture. The discriminating ability of a randomly designed 10-mer primer, HLWL74, was assessed. A total of 60 collection strains of Listeria, encompassing all 7 Listeria species and all known serovars was submitted to **PCR** with the primer HLWL74. Upon agarose gel electrophoresis, 29 different banding profiles were reproducibly obtained. No common profiles were recorded for strains from different Listeria species. For various groups of strains sharing the same serotype (e.g. 4b, 1/2a, 1/2b), **RAPD** analysis could generate further subdivision. On the other hand, some strains from different serotypes produced identical **RAPD** profiles with the primer HLWL74. The **RAPD** typing method from whole cells is proposed as an attractive alternative for other Listeria typing systems, and the 10-mer HLWL74 as a primer to include in a forthcoming set of standard primers for **RAPD** typing of Listeria isolates.

TI: Molecular characterisation of inter- and intra-specific somatic hybrids of potato using randomly amplified polymorphic DNA (RAPD) markers.
AU: BAIRD-E; COOPER-BLAND-S; WAUGH-R; DEMAINE-M; POWELL-W
CS: Cell Mol. Genetics, Scottish Crop Res. Inst., Invergowrie, Dundee DD2 5DA, Scotland
SO: MOLECULAR & GENERAL GENETICS 233(3): 469-475
PY: 1992
LA: English
AB: Protoplast fusion allows the transfer of both mono- and polygenic traits between species that are sexually incompatible. This approach has particular relevance for potato, and somatic hybridisation has been used to introduce a range of disease resistance genes from sexually incompatible wild species into the cultivated potato gene pool. In addition, protoplast fusion allows the resynthesis of tetraploid genotypes from preselected diploid or dihaploid donor parents. A limiting factor for the efficient exploitation of this technology in potato breeding is the difficulty of unequivocally identifying nuclear hybrids (heterokaryons). In order to facilitate the identification of hybrids at an early stage following fusion, Randomly Amplified Polymorphic **DNA** markers (**RAPDs**) have been used to characterise molecularly both inter- and intra-specific somatic hybrids of potato. **RAPD** markers detect naturally occurring polymorphism in the donor genotypes and utilise short oligonucleotide primers of arbitrary nucleotide sequence in combination with the polymerase chain reaction (**PCR**). The exploitation of **RAPDs** in the characterisation of both somatic and sexual hybrids is discussed.

TI: RAPD analysis of Campylobacter isolates: DNA fingerprinting without the need to purify DNA.
AU: MAZURIER-S; VAN-DE-GIESSEN-A; HEUVELMAN-K; WERNARS-K
CS: Lab. Water Food Microbiol., Natl. Inst. Public Health Environ. Protection, PO Box 1, 3720 BA Bilthoven, Neth
SO: LETTERS IN APPLIED MICROBIOLOGY 14(6): 260-262
PY: 1992
LA: English
AB: A method was developed to obtain reproducible **DNA** fingerprints from Campylobacter by **PCR**-based amplification, without the need to isolate total **DNA**. Randomly amplified polymorphic **DNA** (**RAPD**) profiles were generated with three randomly designed 10-mers, using each separately as an amplification primer. A range of C. jejuni serotypes could be typed by **RAPD** analysis. Depending on the primer, the analysis of **RAPD** profiles resulted in different levels of discrimination between the strains. Clear correlations were observed between results of **RAPD** analysis and serotyping. Two of the primers tested generated **RAPD** profiles which allowed discrimination of strains within given Penner and Lior serotypes.

TI: Use of the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to detect DNA polymorphisms in aphids (Homoptera: Aphididae).
AU: BLACK-W-C-IV; DUTEAU-N-M; PUTERKA-G-J; NECHOLS-J-R; PETTORINI-J-M
CS: Dep. Environmental Health, Colo. State Univ., Fort Collins, Colo. 80525
SO: BULLETIN OF ENTOMOLOGICAL RESEARCH 82(2): 151-159
PY: 1992
LA: English
AB: We have used a new technique to identify discrete genetic markers in aphids, a family in which biochemical and morphological genetic polymorphisms are rare. The new technique uses the polymerase chain reaction (**PCR**) to amplify random regions of aphid genomes (random amplified polymorphic **DNA**) and has been termed RPAD-**PCR**. We demonstrate the use of the technique in revealing genetic variation in four aphid species, the greenbug (Schizaphis graminum (Rondani)), the Russian wheat aphid (Diuraphis noxia (Mordvilko)), the pea aphid (Acyrthosiphon pisum (Harris)), and the brown ambrosia aphid (Uroleucon ambrosiae (Thomas)). In contrast with allozyme surveys, **RAPD-PCR** revealed large amounts of genetic variation among individuals in each of these species. Variation was detected among biotypes, populations, colour morphs and even individuals on a single plant. We also explored the utility of **RAPD-PCR** in the detection and identification within aphid bodies of two endoparasitic wasps, Diaeretiella rapae (McIntosh) and Lysiphlebus testaceipes (Cresson). The use of **RAPD-PCR** in species diagnostics, parasitoid detection, and population studies is discussed.

TI: Random sequence oligonucleotide primers detect polymorphic DNA products which segregate in inbred strains of mice.
AU: WOODWARD-S-R; SUDWEEKS-J; TEUSCHER-C
CS: Dep. Microbiol., 873 WIDB, Brigham Young Univ., Provo, Utah 84602, USA
SO: MAMMALIAN GENOME 3(2): 73-78
PY: 1992
LA: English
AB: The random amplification of polymorphic **DNA** (**RAPD**) using primers of arbitrary nucleotide sequence has been extremely valuable in identifying heritable markers in a variety of systems. The present studies examined whether the **RAPD** technique can identify large numbers of polymorphisms that can be used to construct genetic maps in inbred strains of mice. By screening the inbred mouse strains C57BL/6J and DBA/2J with 481 random 10-mer oligonucleotide primers, we identified 95 polymorphisms and mapped 76 of these by use of the BXD series of recombinant inbred (RI) strains. The results clearly demonstrate that the **RAPD** technique allows for the identification of large numbers of **DNA**-based polymorphisms that distinguish these two inbred strains of mice, and that such markers can readily be used to construct molecular genetic linkage maps.

TI: Genetic fingerprinting of Theobroma clones using randomly amplified polymorphic DNA markers.
AU: WILDE-J; WAUGH-R; POWELL-W
CS: Cell Mol. Genetics Dep., Scottish Crop Res. Inst., Invergowrie, Dundee DD2 5DA, UK
SO: THEORETICAL AND APPLIED GENETICS 83(6-7): 871-877
PY: 1992
LA: English
AB: Randomly amplified polymorphic **DNA** (**RAPD**) markers have been used to characterise cocoa clones representing the three main cultivated subpopulations: Criollo, Forastero and Trinitario. The use of single primers arbitrary nucleotide sequence resulted in the selective amplification of **DNA** fragments which were unique to the individual cocoa clones studied. The use of a single primer allowed each of the clones evaluated to be unequivocally characterised. The application of **RAPD** markers for the evaluation of germplasm and cocoa improvement programmes are discussed.

TI: Genetic segregation of random amplified polymorphic DNA in diploid cultivated alfalfa.
AU: ECHT-C-S; ERDAHL-L-A; MCCOY-T-J
CS: Dep. Plant Soil Sci., Montana State University, Bozeman, Montana 59717
SO: GENOME 35(1): 84-87
PY: 1992
LA: English
AB: Polymerase chain reaction was used, with single 10-mer primers of arbitrary sequence, to amplify random regions of genomic **DNA** from a diploid cultivated alfalfa (Medicago sativa) backcross population. Segregation of the random amplified polymorphic **DNA** (**RAPD**) fragments was analyzed to determine if **RAPD** markers are suitable for use as genetic markers. Of the 19 primers tested, 13 amplified a total of 37 polymorphic fragments, of which 28 (76%) segregated as dominant Mendelian traits. **RAPD** markers appear useful for the rapid development of genetic information in species like alfalfa where little information currently exists or is difficult to obtain.

TI: Identification of restriction fragment length polymorphism and random amplified polymorphic DNA markers linked to downy mildew resistance genes in lettuce, using near-isogenic lines.
AU: PARAN-I; KESSELI-R; MICHELMORE-R
CS: Dep. Veg. Crops, Univ. Calif., Davis, Calif. 95616
SO: GENOME 34(6): 1021-1027
PY: 1991
LA: English
AB: Near-isogenic lines were used to identify restriction fragment length polymorphism (RFLP) and random amplified polymorphic **DNA** (**RAPD**) markers linked to genes for resistance to downy mildew (Dm) in lettuce. Two pairs of near-isogenic lines that differed for Dm1 plus Dm3 and one pair of near-isogenic lines that differed for Dm11 were used as sources of **DNA**. Over 500 c**DNA**s and 212 arbitrary 10-mer oligonucleotide primers were screeded for their ability to detect polymorphism between the near-isogenic lines. Four RFLP markers and four **RAPD** markers were identified as linked to the Dm1 and Dm3 region. Dm1 and Dm3 are members of a cluster of seven Dm genes. Marker CL922 was absolutely linked to Dm15 and Dm16, which are part of this cluster. Six **RAPD** markers were identified as linked to the Dm11 region. The use of **RAPD** markers allowed us to increase the density of markers in the two Dm regions in a short time. These regions were previously only sparsely populated with RFLP markers. The rapid screening and identification of tightly linked markers to the target genes demonstrated the potential of **RAPD** markers for saturating genetic maps.

TI: Differentiation of Fusarium solani f. sp. cucurbitae races 1 and 2 by random amplification of polymorphic DNA.
AU: CROWHURST-R-N; HAWTHORNE-B-T; RIKKERINK-E-H-A; TEMPLETON-M-D
CS: DSIR Plant Protection, Private Bag, Auckland, New Zealand
SO: CURRENT GENETICS 20(5): 391-396
PY: 1991
LA: English
AB: We have used a **PCR**-based technique, involving the random amplification of polymorphic **DNA** (**RAPD**), to assess genome variability between 21 isolates from F. solani f. sp. cucurbitae races 1 and 2. Based on **RAPD** marker patterns the isolates fell into two distinct groups corresponding to mating populations MPI and MPV. Four isolates that could not be assigned to one or other mating population by traditional means were distinguished by **RAPD** patterns. Seven polymorphic **RAPD** products were used to probe Southern blotts of MPI and MPV genomic **DNA**. Six of the seven probes hybridized to single-copy sequences and five of the seven probes showed specificity for one or other mating population. We suggest that not

only is the technique a rapid and reliable tool for isolate-typing of fungi but it also provides a rapid method for obtaining species- or race-specific hybridization probes.

TI: Isolation of molecular markers for tomato (Lycopersicon esculentum) using random amplified polymorphic DNA (RAPD).
AU: KLEIN-LANKHORST-R-M; VERMUNT-A; WEIDE-R; LIHARSKA-T; ZABEL-P
CS: Dep. Mol. Biol., Agricultural University, Dreijenlaan 3, NL-6703 HA Wageningen, Netherlands
SO: THEORETICAL AND APPLIED GENETICS 83(1): 108-114
PY: 1991
LA: English
AB: A new **DNA** polymorphism assay was developed in 1990 that is based on the amplification by the polymerase chain reaction (**PCR**) of random **DNA** segments, using single primers of arbitrary nucleotide sequence. The amplified **DNA** fragments, referred to as **RAPD** markers, were shown to be highly useful in the construction of genetic maps ("**RAPD** mapping"). We have now adapted the **RAPD** assay to tomato. Using a set of 11 oligonucleotide decamer primers, each primer directed the amplification of a genome-specific "fingerprint" of **DNA** fragments. The potential of the original **RAPD** assay to generate polymorphic **DNA** markers with a given set of primers was further increased by combining two primers in a single **PCR**. By comparing "fingerprints" of L. esculentum, L. pennellii, and the L. esculentum chromosome 6 substitution line LA1641, which carries chromosome 6 from L. pennellii, three chromosome 6-specific **RAPD** markers could be directly identified among the set of amplified **DNA** fragments. Their chromosomal position on the classical genetic map of tomato was subsequently established by restriction fragment length polymorphism (RFLP) linkage analysis. One of the **RAPD** markers was found to be tightly linked to the nematode resistance gene Mi.

TI: Detection of polymorphic loci in Arachis germplasm using random amplified polymorphic DNAs.
AU: LANHAM-P-G; FENNELL-S; MOSS-J-P; POWELL-W
CS: Cell Molecular Genetics Dep., Scottish Crop Res. Inst., Invergowrie, Dundee DD2 5DA, Scotland
SO: GENOME 35(5): 885-889
PY: 1992
LA: English
AB: The development of easily scoreable genetic markers in Arachis will facilitate the introgression of desirable traits from wild species into adapted germplasm. We have used random amplified polymorphic DNAs (**RAPDs**) to identify polymorphic molecular markers in a range of wild and cultivated Arachis species. From a total of sixty 10-mer oligonucleotide primers, 49 polymorphic loci were identified between a cultivated A. hypogaea type (TMV-2) and a synthetic amphidiploid (B x C)-2 created from a A. batizocoi and A. chacoense cross. The inheritance of polymorphic markers, both in the amphidiploid and in the F-1 progeny in a TMV-2 x (B x C)-2 cross, has also been demonstrated. The potential exploitation of **RAPD** markers in groundnut improvement programs is discussed.

TI: Segregation of random amplified DNA markers in F-1 progeny of conifers.
AU: CARLSON-J-E; TULSIERAM-L-K; GLAUBITZ-J-C; LUK-V-W-K; KAUFFELDT-C; RUTLEDGE-R
CS: Biotechnol. Lab., Univ. British Columbia, 237-6174 University Blvd., Vancouver, B.C., Can. V6T 1Z3
SO: THEORETICAL AND APPLIED GENETICS 83(2): 194-200
PY: 1991
LA: English
AB: The recently developed approach to deriving genetic markers via amplification of random **DNA** segments with single primers of arbitrary nucleotide sequence was tested for its utility in genetic linkage mapping studies with conifers. Reaction conditions were optimized to reproducibly yield clean and specific amplification products. Template **DNA** from several genotypes of Douglas-fir (Pseudotsuga menziesii) and white spruce (Picea glauca) were tested against eight ten-base oligonucleotide primers. Most of the tested primer/parent tree combinations yielded polymorphic **PCR** products ("**RAPD**" markers). Selected primers were then used in **PCR** reactions with template **DNA** isolated from offspring in Douglas-fir and black spruce diallel crosses among the same parental lines. The diallel study confirmed the appropriate inheritance of **RAPD** markers in the F-1 generation. The value of these dominant **RAPD** markers for genetic linkage mapping in trees was established from both theoretical and applied perspectives.

TI: Identification of broccoli and cauliflower cultivars with RAPD markers.
AU: HU-J; QUIROS-C-F
CS: Dep. Veg. Crops, Univ. Calif., Davis, Calif. 95616, USA
SO: PLANT CELL REPORTS 10(10): 505-511
PY: 1991
LA: English
AB: **RAPD** (Random Amplified Polymorphic **DNA**) markers generated by 4 arbitrary 10-mer primers, discriminated 14 broccoli and 12 cauliflower cultivars (Brassica oleracea L.) by banding profiles. The size of the amplified **DNA** fragments ranged from 300 to 2600 base pairs. Twenty-eight percent of the markers were fixed in both broccoli and cauliflower, whereas 12.5% were specific to either crop. The rest were polymorphic in either or both crops. The markers generated by two and three primers were sufficient to distinguish each of the broccoli and cauliflower cultivars, respectively. The average difference in markers was 14.5 between broccoli and cauliflower markers, 5.8 between two broccoli cultivars and 7.9 between two cauliflower cultivars. Large differences of each crop were found between cultivars from different seed companies than within the same company. **RAPD** markers provide a quick and reliable alternative to identify broccoli and cauliflower cultivars.

TI: Development and chromosomal localization of genome-specific markers by polymerase chain reaction in Brassica.
AU: QUIROS-C-F; HU-J; THIS-P; CHEVRE-A-M; DELSENY-M
CS: Dep. Vegetable Crops, Univ. California, Davis, Calif. 95616
SO: THEORETICAL AND APPLIED GENETICS 82(5): 627-632
PY: 1991

LA: English
AB: This paper reports the application of the **RAPD** (random amplification of polymorphic **DNA** sequence) markers in Brassica genetics. Forty-seven arbitrary decamer oligonucleotides were used as primers to amplify genomic **DNA** by polymerase chain reaction. Some of the amplified products were genome specific and could be found in both diploid and derived amphidiploid species. Of a total of 65 such markers, 16 were A genome, 37 B genome, and 12 C genome specific. Of the 37 B-genome-speific markers, 11 were mapped on four indepencent chromosomes of B. nigra with the aid of existing B. napus-nigra disomic alien addition lines.

TI: DNA polymorphisms amplified by arbitrary primers are useful as genetic markers.
AU: WILLIAMS-J-G-K; KUBELIK-A-R; LIVAK-K-J; RAFALSKI-J-A; TINGEY-S-V
CS: Central Res. Dev. Dep., E. I. du Pont de Nemours Co. Inc., Exp. Stn., Wilmington, DE 19880
SO: NUCLEIC ACIDS RESEARCH 18(22): 6531-6536
PY: 1990
LA: English
AB: Molecular genetic maps are commonly constructed by analyzing the segregation of restriction fragment length polymorphisms (RFLPs) among the progeny of a sexual cross. Here we describe a new **DNA** polymorphism assay based on the amplification of random **DNA** segments with single primers of arbitrary nucleotide sequence. These polymorphisms, simply detected as **DNA** segments which amplify from one parent but not the other, are inherited in a Mendelian fashion and can be used to construct genetic maps in a variety of species. We suggest that these polymorphisms be called **RAPD** markers, after Random Amplified Polymorphic **DNA**.

CAMBIA Literature Search Service

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Search criteria were **PARTICLE BOMBARDMENT, MICROPROJECTILE BOMBARDMENT** and **BIOLISTIC**. All these records have plants in the supertaxa field.

TI: Isolation of transgenic progeny of maize by embryo rescue under selective conditions.
AU: WEYMANN-K; URBAN-K; ELLIS-D-M; NOVITZKY-R; DUNDER-E; JAYNE-S; PACE-G
CS: CIBA-GEIGY Agric. Biotechnol., P.O. Box 12257, Research Triangle Park, NC 27709, USA
SO: IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY PLANT 29P(1): 33-37
PY: 1993
LA: English
AB: Fertile transgenic maize plants (T-0) and progeny (T-1) were obtained using **microprojectile bombardment** and callus selection on hygromycin B. To quickly identify progeny expressing the transgene, embryos from T3 generation kernels were excised 20 days after pollination and exposed to different concentrations of hygromycin B. Surviving and non-surviving embryos were assayed for the presence of the hygromycin phosphotransferase (aphIV) gene using polymerase chain reaction. Embryos that germinated and survived on 25, 50, or 100 mg/liter hygromycin possessed the aphIV gene. Embryos that did not germinate lacked the gene. Progeny surviving selection were transferred to the greenhouse and tested for expression of the gene using a leaf disc assay. The results demonstrated that the gene construct was expressed in both embryo and leaf tissue and that selection during germination successfully eliminated progeny lacking the gene of interest. This method is also useful for rapid-cycling of maize generations.

TI: Transformation of four pathogenic Phytophthora spp by microprojectile bombardment on intact mycelia.
AU: BAILEY-A-M; MENA-G-L; HERRERA-ESTRELLA-L
CS: Dep. Ingenieria Genet. Plantas, Cent. Invest. Estudios Avanzados, Unidad Irapuato, Apdo. Postal 629, 36500 Irapuato, Guanajuato, Mex
SO: CURRENT GENETICS 23(1): 42-46
PY: 1993
LA: English
AB: Phytophthora Capsici, Phytophthora citricola, Phytophthora cinnamomi and Phytophthora citrophthora were transformed without the removal of cell walls by **particle** acceleration with plasmids containing the beta-glucuronidase gene and hygromycin B resistance. Transformants were detected by histochemical and fluorometric beta-glucuronidase assays and confirmed by Southern-blot hybridization. It was found that the promoter of a plant virus is functional in Phytophthora. In addition, a method was designed to visually identify homogeneous transformed colonies, derived from zoospores of transformed multinucleated Phytophthora mycelia, based on blue color development on plates containing X-Gluc.

TI: Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize.
AU: VAIN-P; MCMULLEN-M-D; FINER-J-J
CS: Dep. Agron., Ohio State Univ., Wooster, OH 44691, USA
SO: PLANT CELL REPORTS 12(2). 84-88
PY: 1993
LA: English
AB: The effects of osmotic conditioning on both transient expression and stable transformation were evaluated by introducing plasmid DNAs via **particle bombardment** into embryogenic suspension culture cells of Zea mays (A188 times B73). Placement of cells on an osmoticum-containing medium (0.2 M sorbitol and 0.2 M mannitol) 4 h prior to and 16 h after **bombardment** resulted in a statistically significant 2.7-fold increase in transient beta-glucuronidase expression. Under these conditions, an average of approximately 9,000 blue foci were obtained from 100 mu-l packed cell volume of bombarded embryogenic tissue. Osmotic conditioning of the target cells resulted in a 6.8-fold increase in recovery of stably transformed maize clones. Transformed fertile plants and progeny were obtained from several transformed cell lines. We believe the basis of osmotic enhancement of transient expression and stable transformation resulted from plasmolysis of the cells which may have reduced cell damage by preventing extrusion of the protoplasm from bombarded cells.

TI: In vivo analysis of Chlamydomonas chloroplast petD gene expression using stable transformation of beta-glucuronidase translational fusions.
AU: SAKAMOTO-W; KINDLE-K-L; STERN-D-B
CS: Boyce Thompson Inst. Plant Res., Cornell Univ., Itahaca, NY 14853, USA
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 90(2): 497-501
PY: 1993
LA: English
AB: We have used the Escherichia coli beta-glucuronidase (uidA) gene as a reporter gene to localize the promoter and analyze the function of the 5' untranslated region (UTR) of the Chlamydomonas chloroplast petD gene. Using **particle bombardment**, petD-uidA transcriptional and translational fusion genes were introduced into the chloroplast genome in the large inverted repeat flanking the atpB gene. In transformants carrying a petD-uidA transcriptional fusion, uidA mRNA accumulated but was

not translated. However, in a translational fusion that included the entire petD 5' UTR, uidA mRNA accumulated and a high level of beta-glucuronidase activity was detected. When approxeq 70% of the petD 5' UTR was deleted from the translational fusion, uidA mRNA accumulation and beta-glucuronidase activity decreased 4- to 6-fold and 8-fold, respectively. Run-on transcription assays demonstrated that all strains transcribe the uidA gene at equivalent rates. Our results show that sequences essential for translation reside in the petD 5' UTR and also that sequences within the 5' UTR directly or indirectly affect mRNA stability. The expression of beta-glucuronidase under the control of chloroplast transcriptional and translational signals will facilitate further studies of chloroplast gene regulatory mechanisms.

TI: Effect of promoter-leader sequences on transient reporter gene expression in particle bombarded pea (Pisum sativum L.) tissues.
AU: WARKENTIN-T-D; JORDAN-M-C; HOBBS-S-L-A
CS: Plant Biotechnology Inst., National Res. Council Can., Saskatooon, Saskatchewan S7N 0W9, Can
SO: PLANT SCIENCE (LIMERICK) 87(2): 171-177
PY: 1992
LA: English
AB: **Particle bombardment** was used to deliver plasmids containing promoter-beta-glucuronidase (GUS) gene constructs into intact pea embryo axes, leaves and roots. Transient GUS enzyme activity was influenced by the promoter-leading sequence driving the GUS gene, as measured by fluorometric and histochemical assays. In most cases, the untranslated leader sequence from RNA of alfalfa mosaic virus (referred to here as 'AMV') significantly increased GUS activity when included between the promoter (nopaline synthase (NOS), califlower mosaic virus 35S (35S), or the tandem 35S promoter (35S-35S)) and the GUS coding sequence. The 35S-35S-AMV promoter-leading sequence produced 4- to 10-fold greater levels of transient GUS activity in these pea tissues than the 35S promoter alone. **Particle bombardment** is a simple and rapid method for the assessment of vector constructs in pea tissues.

TI: Improvement of plant regeneration and GUS expression in scutellar wheat calli by optimization of culture conditions and DNA-microprojectile delivery procedures.
AU: PERL-A; KLESS-H; BLUMENTHAL-A; GALILI-G; GALUN-E
CS: Dep. Plant Genetics, Weizmann Inst. Sci., Rehovot 76100, Israel
SO: MOLECULAR & GENERAL GENETICS 235(2-3): 279-284
PY: 1992
LA: English
AB: Genetic transformation of cereals by direct DNA delivery via **microprojectile bombardment** has become an established procedure in recent years. But the derivation of functional transgenic plants, especially in wheat, is still problematic, mainly due to low efficiency of DNA delivery and the reduced regeneration capability of **microprojectile**-bombarded tissue. We focussed on these two aspects and found that the regeneration of scutellar calli of wheat can be rendered highly efficient and considerably accelerated by a liquid culture phase in screen rafts. We also found that the expression of a reporter gene following DNA delivery by **microprojectile** can be improved by maintaining the scutellar calli in 0.25 M mannitol before and after **bombardment**, by **bombardment** in the presence of silver thiosulfate and Ca(NO-3)-2 (rather than CaCl-2) and by the elimination of spermidine from the DNA/**microprojectile** mixture. A protocol that includes all these features leads to several-fold higher transient expression of the reporter gene than have previously published procedures.

TI: Regeneration of herbicide resistant transgenic rice plants following microprojectile-mediated transformation of suspension culture cells.
AU: CAO-J; DUAN-X; MCELROY-D; WU-R
CS: Field Botany, Cornell Univ., Ithaca, NY 14853, USA
SO: PLANT CELL REPORTS 11(11): 586-591
PY: 1992
LA: English
AB: Suspension cells of Oryza sativa L. (rice) were transformed, by **microprojectile**, **bombardment**, with plasmids carrying the coding region of Streptomyces hygroscopicus phosphinothricin acetyl transferase (PAT) gene (bar) uner the control of either the 5' region of rice actin 1 gene (Act1) or the cauliflower mosaic virus (CaMV) 35S promoter. Subsequently regenerated plants display detectable PAT activity and are resistant to BASTA-TM, a phosphinothricin (PPT)-base herbicide. DNA gel blot analyses showed that PPT resistant rice plants contain a bar-hybridizing restriction fragment of the expected size. This report shows that expression of the bar gene in transgenic rice plants confers resistance to PPT-base herbicide by suppressing an increase of ammonia in plants after spraying with the herbicide.

TI: Regulation of the maize HRGP gene expression by ethylene and wounding: mRNA accumulation and qualitative expression analysis of the promoter by microprojectile bombardment.
AU: TAGU-D; WALKER-N; RUIZ-AVILA-L; BURGESS-S; MARTINEZ-IZQUIERDO-J-A; LEGUAY-J-J; NETTER-P; PUIGDOMENECH-P
CS: Dep. de Genetica Molecular, CID-CSIC, Jordi Girona 18, 08034 Barcelona, Spain
SO: PLANT MOLECULAR BIOLOGY 20(3): 529-538
PY: 1992
LA: English
AB: The expression of the maize (Zea mays) gene coding for a hydroxyproline-rich glycoprotein (HRGP) has been studied by measuring the mRNA accumulation after wounding or ethylene treatment. RNA blot and in situ hybridization techniques have been used. The temporal and tissue-specific expression has been observed: the cells related to the vascular system show the more intense HRGP mRNA accumulation. Transcriptional constructions of the maize HRGP promoter have been tested on different maize tissues by microbombarding. A 582 bp promoter is able to direct the expression of the gus gene on calli and young leaves. Constructions having shorter promoter sequences lose this ability. The 582 bp construction retains the general specificity of expression observed for the HRGP gene.

TI: Transformation of cucumber tissues by microprojectile bombardment: Identification of plants containing functional and non-functional transferred genes.
AU: CHEE-P-P; SLIGHTOM-J-L
CS: Mol. Biol. Unit, 7242, Upjohn Co., Kalamazoo, Mich. 49007
SO: GENE (AMSTERDAM) 118(2): 255-260
PY: 1992
LA: English
AB: The **microprojectile bombardment** method was used to transfer DNA into embryogenic callus of cucumber (Cucumis sativus), and stably transformed cucumber plant lines were obtained. A total of 107 independently regenerated cucumber plants were assayed for the presence and expression of the transferred Nos-NPTII gene (encoding nopaline synthase-neomycin phosphotransferase II). Genomic blot hybridization analyses showed that a high percentage (16%) of the cucumber plants were transformed with Nos-NPTII; however, only about 25% of these transgenic plants expressed Nos-NPTII. Inactivity of Nos-NPTII in many of the transformed cucumber plants may be associated with the transfer of multiple copies of Nos-NPTII. PCR and genomic blot hybridization analyses were used to show that the transferred gene was inherited in the subsequent plant generation.

TI: New genes for old trees.
AU: MANDERS-G; DAVEY-M-R; POWER-J-B
CS: Plant Genetic Manipulation Group, Dep. Life Sci., Univ. Nottingham, Nottingham NG7 2RD, UK
SO: JOURNAL OF EXPERIMENTAL BOTANY 43(254): 1181-1190
PY: 1992
LA: English
AB: In addition to the extensive improvement programmes within fruit orchards and forest stands, considerable momentum is being generated in the application of genetic manipulation strategies to a variety of woody species. Several transformation approaches have been adopted for the productive of transgenic trees. These include Agrobacterium-mediated gene delivery, chemical and/or electrical stimulated uptake of DNA into protoplasts, and the use of the newer technology of high velocity **bombardment** of plant tissues with DNA-coated particles. Transformation offers advantages over other genetic manipulation techniques, such as somatic hybridization, in that it allows the directed improvement of trees with minimum disruption of the genetic integrity of an elite genome. The information presented here is a comprehensive review of transformation in woody plants. Many of the advances have been made in this area during the last five years.

TI: Transformation of Dendrobium orchid using particle bombardment of protocorms.
AU: KUEHNLE-A-R; SUGII-N
CS: Dep. of Horticulture, Univ. of Hawaii, 3190 Maile Way, Honolulu, Hawaii 96822, USA
SO: PLANT CELL REPORTS 11(9): 484-488
PY: 1992
LA: English
AB: Transformed dendrobium orchids (Dendrobium times Jaquelyn Thomas hybrids) were recovered from protocorms bombarded by particles coated with the plasmid pGA482GG/cpPRV4, which contains the plant expressible Nos-NPT II and papaya ringspot virus (PRV) coat protein (CP) genes. Approximately 280 protocorms from four crosses were bombarded and potentially transformed tissues were identified by growth and green color on half-strength Murashige and Skoog medium supplemented with 2% sucrose and 50-100 mg l-1 kanamycin sulfate. Kanamycin concentrations that prevented growth of nontransformed tissues could not be used for long-term selection because such levels suppressed the regenerations of potentially transformed tissues. PCR and restrictions analysis 21 months after treatment found 13 of 13 plants from two crosses, which appeared kanamycin-tolerant, to contain the NOS-NPT II gene, while the only one of these plants carried the vector-linked PRV CP-gene. These results support use of **particle bombardment** for transformation of this important ornamental monocot.

TI: Genetic transformation of Norway spruce (Picea abies (L.) Karst) using somatic embryo explants by microprojectile bombardment.
AU: ROBERTSON-D; WEISSINGER-A-K; ACKLEY-R; GLOVER-S; SEDEROFF-R-R
CS: Dep. Botany, North Carolina State University, Raleigh, N.C. 27695, USA
SO: PLANT MOLECULAR BIOLOGY 19(6): 925-935
PY: 1992
LA: English
AB: Stable transformation of Norway spruce tissue has been obtained following **bombardment** of mature somatic embryos with pRT99gus, a plasmid that contains neo coding for NPTII, and gusA, coding for beta-glucuronidase, both fused to the CaMV 35S promoter. At least 8 lines have been stably transformed (over 15 months in culture) following **bombardment** and selection on kanamycin. Polymerase chain reaction analyses showed a high frequency of cotransformation of the gusA and neo genes. The frequency of coexpression of the selected and unselected markers was 100%. DNA/DNA hybridization of one transformed line provided conclusive evidence of stable integration and showed copy numbers of over 10 plasmid sequences per genome. None of the transformed lines has remained embryonic.

TI: Transient gene expression in differentiating pine wood using microprojectile bombardment.
AU: LOOPSTRA-C-A; WEISSINGER-A-K; SEDEROFF-R-R
CS: Dep. Forestry, North Carolina State Univ., Box 8008, Raleigh, NC 27695-8008, USA
SO: CANADIAN JOURNAL OF FOREST RESEARCH 22(7): 993-996
PY: 1992
LA: English
AB: We have used **microprojectile bombardment** to obtain transient expression of the reporter gene beta-glucuronidase in differentiating wood (secondary xylem) of loblolly pine (Pinus taedea L.), thereby providing a method for studying expression of introduction DNA in this importance tissue. beta-Glucuronidase activity can be observed in different cells types, including

tracheids, ray parenchyma and axial parenchyma associated with resin canals. **Microprojectile bombardment** can be used to identify active promotes and to compare the relative of different promoters in specific cell types. We have studied the expression of three promoter gene fusions of **bombardment** of loblolly pine stem sections. Wood samples bombarded with an abscisic acid inducible (Em) promoter - beta-glucuronidase fusion contained nearly 10 times the number of stained cells as those bombarded with two more commonly used fusions (nopalinesynthase or cauliflower mosaic virus 35S). Microprojective **bombardment** of differentiating wood should be useful for studying promoters and gene constructs in future attempts to modify wood properties by genetic engineering.

TI: Regulated transcription of the maize Bronze-2 promoter in electroporated protoplasts requires the C1 and R gene products.
AU: BODEAU-J-P; WALBOT-V
CS: Dep. Biol. Sci., Stanford Univ., Stanford, Calif. 94305
SO: MOLECULAR & GENERAL GENETICS 233(3): 379-387
PY: 1992
LA: English
AB: The putative maize transcription factor genes R and C1 are required for expression of reporter genes with promoters from the Bz1 and A1 genes, which encode enzymes required for anthocyanin biosynthesis in maize. Bz2 is another anthocyanin biosynthetic gene; we show that expression of a reporter gene from the Bz2 promoter also requires R and C1 when the fusion construct is introduced into maize kernels by **particle** gun **bombardment**. When electroporated into maize protoplasts from a suspension cell line not synthesizing anthocyanins, reporter genes with Bz2, Bz1, and A1 promoters are expressed only when both R and C1 expression plasmids are co-electroporated. Electroporation of R and C1 expression plasmids also induces the endogenous genes required for anthocyanin synthesis, resulting in pink protoplasts within 24 h. RNase protection analysis demonstrates that accumulation of mRNA from the endogenous Bz1 and Bz2 genes absolutely requires introduced R and C1. In time-course experiments there is a delay of 3-6 h before the Bz2 promoter is activated, supporting the proposed role for R- and C1-encoded proteins in transcriptional control. An excess of R relative to C1 suppresses expression of A1, Bz1, and Bz2 promoters, suggesting an interaction between the R and C1 proteins.

TI: Development of the particle inflow gun for DNA delivery to plant cells.
AU: FINER-J-J; VAIN-P; JONES-M-W; MCMULLEN-M-D
CS: Dep. Agron., Ohio Agric. Res. Dev. Center, Ohio State Univ., Wooster, Ohio 44691, USA
SO: PLANT CELL REPORTS 11(7): 323-328
PY: 1992
LA: English
AB: A simple and inexpensive **particle bombardment** device was constructed for delivery of DNA to plant cells. The **Particle** Inflow Gun (PIG) is based on acceleration of DNA-coated tungsten particles using pressurized helium in combination with a partial vacuum. The particles are accelerated directly in a helium stream rather than being supported by a macrocarrier. **Bombardment** parameters were partially optimized using transient expression assays of a beta-glucuronidase gene in maize embryogenic suspension culture and cowpea leaf tissues. High levels of transient expression of the beta-glucuronidase gene were obtained following **bombardment** of embryogenic suspension cultures of corn and soybean, and leaf tissue of cowpea. Stable transformation of embryogenic tissue of soybean has also been obtained using this **bombardment** apparatus.

TI: Expression of an abscisic acid responsive promoter in Picea abies (L.) Karst. following bombardment from an electric discharge particle accelerator.
AU: NEWTON-R-J; YIBRAH-H-S; DONG-N; CLAPHAM-D-H; VON-ARNOLD-S
CS: Dep. Forest Sci., Texas Agric. Experiment Station, Texas A and M Univ. System, College Station, Tex. 77843-2135
SO: PLANT CELL REPORTS 11(4): 188-191
PY: 1992
LA: English
AB: The 1.5 kilobase promoter sequence upstream of Dc8, a late embryo abundant gene of Daucus, fused to the reporter beta-glucuronidase gene was introduced into several tissues of Picea abies via a custom-made electric-discharge **particle** accelerator. Transient expression was measured histochemically as spot number 2 d after **bombardment**. Embryogenic suspensions gave higher levels of expression depending upon cell line than embryogenic callus or zygotic embryos. Expression was enhanced when cultures were treated with abscisic acid for 3 d before **bombardment**. A mean and maximum of 17 and 34 spots/disk, respectively, were observed with the best cell line, which was comparable with the level of expression driven byu an enanced 35S promoter.

TI: Transient expression from cab-m1 and rbcS-m3 promoter sequences is different in mesophyll and bundle sheath cells in maize leaves.
AU: BANSAL-K-C; VIRET-J-F; HALEY-J; KHAN-B-M; SCHANTZ-R; BOGORAD-L
CS: Dep. Cellular Developmental Biology, Harvard University, 16 Divinity Avenue, Cambridge, Mass. 02138
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 89(8): 3654-3658
PY: 1992
LA: English
AB: Cell-specific and light-regulated expression the beta-glucuronidase (GUS) reporter gene from maize cab-m1 and rbcS-m3 promoter sequences we studied in maize leaf segments by using an in situ transient expression **microprojectile bombardment** assay. The cab-m1 gene is known to be strongly photoregulated and to be expressed almost exclusively in mesophyll cells (MC) but not in bundle sheath cells (BSC). Expression of GUS from a 1026-base-pair 5' promoter fragment is increased about 10-fold upon illumination of dark-grown leaves. In illuminated leaves, the ratio of GUS expression in MC vs. BSC is about 10:1. The cab-m1 region between 868 and 1026 base pairs 5' to the translation start confers strong MC-preferred expression on the remainder of the chimeric gene in illuminated leaves, but a region between -39 and 359 from the translation start is required from photoregulated expression. Transcripts of rbc-S-m3 are found in BSC but not in MC and are about double

in BSC of greening dark-grown seedlings. In contrast to the behavior of the cab-m1-GUS construct, GUS expression driven by 2.1 kilobase pairs of the rbcS-m3 5' region was about twice as high in MC as in BSC of umilluminated dark-grown maize leaves. The number of BSC, but not MC, expressing GUS nearly doubled upon greening of bombarded etioloated leaves. These data suggest that the 5'region of rbc-S-m3 used here could be responsible for most of the light-dependent increase in rbcS-me transcripts observed in BSC of greening leaves and that transcriptional or postranscriptional mechanisms are responsible for the lack of rbcS-m3 transcripts in MC.

TI: Two types of chloroplast gene promoters in Chlamydomonas reinhardtii.
AU: KLEIN-U; DE-CAMP-J-D; BOGORAD-L
CS: Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Mass. 02138
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 89(8): 3453-3457
PY: 1992
LA: English
AB: Structures of the promoters of Chlamydomonas reinhardtii plastid atpB and 16S rRNA-encoding genes were analyzed in vivo. Chimeric constructs, containing the Chlamydomonas chloroplast atpB or 16S rRNA-encoding gene promoter coupled to the Escherichia coli uidA (beta-glucuronidase, GUS) reporter gene and bordered by C.reinhardtii chloroplast sequences, were stably introduced into the chloroplast of Chlamydomonas by **microprojectile bombardment**. Activity of the promoters in the chloroplast of GUS gene-positive transformants was assayed by measuring the abundance of GUS transcripts and determining the relative rates of GUS transcription in vivo. Deletion analyses of the 16S rRNA gene and atpB promoter fragments showed that the two promoters differ structurally. The 16S rRNA gene promoter resembles the bacterial sigma-70 type with typical -10 and -25 elements. The atpB promoter, on the other hand, lacks a conserved motif in the -35 region but contains, in the -10 region, a characteristic octameric palindrome (TATAATAT) that is conserved in the promoter sequences of some other C. reinhardtii chloroplast genes. For maximum activity, the atpB promoter requires sequences of apprxeq 22 base pairs upstream and apprxeq 60 base pairs downstream of the trancription start site.

TI: Expression of the GUS-gene in the monocot tulip after introduction by particle bombardment and Agrobacterium.
AU: WILMINK-A; VAN-DE-VEN-B-C-E; DONS-J-J-M
CS: Dep. Dev. Biol., Cent. Plant Breeding and Reproduction Res., P.O. Box 16, 6700 AA Wageningen, Neth
SO: PLANT CELL REPORTS 11(2): 76-80
PY: 1992
LA: English
AB: Gene transfer to the monocotyledon tulip (Tulipa sp. L.) was obtained both by **particle bombardment** and Agrobacterium transformation. Using a **Particle** Delivery System, transient expression of the reporter gene for beta-glucuronidase was demonstrated. It was shown that the CAMV 35S as well as the TR2' promoter were active in flower stem explants. Various wildtype and disarmed Agrobacterium strains, harbouring the 35S GUSintron gene on a binary plasmid, were used for infection of flower stem explants of 7 cultivars and 7 botanical Tulipa species. In nine genotypes the GUSintron gene was expressed, despite the fact that tulip tissue did not produce detectable amounts of virulence-inducing substances. Agrobacterium rhizogenes appeared to be most effective in gene transfer to tulip tissue.

TI: Expression of foreign genes in transgenic yellow-poplar plants.
AU: WILDE-H-D; MEAGHER-R-B; MERKLE-S-A
CS: Sch. Forest Resources, University Georgia, Athens, Ga. 30602
SO: PLANT PHYSIOLOGY (BETHESDA) 98(1): 114-120
PY: 1992
LA: English
AB: Cells of yellow-poplar (Liriodendron tulipifera L.) were transformed by direct gene transfer and regenerated into plants by somatic embryogenesis. Plasmid DNA bearing marker genes encoding beta-glucuronidase (GUS) and neomycin phosphotransferase (NPT II) were introduced by **microprojectile bombardment** into single cells and small cell clusters isolated from embryogenic suspension cultures. The number of full-length copies of the GUS gene in independently transformed callus lines ranged from approximately 3 to 30. An enzyme-linked immunosorbent assay for NP II and a fluorometric assay for GUS showed that the expression of both enzymes varied by less than fourfold among callus lines. A histochemical assay for GUS activity revealed a heterogeneous pattern of staining with the substrate 5-bromo-4-chloro-3-indoyl-beta-D-glucuronic acid in some transformed cell cultures. However, cell clusters reacting positively (blue) or negatively (white) with 5-bromo-4-chloro-3-indoyl-beta-D-glucuronic acid demonstrated both GUS activity and NPT II expression in quantitative assays. Somatic embryos induced from transformed cell cultures were found to be uniformly GUS positive by histochemical analysis. All transgenic plants sampled expressed the two markers genes in both root and shoot tissues. GUS activity was found to be higher in leaves than roots by fluorometric and histochemical assays. Conversely, roots expressed higher levels of NPT II than leaves.

TI: Microprojectile bombardment of plant tissues increases transformation frequency by Agrobacterium tumefaciens.
AU: BIDNEY-D; SCELONGE-C; MARTICH-J; BURRUS-M; SIMS-L; HUFFMAN-G
CS: Pioneer Hi-Bred International Inc., Dep. Biotechnology Res., 7300 N.W. 62nd Avenue, Johnston, IA 50131, USA
SO: PLANT MOLECULAR BIOLOGY 18(2): 301-314
PY: 1992
LA: English
AB: **Bombardment** of plant tissues with microprojectiles in an effective method of wounding to promote Agrobacterium-mediated transformation. Tobacco (Nicotiana tabacum) cv. Xanthi leaves and sunflower (Helianthus annuus) apical meristems were wounded by **microprojectile bombardment** prior to application of Agrobacterium tumefaciens strains containing genes within the T-DNA encoding GUS (beta-glucuronidase) or NPTII (neomycin phosphotransferase). Stable kanamycin-resistant tobacco transformants were obtained using an NPTII construct from **particle**/plasmid, **particle**-wounded/Agrobacterium-treated or scalpel-wounded /Agrobacterium-treated potato leaves. Those leaves bombarded with particles suspended in Te buffer prior

to Agrobacterium treatment produced at least 100 times more kanamycin-resistant colonies than leaves treated by the standard **particle** gun transformation protocol. In addition, large sectors of GUS expression, indicative of meristem cell transformation, were observed in plants recovered from sunflower apical explants only when the meristems were wounded first by **particle bombardment** prior to Agrobacterium treatment. Similar results in two different tissue types suggest that (1) particles may be used as a wounding mechanism to enhance Agrobacterium tranformation frequencies, and (2) Agrobacterium mediation of stable transformation is more efficient than the analogous **particle**/plasmid protocol.

TI: Dissection of a pollen-specific promoter from maize by transient transformation assays.
AU: HAMILTON-D-A; ROY-M; RUEDA-J; SINDHU-R-K; STANFORD-J; MASCARENHAS-J-P
CS: Dep. Biol. Sci., Cent. Mol. Genet., State Univ. N.Y. at Albany, Albany, NY 12222, USA
SO: PLANT MOLECULAR BIOLOGY 18(2): 211-218
PY: 1992
LA: English
AB: We have previously reported the isolation and characterization of a gene (Zm13) from Zea mays which shows a pollen-specific pattern of expression. Stably transformed tobacco plants containing a reporter gene linked to portions of the Zm13 5' flanking region show correct temporal and spatial expression of the gene. Here we present a more detailed analysis of the 5' regions responsible for expression in pollen by utilizing a transient expression system. Constructs containing the beta-glucuronidase (GUS) gene under the control of various sized fragments of the Zm 13 5' flanking region were introduced into Tradescantia and Zea mays pollen via high-velocity **microprojectile bombardment**, and monitored both visually and with a fluorescence assay. The results suggest that sequences necessary for expression in pollen are present in a region from -100 to -54, while other sequences which amplify that expression reside betwen -260 and -100. The replacement of the normal terminator with a portion of the Zm 13 3' region containing the putative polyadenylation signal and site also increased GUS expression. While the -260 to -100 region contains sequences similar to other protein-binding domains reported for plants, the -100 to -54 region appears to contain no significant homology to other known promoter fragments which direct pollen-specific expression. The **microprojectile bombardment** of Tradescantia pollen appears to be a good test system for assaying maize and possibly other monocot promoter constructs for pollen expression.

TI: Segregation of transgenes in maize.
AU: SPENCER-T-M; O'BRIEN-J-V; START-W-G; ADAMS-T-R; GORDON-KAMM-W-J; LEMAUX-P-G
CS: Discovery Res., DEKALB Plant Genet., Eastern Point Road, Groton, CT 06340, USA
SO: PLANT MOLECULAR BIOLOGY 18(2): 201-210
PY: 1992
LA: English
AB: Progeny recovered from backcrossed transgenic maize tissue culture regenerants (R-0) were analyzed to determine the segregation, expression, and stability of the introduced genes. Transgenic A188 times B73 R-0 plants (regenerated from embryogenic suspension culture cells transformed by **microprojectile bombardment**; see (9)) were pollinated with nontransformed B73 pollen. Inheritance of a selectable marker gene, bar, and a nonselectable marker gene, uidA, was analyzed in progeny (R-1) representing four independent transformation events. Activity of the bar gene product, phosphinothricin acetyltransferase (PAT), was assessed in plants comprising the four R-1 populations. The number of R-1 plants containing PAT activity per total number of R-1 plants recovered for each population was 2/7, 19/34, 3/14 and 73/73. Molecule analysis confirmed the segregation of bar in three R-1 populations and the lack of segregation in one R-1 population. Cosegregation analysis indicated genetic linkage of bar and uidA in all four R-1 populations. Analysis of numerous R-2 plants derived from crossing transformed R-1 plants with nontransformed inbreds revealed 1:1 segregation of PAT activity in three of four lines, including the line that failed to segregate in the R-1 generation. Integrated copies of bar in one line appeared to be unstable or poorly transmitted.

TI: Transformation and inheritance of a hygromycin phosphotransferase gene in maize plants.
AU: WALTERS-D-A; VETSCH-C-S; POTTS-D-E; LUNDQUIST-R-C
CS: Plant Sci. Res. Inc., 10320 Bren Road East, Minnetonka, MN 55343, USA
SO: PLANT MOLECULAR BIOLOGY 18(2): 189-200
PY: 1992
LA: English
AB: Embryogenic maize (Zea mays L.) callus cultures were transformed by **microprojectile bombardment** with a chimeric hygromycin phosphotransferase (HPT) gene and three transformed lines obtained by selecting for hygromycin resistance. All lines contained one or a few copies of the intact HPT coding sequence. Fertile, transgenic plants were regenerated and the transmission of the chimeric gene was demonstrated through two complete generations. One line inherited the gene in the manner expected for a single, dominant locus, whereas two did not.

TI: In vitro self-splicing reactions of the chloroplast group I intron Cr.LSU from Chlamydomonas reinhardtii and in vivo manipulation via gene-replacement.
AU: THOMPSON-A-J; HERRIN-D-L
CS: University Texas Austin, Dep. Botany, Austin, Tex. 78713, USA
SO: NUCLEIC ACIDS RESEARCH 19(23): 6611-6618
PY: 1991
LA: English
AB: The group I intron from the chloroplast rRNA large subunit of Chlamydomonas reinhardtii (Cr.LSU) undergoes autocatalytic splicing in vitro. Cr.LSU displays a range of reactions typical of other group I introns. Under optimal conditions, the 5' cleavage step proceeds rapidly, but the exon-ligation step is relatively slow, and no pH dependent hydrolysis of the 3' splice site occurs. A requirement for high temperature and high (Mg-2+) suggests involvement of additional splicing factors in vivo. The positions of three cyclization sites of the free intron have been mapped; two of these sites represent reactions analogous to 5'-splice site cleavage, whereas the third is an example of G-exchange. Cr.LSU contains an open reading frame (ORF) potentially encoding an 163 amino acid polypeptide. ORF function has been investigated by using chloroplast gene replacement via **particle**

bombardment. We have shown that the ORF can be deleted from Cr.SU without affecting splicing in vivo and it thus does not encode an essential splicing factor.

TI: Transformation of maize using microprojectile bombardment: An update and perspective.
AU: GORDON-KAMM-W-J; SPENCER-T-M; O'BRIEN-J-V; START-W-G; DAINES-R-J; ADAMS-T-R; MANGANO-M-L; CHAMBERS-S-A; ZACHWIEJA-S-J; ET-AL
CS: Inq: C. J. Mackey, DEKALB Plant Genetics, Discovery Res., Eastern Point Road, Groton, Connecticut 06340, USA
SO: IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY PLANT 27P(1): 21-27
PY: 1991
LA: English
AB: Using **microprojectile bombardment** of maize suspension cultures and bialaphos selection, transformed embryogenic calli have been recovered in numerous independent experiments. Fertile transgenic plants have been regenerated from several transformed callus lines. Stable inheritance and expression of bar and functional activity of the enzyme phosphinothricin acetyl transferase were observed in three subsequent generations of transformed plants. Evidence to date indicates that the transformation process and the presence of the foreign gene per se do not detrimentally influence either plant vigor or fertility. This represents a practical method for introducing foreign genes into maize, which may be applicable to other monocot species.

TI: Deletion analysis of pollen-expressed promoters.
AU: MCCORMICK-S; YAMAGUCHI-J; TWELL-D
CS: Plant Res. Cent., Agric. Can., Ottawa, Ont. K17 OC6, Can
SO: IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY PLANT 27P(1): 15-20
PY: 1991
LA: English
AB: We have used **microprojectile bombardment** of tobacco pollen to study the DNA sequences involved in the expression of pollen-expressed genes. Promoter-reporter gene fusions constructed with the promoters of three different pollen-expressed genes from tomato (LAT52, LAT56, and LAT59) and either the beta-glucuronidase orluciferase reporter genes were assayed by bombarding hydrated tobacco pollen with the gene constructs precipitated onto tungsten microprojectiles. Reporter gene expresssion can be assayed within 30 min, with the maximal level of expression between 6 and 12 h after **bombardment**. By constructing and assaying promoter deletion derivatives, we have been able to delimit regions of the promoters that are necessary for high level expression in pollen. We also demonstrate that results with this transient expression system parallel the expression levels seen in pollen from stably transformed transgenic plants. The **microprojectile bombardment** assay can be used to rapidly test constructs for pollen expression before Agrobacterium-mediated plant transformation. Furthermore, it may be possible to adapt the **microprojectile bombardment** technique to achieve stable transformation of pollen.

TI: Transformation of soybean via particle bombardment of embryogenic suspension culture tissue.
AU: FINER-J-J; MCMULLEN-M-D
CS: Dep. Agronomy, Ohio State Biotechnology Center, Ohio State University, Wooster, Ohio 44691
SO: IN VITRO CELLULAR & DEVELOPMENTAL BIOLOGY PLANT 27P(4): 175-182
PY: 1991
LA: English
AB: Embryogenic suspension culture tissue of soybean (Glycine max Merrill.) was bombarded with particles coated with plasmid DNAs encoding hygromycin resistance and beta-glucuronidase (GUS). One to two weeks after **bombardment**, embryogenic tissue was placed in a liquid proliferation medium containing hygromycin. Four to six weeks after **bombardment**, lobes of yellow-green, hygromycin-resistant tissue, which began as outgrowths on brown clumps of hygromycin-sensitive tissue, were isolated and cultured to give rise to clones of transgenic embryogenic material. In vivo GUS assays of hygromycin-resistant clones showed that the early outgrowths could be negative, sectored, or positive for GUS activity. Transgenic, fertile plants could be routinely produced from the proliferating transgenic embryogenic clones. Southern hybridization analyses confirmed stable transformation and indicated that both copy number and integration pattern of the introduced DNA varied among independently transformed clones. Hybridization analysis of DNA from progeny plants showed genetic linkage of multiple copies of introduced DNA. An average of three transgenic clones were obtained per **bombardment** making this procedure very suitable for transformation of soybean.

TI: Gene transfer using electric discharge particle bombardment and recovery of transformed cranberry plants.
AU: SERRES-R; STANG-E; MCCABE-D; RUSSELL-D; MAHR-D; MCCOWN-B
CS: Dep. Hortic., Univ. Wisconsin-Madison, Madison, Wis. 53706
SO: JOURNAL OF THE AMERICAN SOCIETY FOR HORTICULTURAL SCIENCE 117(1): 174-180
PY: 1992
LA: English
AB: Genetic transformation of the American cranberry, Vaccinium macrocarpon Ait., was accomplished using electric discharge **particle** acceleration. Plasmid DNA containing the genes GUS (beta-glucuronidase), NPTII (neomycin phosphotransferase II), and BT (Bacillus thuringiensis subsp. kurstaki crystal protein) was introduced into stem sections, derived from in vitro cultures, that had been induced to form adventitious buds. The stage of development of these adventitious buds was critical for efficient initial expression. After exposure to electric discharge **particle** acceleration, stem sections were cultured on a solid-phase bud-inducing medium containing 300 mg kanamycin/liter. In addition, a thin overlay of 300 mg kanamycin/liter in water was added to inhibit growth of nontransformed cells. Within 7 weeks, green shoots emerged amidst kanamycin-inhibited tissue. No escape (nontransformed) shoots were recovered, and 90% of the transformed shoots were shown through PCR and Southern blot analysis to contain all three introduced genes. GUS expression varied markedly among various transformed plants. Preliminary bioassays for efficacy of the BT gene against the feeding of an economically important lepidopteran cranberry pest have shown no consistently effective control. Potential with the expression of the BT and GUS genes are discussed

TI: Efficiency of particle-bombardment-mediated transformation is influenced by cell cycle stage in synchronized cultured cells of tobacco.
AU: LIDA-A; YAMASHITA-T; YAMADA-Y; MORIKAWA-H
CS: Dep. Biological Sci., Fac. Sci., Hiroshima University, Higashi-Hiroshima 724, Japan
SO: PLANT PHYSIOLOGY (BETHESDA) 97(4): 1585-1587
PY: 1991
LA: English
AB: Plasmid DNA pB1221 harboring beta-glucuronidase gene was delivered to synchronized cultured tobacco (Nicotiana tabacum L. cv Bright Yellow-2) cells of different cell cycle stages by a pneumatic **particle** gun. The cells bombarded at M and G-2 phases gave 4 to 6 times higher transformation efficiency than those bombarded at the S and G-1 phases.

TI: Transgenic Arabidopsis thaliana plants obtained by particle-bombardment-mediated transformation.
AU: SEKI-M; SHIGEMOTO-N; KOMEDA-Y; IMAMURA-J; YAMADA-Y; MORIKAWA-H
CS: Dep. Biol. Sci., Fac. Sci., Hiroshima Univ., Hiroshima 730, Jpn
SO: APPLIED MICROBIOLOGY AND BIOTECHNOLOGY 36(2): 228-230
PY: 1991
LA: English
AB: Stable transformation of Arabidopsis thaliana with plasmid DNA (pCaMVNEO) harbouring the neomycin phosphotransferase II (npt-II) gene was achieved using a previously described pneumatic **particle** gun device driven by compressed air. Transgenic A. thaliana plants were regenerated from root sections bombarded with DNA-coated gold particles accelerated by the device. Enzyme assays and Southern blot hybridization confirmed the expression of the foreign gene and its stable integration into the Arabidopsis genome. The analysis for kanamycin resistance in R-1 plants from a self-pollinated transformant indicated transmission of the npt-11 gene to R-1 progeny.

TI: Development and optimisation of microprojectile systems for plant genetic transformation.
AU: BIRCH-R-G; FRANKS-T
CS: Botany Dep., Univ. Queenland, Qld 4072, Aust
SO: AUSTRALIAN JOURNAL OF PLANT PHYSIOLOGY 18(5): 453-470
PY: 1991
LA: English
AB: The recently developed **microprojectile** method for gene transfer to intact cells has been successfully used to transform plant species including some which previously resisted attempts using Agrobacterium and protoplasts mediated techniques. In addition, **microprojectile bombardment** has already proved uniquely suitable for other applications including direct transformation of organelle genomes and rapid assessment of transient expression of genetic constructs introduced into cells of intact tissues. Here we describe various **microprojectile** acceleration devices and the steps necessary to develop an effective **microprojectile** mediated transformation system for any plant species. We emphasise the need the optimise the delivery of DNA into cells, and to tailor strategies for generating stably transformed plants based on the nature of the target tissue, behaviour in tissue culture, and available marker genes. Pattern of cotransformation and coexpression of introduced genes in stable nuclear transformants generated with microprojectiles are summarised, and other applications including organelle transformation are briefly discribed. We mention technical limitation to the application of **microprojectile**-mediated gene transfer which need to be overcome if the method is to achieve its full potential as a near-universal gene transfer gene transfer technique with exciting applications in basic plant molecular biology and practical plant improvement.

TI: Evidence that more than 90 percent of beta-glucuronidase-expressing cells after particle bombardment directly receive the foreign gene in their nucleus.
AU: YAMASHITA-T; LIDA-A; MORIKAWA-H
CS: Dep. Biol. Sci., Fac. Sci., Hiroshima Univ., Hiroshima 730, Jpn
SO: PLANT PHYSIOLOGY (BETHESDA) 97(2): 829-831
PY: 1991
LA: English
AB: Plasmid DNA harboring the beta-glucuronidase (GUS) gene, coated on gold particles, was delivered into cultured tobacco (Nicotiana tabacum L. cv Bright Yellow-2) cells using a pneumatic **particle** gun. Cytological analyses of intracellular location of the introduced gold particles before and after GUS expression assay indicated that more than 90% of GUS-expressing cells after **bombardment** received a DNA-coated **particle** in their nucleus.

TI: Gene transfer into intact sugarcane cells using microprojectile bombardment.
AU: FRANKS-T; BIRCH-R-G
CS: Dep. Botany, Univ. Queensland, Qld. 4072, Aust
SO: AUSTRALIAN JOURNAL OF PLANT PHYSIOLOGY 18(5): 471-480
PY: 1991
LA: English
AB: A **microprojectile** accelerator has been constructed and used to bombard cultured sugarcane tissues with GUS reporter gene constructs. Design features useful to minimise target tissue damage and variation between shots are secribed. Transient expression of GUS occurred in pEmuGN-bombarded cells of nonregenerable suspension culture as well as in regenerable embryogenic callus of commercial sugarcane cultivar Q63, and in suspension cultures of regeneration to plants. Parameters yielding transient GUS expression in up to 1055 cells per **bombardment** in homogenous suspension cultures of sugarcane have been established with a mean of 206 expressing cells per **bombardment** over a series of 8 independent experiments. Approximately 4% of these transiently expressing cells continued to express GUS for extended periods, indicating probable stable transformation of intact cells of the commericial sugarcane cultivar. **Microprojectile bombardment** appears the most promising of the available gene transfer techniques for practical genetic transformation of sugarcane because most commercial cultivars readily form regenerable callus suitable for **bombardment**.

TI: Transient expression of foreign genes introduced into barley endosperm protoplasts by PEG-mediated transfer or into intact endosperm tissue by microprojectile bombardment.
AU: LEE-B-T; MURDOCH-K; TOPPING-J; JONES-M-G-K; KREIS-M
CS: Ciba-Geigy Seeds, Rosental R1001A.1.44 CH-4002 Basle, Switzerland
SO: PLANT SCIENCE (LIMERICK) 78(2): 237-246
PY: 1991
LA: English
AB: Starchy endosperm protoplasts from developing barley (Hordeum vulgare) grains were isolated 8-15 days post-anthesis. These endosperm protoplasts were incubated in hormone free medium containing sucrose as a carbon source and glutamine as a nitrogen source. In this medium the endosperm protoplasts remained viable for several days and starch synthesis was also observed. Transient expression of chloramphenicol acetyl transferase (CAT) and beta-glucuronidase (GUS) reporter genes linked to the CaMV 35S promoter was detected 18-20 h after PEG induced DNA uptake. GUS fusions to two endosperm specific promoters, a wheat high molecular weight (HMW) glutenin and a barley chymotrypsin-inhibitor 2 were also functional in the endosperm protoplasts. Similarly, GUS activity could be detected when either the CaMV 35S or HMW glutenin promoters linked to the GUS gene were introduced into intact endosperm tissues of 15-20 days post-anthesis barley grains by **microprojectile bombardment**.

TI: Transient gene expression in bean tissues by high-velocity microprojectile bombardment.
AU: GENGA-A; CERIOTTI-A; BOLLINI-R; BERNACCHIA-G; ALLAVENA-A
CS: Ist. Biosintesi Vegetali, CNR, via Bassini 15, 20133 Milano, Italy
SO: JOURNAL OF GENETICS & BREEDING 45(2): 129-134
PY: 1991
LA: English
AB: A home made high-velocity **microprojectile bombardment** device has been constructed and set up. The efficiency of gene delivery was tested in cotyledons and embryo axes of immature bean seeds and in tobacco leaves. Plasmid pBI 221, carrying the beta-glucuronidase (GUS) gene under the control of CaMV 35S promoter was used. In Phaseolus coccineus 400 +- 178 units (hivin x +- CL) transiently expressing the GUS gene were found per shot. On average, 60% of the apical meristems of both P. coccineus and P. vulgaris embryo axes showed at least one GUS expressing unit after three shots. The choice of the target for attempting stable transformation of bean will depend, for each species of cultivar, on the ability of their tissues to regenerate full plants.

TI: CIS-acting elements in the pyruvate, orthophosphate dikinase gene from maize.
AU: MATSUOKA-M; NUMAZAWA-T
CS: Dep. Plant Physiology, Natl. Inst. Agrobiol. Resources, 2-1-2 Kannondai, Tsukuba, Ibaraki 305, Jpn
SO: MOLECULAR & GENERAL GENETICS 228(1-2): 143-152
PY: 1991
LA: English
AB: To investigate the mechanisms that control expression of the gene for pyruvate, orthophosphate dikinase (PPDK) in maize, the 5' flanking region of the gene was analyzed for interactions with nuclear extracts. Gel retardation assay showed that there are several sites in the promoter region which bind to protein factors. In this report we describe further study of one of these sites, designated the PPD-1 binding site The nuclear binding factor, PPD-1, is restricted to nuclear extracts from green leaves where the PPDK gene is expressed. No binding of PPD-1 was detected in tissues such as roots or etiolated leaves where the gene is not expressed in vivo. Gel retardation assays using deletion fragments from the promoter region and synthetic oligonucleotides, as well as exonuclease III protection assays, revelaed that the site of PPD-1 binding lies between positions -301 and -296. To identify the functional role of the interaction between PPD-1 and its binding site, a deletion series of the promoter region was joined to a reporter gene, beta-glucuronidase. These constructs were introduced into green leaves of maize by **microprojectile bombardment**. Expression of the reporter gene occurred if the PPD-1 binding site remained in the promoter region of the chimeric genes but deletion of the binding site caused a drastic reduction in expression levels. These data indicate that interaction between PPD-1 and its binding site is essential for active transcription of the PPDK gene.

TI: Primary structure of a novel barley gene differentially expressed in immature aleurone layers.
AU: KLEMSDAL-S-S; HUGHES-W; LONNEBORG-A; AALEN-R-B; OLSEN-O-A
CS: Plant Molecular Biol., Lab. NLVF, P.O. Box 51, 1432 As-NLH, Norw
SO: MOLECULAR & GENERAL GENETICS 228(1-2): 9-16
PY: 1991
LA: English
AB: As a direct approach to elucidate the molecular biology of barley aleurone cell development, we differentially screened an aleurone cDNA library made from poly(A)+ RNA of immature grains for clones representing transcripts present in the aleurone but not in the starchy endosperm. For one of these clones, B22E, which hybridies to a 0.7 kb transcript, Northern and in situ hybridization revealed that expression is under complex spatial, temporal and hormonal control in barley grains. cDNAs corresponding to B22E transcripts were isolated from aleurone/pericarp and embryo of developing grains, and from germinating scutella. Among these were the nearly full-length aleurone/pericarp clone pB22E.a16 (541 bp). cDNAs matching the sequence of this clone (type 1 transcript) were found for all tissues investigated. In addition, cDNAs with an extra 12 bp insertion (type 2 transcript) were obtained from germinating scutella. The two different transcripts can encode novel barley proteins of 115 and 119 amino acids, respectively. A gene designated B22EL8 was isolated and sequenced; it encodes the type 1 B22E transcript and contains two introns of 145 and 125 bp. **Particle bombardment** of barley aleurone with a B22EL8 promoter-GUS (beta-glucuronidase) construct demonstrates that the promoter (3 kb) is active in developing barley grains. The promoter is not, however, active in the seeds of tobacco plants transgenic for the B22EL8 gene, indicating the existence of sequences specific for monocots. A comparison of 1.4 kb of upstream sequence of B22E with the maize c1 promoter reveals a number of short, identical sequences which may be responsible for aleurone cell-specific gene transcription.

TI: Expression of inducible angiosperm promoters in a gymnosperm, Picea glauca (white spruce).
AU: ELLIS-D-D; MCCABE-D; RUSSELL-D; MARTINELL-B; MCCOWN-B-H
CS: Dep. Hortic., Univ. Wis.-Madison, Madison, Wis. 53706, USA
SO: PLANT MOLECULAR BIOLOGY 17(1): 19-28
PY: 1991
LA: English
AB: Electrical discharge **particle** acceleration was used to test the transient expression of numerous inducible angiosperm promoters in a gymnosperm Picea glauca (white spruce). Promoter expression was assayed in three different tissues capable of in vitro regeneration, zygotic embryos, seedlings and embryogenic callus. The promoters tested include the light-inducible Arabidopsis and soybean ribulose-1,5-bisphosphate small subunit promoters and a maize phosphoenolpyruvate carboxylase promoter; a soybean heat-shock-inducible promoter, a soybean auxin inducible promoter and a maize alcohol dehydrogenase promoter. Promoters were cloned into a promoter-less expression vector to form a promoter-beta-glucuronidase-nopaline synthase 3' fusion. A similar construct was made using the cauliflower mosaic virus 35S (CaMV 35S) promoter as a control. All promoters were expressed in white spruce embryos, yet at levels lower than CaMV 35S. In addition, in the embryos the heat-shock and the alcohol dehydrogenase promoters showed inducible expression when given the proper induction stimulus. In seedlings, expression of all promoters was lower than in the embryos and expression was only inducible with the heat-shock promoter in the cotyledons. Of the tissues tested, the expression level of all promoters was lowest in embryogenic callus. Interestingly, the expression of the beta-glucuronidase gene in embryogenic callus was restricted to the proembryonal head cells regardless of the promoter used. These results clearly demonstrate the use of **particle bombardment** to test the transient expression of heterologous promoters in organized tissue and the expression of angiosperm promoters in a gymnosperm.

TI: Transient expression from microprojectile-mediated DNA transfer in Pinus taeda.
AU: STOMP-A-M; WEISSINGER-A; SEDEROFF-R-R
CS: Dep. Forest., N.C. State Univ., Raleigh, N.C. 27695, USA
SO: PLANT CELL REPORTS 10(4): 187-190
PY: 1991
LA: English
AB: Transfer of plasmid DNA to Pinus taeda L. (loblolly pine) cotyledon cells by **microprojectile bombardment** has been demonstrated using beta-glucuronidase (GUS). GUS histochemical staining indicated active enzyme in localized centers (blue spots) 24 hours after **bombardment**. GUS expression declined during subsequent culture, but remained detectable in meristematic tissue 62 days post-**bombardment**, however, transgenic shoots were not recovered. Localized GUS expression events resulted predominantly from single-cell events containing one **microprojectile**. The staining pattern was complex, with indigo found both in the central target cells and in adjacent cells. Cellular damage sustained by GUS-positive cells ranged from undetectable to sufficiently extensive to cause cell death. **Microprojectile bombardment** provides a useful method to assay transient gene expression in loblolly pine and has potential for the production of transgenic plants in pine.

TI: Transient expression of marker genes in immature zygotic embryos of spring wheat (Triticum aestivum) through microprojectile bombardment.
AU: CHIBBAR-R-N; KARTHA-K-K; LEUNG-N; QURESHI-J; CASWELL-K
CS: Plant Biotechnology Institute, National Research Council, 110 Gymnasium Road, Saskatoon, Sask. S7N OW9, Canada
SO: GENOME 34(3): 453-460
PY: 1991
LA: English
AB: Transient expression of marker genes (cat and uidA) delivered by the Biolistics **microprojectile bombardment** technique has been detected in immature zygotic embryos of wheat (Triticum aestivum L.). The DNA expression vectors that gave maximal expression of both cat (pCaMVI-1CN) and uidA (pCaMVI-1GusN) genes had an alcohol dehydrogenase (AdhI) intron 1 cloned in between the cauliflower mosaic virus (CaMV35S) promoter and the coding region of the gene. Detection of chloramphenicol acetyltransferase (CAT) activity in response to cat gene was complicated by the presence of an inhibitor of CAT activity as well as an endogenous CAT-like activity. The results of enzymatic assays were confirmed by an ELISA technique using CAT-specific antibodies, whereas the beta-glucuronidase (GUS) activity following the introduction of the uidA gene was confirmed by both histochemical and fluorometric techniques.

TI: Histology of, and physical factors affecting, transient GUS expression in pearl millet (Pennisetum glaucum (L.) R. Br.) embryos following microprojectile bombardment.
AU: TAYLOR-M-G; VASIL-I-K
CS: Lab. Plant Cell Molecular Biology, Dep. Vegetable Crops, University Florida, Gainesville, Fla. 32611
SO: PLANT CELL REPORTS 10(3): 120-125
PY: 1991
LA: English
AB: Transient GUS (beta-glucuronidase) expression was visualized in whole and sectioned embryos of Pennisetum glaucum (L.) R. Br. (pearl millet) after **microprojectile bombardment** with pMON 8678 DNA. Strongest GUS expression occurred in cells located in the center of GUS positive spots with decreasing intensity in surrounding cells. GUS positive cells could be seen up to 12 cell layers beneath the epidermis. Needle-like crystals of the GUS assay product were found throughout the cytoplasm of GUS positive cells. The number of GUS positive spots was correlated to the **microprojectile** spread pattern on the medium surface. Shorter **bombardment** distances (6.6 and 9.8 cm) and the standard accelerator speed gave the best results for transient expression but also caused maximum tissue damage. The speed and distance, however, had little influence on the ability of bombarded embryos to form compact callus. The developmental stage of the bombarded immature embryos was the determining factor in the formation of compact callus, from which plants were regenerated.

TI: Deletion analysis of a phytochrome-regulated monocot rbcS promoter in a transient assay system.
AU: ROLFE-S-A; TOBIN-E-M
CS: Dep. Biol., University California, 404 Hilgard Avenue, Los Angeles, Calif. 90024-1606
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 88(7): 2683-2686
PY: 1991
LA: English
AB: We have developed a transient gene expression assay system in the aquatic monocot Lemna gibba in which DNA was introduced into intact tissue by **particle bombardment**. Constructs based on the Lemna rbcS gene SSU5B, which is positively regulated by phytochrome in vivo, also showed phytochrome regulation in the transient assay system. Reporter gene expression increased 12-fold over dark levels in response to a single treatment with red light. This increase was not observed if far-red light was immediately followed by the red light. A 5' deletion analysis of the promoter defined a region from position -205 to position -83 relative to the start of transcription as necessry to observe the phytochrome response. This region contains the binding site for the light-induced binding activity (LRF-1) found in Lemna nuclear extracts. Upstream of position -205, we found evidence for the presence of at least two upstream activating sequences and a silencer.

TI: Engineering the chloroplast genome: Techniques and capabilities for chloroplast transformation in Chlamydomonas reinhardtii.
AU: KINDLE-K-L; RICHARDS-K-L; STERN-D-B
CS: Plant Sci. Cent., Biotechnol. Building, Cornell Univ., Ithaca, N.Y. 14853
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 88(5): 1721-1725
PY: 1991
LA: English
AB: Chloroplast transformation of Chlamydomonas reinhardtii has been accomplished by agitating cell wall-deficient cells in the presence of glass beads and DNA. By using the atpB gene as the selected marker and cells grown in 0.5 mM 5-fluorodeoxyuridine, we have recovered up to 50 transformants per microgram of DNA. This method is easy and does not require specialized equipment, although it is not as efficient as the tungsten **particle bombardment** method (Boynton, J. E., Gillham, N. W., Harris, E. H., Hosler, J. P., Johnson, A. M., Jones, A. R., Randolph-Anderson, B. L., Robertson, D., Klein, T. M., Shark, K. B. & Sanford, J. C. (1988) Science 240, 1534-1537). By using **particle bombardment**, we have developed a cotransformation approach in which spectinomycin-resistant 16S rRNA-encoding DNA is the selected marker, and we have demonstrated that cotransformation of an unselected marker on an independent replicon is very efficient. We have used this strategy (i) to recover transformants with partially depleted atpB genes that could not otherwise have been selected since they did not restore photosynthetic capability to a recipient carrying a more extensive atpB deletion and (ii) to generate specific deletion mutations in a wild-type recipient. This methodology should allow the introduction of any desired change into the chloroplast genome, even in the absence of phenotypic selection, and thus a detailed functional analysis of any chloroplast DNA sequence should be possible.

TI: Cis-acting elements involved in photoregulation of an oat phytochrome promoter in rice.
AU: BRUCE-W-B; QUAIL-P-H
CS: Univ. Calif. Berkeley/United States Dep. Agric., Plant Gene Expression Cent., 800 Buchanan St., Albany, Calif. 94710
SO: PLANT CELL 2(11): 1081-1090
PY: 1990
LA: English
AB: Phytochrome negatively regulates the transcripts of its own phyA genes. High levels of Pfr, the active, far-red-light absorbing form of phytochrome, repress phyA transcription; low Pfr levels result in derepression. We have utilized **microprojectile**-mediated gene transfer to identify regions of an oat phyA 3 gene involved in this autoregulation. Chimeric constructs containing various deletion and sequence substitution mutants of the oat phyA3 gene fused to a chloramphenicol acetyltransferase reporter (phyA3/CAT) have been introduced into etiolated rice seedlings by **particle bombardment**. Low Pfr concentrations induce high phyA3/CAT expression, whereas high Pfr represses activity to near basal levels. Removal of phyA3 sequences 3' to the transcription start site reduces expression about fivefold, suggesting that intron 1 of the phyA3 gene may be required for high activity. The degree of high-Pfr-imposed repression is unaffected by any of a series of deletions or sequence substitutions in the phyA3 promoter, thus providing no evidence of any Pfr-activated negative elements. In contrast, 5' and internal deletions identify a minimum of three major positive promoter elements, designated PE1 (-381 base pairs (bp) to -348 bp), identify a minimum of three major positive promoter elements, designated PE1 (-391 base pairs bp), PE2 (-635 bp to -489 bp), and PE3 (-110 bp to -76 bp) that are necessary for high-level expression in low-Pfr cells. The data indicate that PE1 and PE2 are functionally redundant, but that PE3 is required in conjunction with either PE1 or PE2 for activity. PE3 contains a sequence element that is highly conserved between monocot phyA promoters, indicative of a critical role in phyA expression.

TI: Stable transformation of plastids in higher plants.
AU: SVAB-Z; HAJDUKIEWICZ-P; MALIGA-P
CS: Waksman Inst., Rutgers, State University New Jersey, Piscataway, N.J. 08855-0759
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 87(21): 8526-8530
PY: 1990
LA: English
AB: Stable genetic transformation of the plastid genome is reported in a higher plant, Nicotiana tabacum. Plastid transformation was obtained after **bombardment** of leaves with tungsten particles coated with pZS148 plasmid DNA. Plasmid pZS148 (9.6 kilobases) contains a 3.7-kilobase plastid DNA fragment encoding the 16S rRNA. In the 16S rRNA-encoding DNA (rDNA) a spectinomycin resistance mutation is flanked on the 5' side by a streptomycin resistance mutation and on the 3' side by a Pst I site generated by ligating an oligonucleotide in the intergenic region. Transgenic lines were selected by spectinomycin resistance and distinguished from spontaneous mutants by the flanking, cotransformed streptomycin resistance and Pst I

markers. Regenerated plants are homoplasmic for the spectinomycin resistance and the Pst I markers and heteroplasmic for the unselected streptomycin resistance trait. Transgenic plastid traits are transmitted to the seed progeny. The transgenic plastid genomes are products of a multistep process, involving DNA recombination, copy correction, and sorting out of plastid DNA copies.

TI: Transient expression of exogenous DNA in intact, viable wheat embryos following particle bombardment.
AU: LONSDALE-D; ONDE-S; CUMING-A
CS: Dep. Genetics, Univ. Leeds, Leeds LS2 9JT, UK
SO: JOURNAL OF EXPERIMENTAL BOTANY 41(230): 1161-1166
PY: 1990
LA: English
AB: Expression of foreign DNA has been detected in intact, germinating wheat embryos (Triticum aestivum L.) following **bombardment** with tungsten particles complexes with a reporter gene encoding the bacterial enzyme beta-glucuronidase ('GUS': E.C. 3.2.1.31). Expression was detected in situ in individual cells and groups of cells, by supplying the germinating embryos with the chromogenic substrate of the GUS enzyme, 'X-gluc'. Expression was dependent on the presence of a constitutive plant promoter, the Cauliflower Mosaic Virus '35S' promoter, fused to the GUS structural coding sequence. The relative simplicity of this technique recommends its future use for the assay of regulatory elements which control the spatial and temporal specificity of genes expressed during embryo development.

TI: Stable transformation of papaya via microprojectile bombardment.
AU: FITCH-M-M-M; MANSHARDT-R-M; GONSALVES-D; SLIGHTOM-J-L; SANFORD-J-C
CS: Dep. Horticulture, Univ. Hawaii, Honolulu, Hawaii 96822, USA
SO: PLANT CELL REPORTS 9(4): 189-194
PY: 1990
LA: English
AB: Stable transformation of papaya (Carica papaya L.) has been achieved following DNA delivery via high velocity microprojectiles. Three types of embryogenic tissues, including immature zygotic embryos, freshly explanted hypocotyl sections, and somatic embryos derived from both, were bombarded with tungsten particles carrying chimeric NPTII and GUS genes. All tissue types were cultured prior to and following **bombardment** on half-strength MS medium supplemented with 10 mg l-1 2,4-D, 400 mg l-1 glutamine, and 6% sucrose. Upon transfer to 2,4-D-free medium containing 150 mg l-1 kanamycin sulfate, ten putative transgenic isolates produced somatic embryos and five regenerated leafy shoots. Leafy shoots were produced six to nine months following **bombardment**. Tissues from 13 of these isolates were assayed for NPTII (neomycin phosphotransferase) activity, and 10 were positive. Six out of 15 isolates assayed for GUS (beta-glucuronidase) expression were positive. Three isolates were positive for both NPTII and GUS.

TI: Transformation of maize cells and regeneration of fertile transgenic plants.
AU: GORDON-KAMM-W-J; SPENCER-T-M; MANGANO-M-L; ADAMS-T-R; DAINES-R-J; START-W-G; O'BRIEN-J-V; CHAMBERS-S-A; ADAMS-W-R-JR; ET-AL
CS: Inq.: Catherine J. Mackey, Discovery Res., DeKalb Plant Genetics, Eastern Point Road, Groton, Conn. 06340
SO: PLANT CELL 2(7): 603-618
PY: 1990
LA: English
AB: A reproducible system for the generation of fertile, transgenic maize plants has been developed. Cells from embryogenic maize suspension cultures were transformed with the bacterial gene bar using **microprojectile bombardment**. Transformed calli were selected from the suspension cultures using the herbicide bialaphos. Integration of bar and activity of the enzyme phosphinothricin acetyltransferase (PAT) encoded by bar were confirmed by all bialaphos-resistant callus lines. Fertile transformed maize plants (R-0) were regenerated, and of 53 progeny, (R-1) tested, 29 had PAT activity. All PAT-positive progeny analyzed contained bar. Localized application of herbicide to leaves of bar-transformed R-0 and R-1 plants resulted in no necrosis, confirming functional activity of PAT in the transgenic plants. Cotransformation experiments were performed using a mixture of two plasmids, one encoding PAT and one containing the nonselected gene encoding beta-glucuronidase. R-0 plants regenerated from cotransformed callus expressed both genes. These results describe and confirm the development of a system for introduction of DNA into maize.

TI: The pFF plasmids: Cassettes utilizing CaMV sequences for expression of foreign genes in plants.
AU: TIMMERMANS-M-C-P; MALIGA-P; VIEIRA-J; MESSING-J
CS: Waksman Inst., Rutgers, State University New Jersey, Piscataway, NJ 08855-0759
SO: JOURNAL OF BIOTECHNOLOGY 14(3-4): 333-344
PY: 1990
LA: English
AB: A plant expression cassette was constructed using the cauliflower mosaic virus 35S 5' regulatory region with the enhancer duplicated and the 35S polyadenylation signal. Insertion of a polylinker between the transcription initiation and polyadenylation sites allows for easy cloning of genes. To test the usefulness of the cassette chimeric bacterial genes were prepared. The constructs were introduced into Nicotiana tabacum suspension culture cells by the **particle bombardment** process. Expression of the beta-glucuronidase reporter gene was verified by histochemical staining. Stable kanamycin and hygromycin resistant transgenic lines were obtained after introduction of chimeric genes encoding the enzymes neomycin phosphotransferase and hygromycin B phosphotransferase, respectively. The number of stable transformants was approximately 2% of the cells that transiently expressed the beta-glucuronidase reporter gene.

TI: Agrobacterium and microprojectile, mediated viral DNA delivery into barley microspore-derived cultures.
AU: CREISSEN-G; SMITH-C; FRANCIS-R; REYNOLDS-H; MULLINEAUX-P
CS: John Innes Inst., Colney Lane, Norwich NR4 7UH, UK
SO: PLANT CELL REPORTS 8(11): 680-683

PY: 1990
LA: English
AB: Anther cultures of barley (Hordeum vulgare L. var. "Igri") were used as targets for Agrobacteriuim-mediated DNA transfer and direct DNA uptake by **particle bombardment**. A wheat dwarf virus construct which can replicate to a high copy number in cereal cells provided a sensitive marker for successful DNA delivery. Although DNA delivery was achieved using both procedures, **particle bombardment** gave more reproducible and higher levels of infection. The ability to deliver DNA into cereal cells which have a high regeneration capacity may provide a route for stable transformation.

TI: Transgenic tobacco plants and their progeny derived by microprojectile bombardment of tobacco leaves.
AU: TOMES-D-T; WEISSINGER-A-K; ROSS-M; HIGGINS-R; DRUMMOND-B-J; SCHAAF-S; MALONE-SCHONEBERG-J; STAEBELL-M; FLYNN-P; ANDERSON-J; HOWARD-J
CS: Dep. Biotechnol., Res. Pioneer Hi-Bred Int. Inc., Johnston, Iowa 50131, USA
SO: PLANT MOLECULAR BIOLOGY 14(2): 261-268
PY: 1990
LA: English
AB: Transgenic tobacco plants (Nicotiana tabacum) and progency carrying coding sequences for neomycin phosphotransferase II (NPTII) and beta-glucuronidase (GUS) were recovered following **microprojectile bombardment** of tobacco leaves. Transgenic plants were regenerated from bombarded leaf pieces of tobacco cvs. 'Xanthi' and 'Ky 17' which were cultured in the presence of 100 or 200 mu-g/ml kanamycin for six to eight weeks. Among 160 putative transgenic plants from at least 16 independent transformation events 76% expressed NPTII, and 50% expressed GUS. Southern analysis of plants expressing either one or both of the enzymes indicated DNA in high molecular weight DNA in 8 of 9 independent transformants analyzed. Two independent transformants and their progeny were analyzed in detail. Analysis of progeny for quantitative enzyme levels of NPTII and GUS, and Southern analysis of parents and progeny clearly demonstrated that the genes were transmitted to progeny. One transformant demonstrated Mendelian ratios for seed germination on kanamycin-containing medium while the other transformant had non-Mendelian ratios. DNA analysis of progeny indicate complex integration of the plasmid DNA, and suggest that rearrangements of this DNA has occurred. These results are consistent with other methods of direct DNA uptake into cells, and verify that the **microprojectile bombardment** method is capable of DNA delivery into intact plant cells which can give rise to transgenic plants and progeny.

TI: Transformation of cotton (Gossypium hirsutum L.) via particle bombardment.
AU: FINER-J-J; MCMULLEN-M-D
CS: Dep. Agronomy, Ohio State Biotechnol. Cent., Ohio Agric. Res. Dev. Cent., Ohio State Univ., Wooster, Ohio 44691, USA
SO: PLANT CELL REPORTS 8(10): 586-589
PY: 1990
LA: English
AB: Embryogenic suspension cultures of cotton (Gossypium hirsutum L.) were subjected to **particle bombardment**, where high density particles carrying plasmid DNA were accelerated towards the embryogenic plant cells. The plasmid DNA coating the particles encoded hygromycin resistance. One to two weeks following **bombardment**, embryogenic cotton cells were placed in proliferation medium containing 100 mu-g/ml hygromycin. Clumps of tissue which grew in the presence of hygromycin were subcultured at low density into fresh hygromycin-containing proliferation medium. Following sequential transfer of embryogenic tissue to development and then germination media, plants were recovered from transgenic embryogenic tissue. Southern hybridization cofirmed the presence of the hygromycin resistance gene in embryogenic suspension culture tissue and regenerated plants.

TI: Transient expression of chimeric genes delivered into pollen by microprojectile bombardment.
AU: TWELL-D; KLEIN-T-M; FROMM-M-E; MCCORMICK-S
CS: US Dep. Agric.-Agric. Res. Serv., Plant Gene Expression Cent., Albany, Calif. 94710, USA
SO: PLANT PHYSIOLOGY (BETHESDA) 91(4): 1270-1274
PY: 1989
LA: English
AB: Chimeric genes containing a pollen-specific promoter from tomato (Lycopersicon esculentum) or the CaMV35S promoter were transiently expressed following their introduction into tobacco (Nicotiana tabacum) pollen using high velocity microprojectiles. Transient expression of the **microprojectile**-introduced genes in leaves and pollen was similar to that observed for these genes in stably transformed tobacco plants.

TI: Transient expression of chloramphenicol acetyltransferase (CAT) gene in barley cell cultures and immature embryos through microprojectile bombardment.
AU: KARTHA-K-K; CHIBBAR-R-N; GEORGES-F; LEUNG-N; CASWELL-K; KENDALL-E; QURESHI-J
CS: Plant Biotechnol. Inst., Natl. Res. Council Can., 110 Gymnasium Road, Saskatoon S7N 0W9, Saskatchewan, Can
SO: PLANT CELL REPORTS 8(8): 429-432
PY: 1989
LA: English
AB: Transient expression of chloramphenicol acetyl transferase gene has been detected in cultured barley (Hordeum vulgare L. cv. Heartland) cells and freshly isolated immature zygotic embryos (cv. Ellice) following the introduction of the gene by **microprojectile bombardment**. The DNA expression vector used to introduce the CAT gene, pCaMVI-1CN is a pUC8 derivative and consisted of a CaMV35S promoter, a fragment of alcohol dehydrogenase intron1, a CAT coding region and NOS polyadenylation region. The inclusion of the Adh1 intron1 was essential for the expression of CAT activity in cultured cells as well as immature zygotic embryos. Expression of CAT activity, which was dependent upon the DNA concentration used, could be detected as early as 20 h after **bombardment**. The results also suggested tha the recipient cells have to be in an active state of cell division in order for the introduced gene to be expressed since mature zygotic as well as somatic embryos failed to reveal any gene expression. The effect of other parameters which influence the expression of the introduced gene as well as the potential of this novel technology for cereal transformation are also discussed.

TI: Photoregulation of a phytochrome gene promoter from oat transferred into rice by particle bombardment.
AU: BRUCE-W-B; CHRISTENSEN-A-H; KLEIN-T; FROMM-M; QUAIL-P-H
CS: Univ. California, Berkeley/US Dep. Agric. Plant Gene. Expression Cent., 800 Buchanan St., Albany, Calif. 94710
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 86(24): 9692-9696
PY: 1989
LA: English
AB: The regulatory photoreceptor phytochrome controls the transcription of its own phy genes in a negative feedback fashion. We have exploited **microprojectile**-mediated gene transfer to develop a rapid transient expression assay system for the study of DNA sequences involved in the phytochrome-regulated expression of these genes. The 5'-flanking sequence and part of the structural region of an oat phy gene have been fused to a reporter coding sequence (chloramphenicol acetyltransferase, CAT) and introduced into intact dark-grown seedlings by using high-velocity microprojectiles. Expression is assayable in lt 24 hr from **bombardment**. The introduced oat phy-CAT fusion gene is expressed and down-regulated by white light in barley, rice, and oat, whereas no expression is detected in three dicots tested, tobacco, cucumber, and Arabidopsis thaliana. In bombarded rice shoots, red/far-red light-reversible repression of expression of the heterologous oat phy-CAT gene shows that it is regulated by phytochrome in a manner parallel to that of the endogenous rice phy genes. These data indicate that the transduction pathway components and promoter sequences involved in autoregulation of phy expression have been evolutionarily conserved between oat and rice. The experiments show the feasibility of using high-velocity **microprojectile**-mediated gene transfer for the rapid analysis of light-controlled monocot gene promoters in monocot tissues that until now have bene recalcitrant to such studies.

TI: Stable nuclear transformation of Chlamydomonas using the Chlamydomonas gene for nitrate reductase.
AU: KINDLE-K-L; SCHNELL-R-A; FERNANDEZ-E; LEFEBVRE-P-A
CS: Sect. Biochem. Mol. and Cell Biol., Cornell Univ., Ithaca, New York 14853
SO: JOURNAL OF CELL BIOLOGY 109(6 PART 1): 2589-2602
PY: 1989
LA: English
AB: We have developed a nuclear transformation system for Chlamydomonas reinhardtii, using **microprojectile bombardment** to introduce the gene encoding nitrate reductase into a nit1 mutant strain which lacks nitrate reductase activity. By using either supercoiled or linear plasmid DNA, transformants were recovered consistently at a low efficiency, on the order of 15 transformants per microgram of plasmid DNA. In all cases the transforming DNA was integrated into the nuclear genome, usually in multiple copies. Most of the introduced copies were genetically linked to each other, and they were unlinked to the original nit1 locus. The transforming DNA and nit+ phenotype were stable through mitosis and meiosis, even in the absence of selection. nit1 transcripts of various sizes were expressed at levels equal to or greater than those in wild-type nit+ strains. In most transformants, nitrate reductase enzyme activity was expressed at approximately wild-type levels. In all transformants, nit1 mRNA and nitrate reductase enzyme activity were repressed in cells grown on ammonium medium, showing that expression of the integrated nit1 genes was regulated normally. When a second plasmid with a nonselectable gene was bombarded into the cells along with the nit1 gene, transformants carrying DNA from both plasmids were recovered. In some cases, expression of the unselected gene could be detected. With the advent of nuclear transformation in Chlamydomonas, it becomes the first photosynthetic organism in which both the nuclear and chloroplast compartments can be transformed.

TI: Genetic transformation of maize cells by particle bombardment.
AU: KLEIN-T-M; KORNSTEIN-L; SANFORD-J-C; FROMM-M-E
CS: Plant Gene Expression Cent., USDA,ARS/Univ. Calif., Berkeley, Calif. 94710
SO: PLANT PHYSIOLOGY (BETHESDA) 91(1): 440-444
PY: 1989
LA: English
AB: Intact maize (Zea mays) cells were bombarded with microprojectiles bearing plasmid DNA coding for selectable (neomycin phosphotransferase (NPT II)) and screenable (beta-glucuronidase (GUS)) marker genes. Kanamycin-resistant calli were selected from bombarded cells, and these calli carried copies of the NPT II and GUS genes as determined by Southern blot analysis. All such calli expressed GUS although the level of expression varied greatly between transformed cell lines. These results show that intact cells of important monocot species can be stably transformed by microprojectiles.

TI: Stable genetic transformation of intact Nicotiana cells by the particle bombardment process.
AU: KLEIN-T-M; HARPER-E-C; SVAB-Z; SANFORD-J-C; FROMM-M-E; MALIGA-P
CS: Plant Gene Expression Cent., U.S. Dep. Agric., Agric. Res. Serv., 800 Buchanan St., Albany, Calif. 94710
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 85(22): 8502-8505
PY: 1988
LA: English
AB: We show that the genetic transformation of Nicotiana tabacum can be achieved by bombarding intact cells and tissues with DNA-coated particles. Leaves or suspension culture cells were treated with tungsten microprojectiles carrying plasma DNA containing a neomycin phosphotransferase gene. Callus harboring the foreign gene was recovered from the bombarded tissue by selection on medium containing kanamycin. Kanamycin-resistant plants have subsequently been regenerated from the callus derived from leaves. Transient expression of an introduced beta-glucuronidase gene was used to assess the efficiency of DNA delivery by microprojectiles. The frequency of cells that were stably transformed with the neomycin phosphotransferase gene was a few percent of the cells that transiently expressed the beta-glucuronidase gene. These results show that gene transfer by high-velocity microprojectiles is a rapid and direct means for transforming intact plant cells and tissues that eliminates the need for production of protoplasts or infection by Agrobacterium.

TI: Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment.
AU: WANG-Y-C; KLEIN-T-M; FROMM-M; CAO-J; SANFORD-J-C; WU-R
CS: Sect. Biochem. Mol. Cell Biol., Cornell Univ., Ithaca, N.Y. 14853
SO: PLANT MOLECULAR BIOLOGY 11(4): 433-440
PY: 1988
LA: English
AB: The development of an efficient transformation system is a prerequisite for the molecular analysis of gene expression in plants. In crop plants, this development has been hindered by difficulties encountered both in whole plant regeneration from protoplasts and in the general insusceptibility of monocots to Agrobacterium-mediated transformation. We have circumvented these difficulties by transferring foreign genes directly into the intact cells (with cell walls) to three important crop plants including rice, wheat and soybean by a **particle bombardment** device. Oryza sativa and Triticum monococcum cells were bombarded with accelerated tungsten particles coated with plasmids containing a beta-glucuronidase gene as the reporter. Blue transformed cells were detected in an in situ enzyme assay. The number of blue cells was next used as a convenient criterion to study several factors affecting gene transfer efficiency. After optimal conditions were defined, gene transfer into intact cells of O. sativa, T. monococcum and Glycine max was successfully carried out with chloramphenicol acetyltransferase (CAT) gene as the reporter.

TI: Plasmids can stably transform yeast mitochondria lacking endogenous mitochondrial DNA.
AU: FOX-T-D; SANFORD-J-C; MCMULLIN-T-W
CS: Sect. Genetics Dev., Cornell Univ., Ithaca, N.Y. 14853
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 85(19): 7288-7292
PY: 1988
LA: English
AB: The mitochondrial gene oxi1, carried on a bacterial plasmid, has been used to transform the mitochondria of a yeast strain lacking mtDNA (rho-0). The plasmid DNA behaved in a manner entirely consistent with the known properties of normal yeast rho- mtDNA after its introduction by high-velocity **microprojectile bombardment**. Like the mtDNA sequences retained in natural rho- strains, the plasmid DNA in the transformants was reiterated into concatemers whose size was indistinguishable from that of wild-type mtDNA. The oxi1 sequences in the transformants were surrounded by restriction sites derived from the plasmid that were not present in wild-type mtDNA. oxi1 genetic information in these "synthetic rho-" strains could be expressed in diploids either after "marker rescue" by recombination with rho+ mtDNA carrying an appropriate oxi1 point mutation or in trans during the growth of diploids heteroplasmic for both the plasmid-derived oxi1 sequences and rho+ mtDNA with oxi1 deleted. The ability to generate such "synthetic rho-" strains by transformation will allow transfer of mutations generated in vitro to wild-type rho+ mtDNA as well as examination of the function of altered genes in trans.

TI: Microprojectile-DNA delivery in conifer species: Factors affecting assessment of transient gene expression using the beta-glucuronidase reporter gene.
AU: CHAREST-P-J; CALERO-N; LACHANCE-D; DATLA-R-S-S; DUCHESNE-L-C; TSANG-E-W-T
CS: Molecular Genetics Tissue Culture Group, Petawawa Natl. Forestry Inst., Forestry Canada, Chalk River, ON K0J 1J0, Can
SO: PLANT CELL REPORTS 12(4): 189-193
PY: 1993
LA: English
AB: The **Biolistic microprojectile** DNA-delivery method was used to test the usefulness in conifers of eight gene constructs based on the 35S promoter, the AMV translational enhancer, and gene fusion between the beta-glucuronidase and the neomycin phosphotransferase II genes. The evaluation was done with embryogenic cells of Picea glauca, where the relative strengths of the promoters were 35S-35S-AMVE gt 35S-AMVE gt 35S-35S gt 35S as evaluated by transient gene expression. The fusion gene of GUS and NPT II gave lower levels of transient gene expression that the unfused GUS gene as detected by X-GLU histochemical assays. Experiments comparing the EM promoter of wheat and the 35S-35S-AMVE promoter (with and without fusion between GUS and NPT II) were done in Picea rubens, P. P. mariana, P. glauca, and Larix times eurolepis. The unfused gene with the 35S-35S-AMVE promoter gave higher levels of transient gene expression than the fused GUS-NPT II gene. The fluorescent MUG assay was more sensitive than the histochemical X-GLU assay to detect the activity of the beta-glucuronidase gene.

TI: High-frequency plastid transformation in tobacco by selection for a chimeric aadA gene.
AU: SVAB-Z; MALIGA-P
CS: Waksman Inst., Rutgers, State Univ. New Jersey, Piscataway, NJ 08855-0759
SO: PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA 90(3): 913-917
PY: 1993
LA: English
AB: We report here a 100-fold increased frequency of plastid transformation in tobacco by selection for a chimeric aadA gene encoding aminoglycoside 3"-adenylyltransferase, as compared with that obtained with mutant 16S rRNA genes. Expression of aadA confers resistance to spectinomycin and streptomycin. In transforming plasmid pZS197, a chimeric aadA is cloned between rbcL and open reading frame ORF512 plastid gene sequences. Selection was for spectinomycin resistance after **biolistic** delivery of pZS197 DNA into leaf cells. DNA gel-blot analysis confirmed incorporation of the chimeric aadA gene into the plasmid genome by two homologous recombination events via the flanking plastid gene sequences. The chimeric gene became homoplasmic in the recipient cells and is uniformly transmitted to the maternal seed progeny. The ability to transform routinely plastids of land plants opens the way to manipulate the process of photosynthesis and to incorporate novel genes into the plastid genome of crops

TI: Genetic engineering: An addition tool for plant improvement.
AU: JAIN-S-M; OKER-BLOM-C; PEHU-E; NEWTON-R-J
CS: Univ. Helsinki, Dep. Plant Production, SF-00710 Helsinki, Finland
SO: AGRICULTURAL SCIENCE iN FINLAND 1(3): 323-338
PY: 1992
LA: English
AB: Advances in gene transfer technologies have enabled the production of both monocot and dicot transgenic plants. With the **biolistic** method, genes can be transferred in recalcitrant crop plants and forest trees, independent of their genotype. Inexpensive methods for both stable and transient gene transfers-ultrasonication, direct DNA insertion during imbibition using somatic embryos, and silicon carbide fibres-have been developed. The frequency of Agrobacterium-mediated transformation rates of cloned genes can be enhanced in plant cells. The analysis of molecular markers (RFLPs, RAPDs, DNA fingerprints) can accomplished the characterization, gene mapping and identification and certification and patent protection of cultivars. With PCR, selective amplification of a specific DNA segment from a small amount of an organism's total DNA can be used to identify transgenic cultivars. The expression of a target gene can be inhibited with antisense RNA. So far, a limited number of genes have been identified and cloned with genetic engineering. With specific gene transfers, many goals such as biological control of insect pests and fungi, male sterility, virus resistance, improving seed protein, and production of transgenic plants as "bioreactors" can be accomplished. T-DNA mutagenesis may lead to learning more about the genetic control of plant development and morphogenesis, and isolation of useful mutants. Before genetic engineering becomes a reliable tool of plant breeding, more attention is needed to explore: (a) new plant genetic resources in order to identify and clone new genes, (b) fate of selective and scorable marker genes, and (c) field evaluation of transgenes in transgenic plants.

TI: Regulation and interaction of multiple protein factors with the proximal promoter regions of a rice high pI alpha-amylase gene.
AU: KIM-J-K; CAO-J; WU-R
CS: Field Botany, Div. Biological Sci., Cornell Univ., Ithaca, N.Y. 14853, USA
SO: MOLECULAR & GENERAL GENETICS 232(3): 383-393
PY: 1992
LA: English
AB: The alpha-amylase gene is known to be regulated by the plant hormone gibberellin (GA) in cereal aleurone cells. The accumulation of the mRNA corresponding to a rice high pI alpha-amylase gene, OSamy-c, was stimulated 20-fold by exogenous GA-3, in half-seeds lacking embryos. Regulatory regions in the promoter of this high pI subfamily were analyzed. The OSamy-c 5'-flanking sequence, spanning positions -231 to +29, was fused upstream of the beta-glucuronidase (GUS) gene coding region. The delivery of this plasmid into rice aleurone cells by the **biolistic** method resulted in a GA-stimulated synthesis of GUS. Gel retardation assays were performed to study protein-DNA interactions between putative regulatory sequences of OSamy-c and partially purified rice seed extracts. We identified multiple seed-specific protein factors that bind to proximal regions of the OSamy-c promoter between positions -231 and 162. Five different proteins were distinguished based on competitive binding studies. Three protein binding regions were located by footprinting analyses, one of which is located in the conserved sequence also found upstream of other GA-inducible genes. Two protein factors in rice aleurone cells that interact with the putative regulatory sequence do not require GA induction.

TI: Effect of promoter sequence on transient expression of the beta-glucuronidase gene in embryogenic calli of Larix X eurolepis and Picea mariana following microprojection.
AU: DUCHESNE-L-C; CHAREST-P-J
CS: Forestry Canada, Petawawa National Forestry Institute, P.O. Box 2000, Chalk River, Ont., Can. K0J 1J0
SO: CANADIAN JOURNAL OF BOTANY 70(1): 175-180
PY: 1992
LA: English
AB: The transient expression of the beta-glucuronidase reporter gene was compared in embryogenic cell lines of Larix times eurolepis (L. decidua times L. leptolepis) and Picea mariana after introduction of eight vectors containing different promoter sequences using the Dupone **Biolistic**-TM **particle** delivery system. Transient beta-glucuronidase gene expression was highest in cells of both species after **bombardment** using the wheat abscisic acid inducible Em gene promoter. Transient beta-glucuronidase gene expression was comparable in P. mariana and L. times eurolepis for all vectors, with the exception of the rice actin promoter that yielded higher activity in P. mariana than in L. times eurolepis. The Em gene promoter proved inducible by abscisic acid; upon the addition of abscisic acid to the culture medium, beta-glucuronidase gene expression was increased 2.3- and 4.4-fold for L. times eurolepis and P. mariana, respectively. Investigation of beta-glucuronidase gene expression over time showed that all transient activity disappeared 16 days after microprojection.

TI: Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation.
AU: RUSSELL-J-A; ROY-M-K; SANFORD-J-C
CS: Dep. Horticultural Sciences, Cornell Univ., Geneva, New York 14456
SO: PLANT PHYSIOLOGY (BETHESDA) 98(3): 1050-1056
PY: 1992
LA: English
AB: A cell suspension culture of tobacco (Nicotiana tabacum L.) was used as a model to study injury to cells during **biolistic** transformation. Lawns of cells were bombarded with tungsten particles that were coated with a plasmid containing the beta-glucuronidase and the neomycin phosphotransferase II genes. When a gunpowder-driven **biolistic** device was used, numerous transiently expressing cells were focused around the epicenter of the blast which was manifested by hole blown in the filter paper supporting the cells. However, transformed cells nearest the blast epicenter were injured and could not be recovered as stable transformants. The injury was primarily caused by physical trauma to the cells from gas blast and acoustic shock generated by the device. Postlaunch baffles or meshes placed in the gunpowder device reduced cell injury and increased the recovery of kanamycin-resistant colonies 3.5- and 2.5-fold, respectively. A newly developed helium-derived device was more gentle to the cells and also increased the number of transformants. Cell injury could be further moderated by using a mesh and

a prelaunch baffle in the helium device. Toxicity of the tungstem microprojectiles also contributed to cell injury. Gold microprojectiles were not toxic and resulted in fourfold more kanamycin-resistant colonies than when similar quantities of similarly sized tungsten particles were used.

TI: Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation.
AU: STAUB-J-M; MALIGA-P
CS: Waksman Inst., Rutgers, State Univ. New Jersey, Piscataway, N.J. 08855-0759
SO: PLANT CELL 4(1): 39-46
PY: 1992
LA: English
AB: We investigated the size of flanking DNA incorporated into the tobacco plastid genome alongside a selectable antibiotic resistance mutation. The results showed that integration of a long uninterrupted region of homologous DNA, rather than of small fragments as previously thought, is the more likely event in plastid transformation of land plants. Transforming plasmid pJS75 contains a 6.2-kb DNA fragment from the inverted repeat region of the tobacco plastid genome. A spectinomycin resistance mutation is encoded in the gene of the 16S rRNA and, 3.2 kb away, a streptomycin resistance mutation is encoded in exon II of the ribosomal protein gene rps12. Transplastomic lines were obtained after introduction of pJS75 DNA into leaf cells by the **biolistic** process and selection for the spectinomycin resistance marker. Homologous replacement of resident wild-type sequences resulted in integration of all, or almost all, of the 6.2-kb plastid DNA sequence from pJS75. Plasmid pJS75, which contains engineered cloning sites between two selectable markers, can be used as a plastid insertion vector.

TI: Transient gene expression in cassava using high-velocity microprojectiles.
AU: FRANCHE-C; BOGUSZ-D; SCHOPKE-C; FAUQUET-C; BEACHY-R-N
CS: ORSTOM, Saint Louis, Mo. 63130
SO: PLANT MOLECULAR BIOLOGY 17(3): 493-498
PY: 1991
LA: English
AB: The bacterial gene encoding beta-glucuronidase (GUS) was transiently expressed in Cassava (Manihot esculenta) leaves following the introduction of the gene by microparticle **bombardment**. The DNA expression vector used to introduce the reporter gene is a pUC 19 derivative and consisted of a CaMV 35S promoter (P35S), the GUS coding region and 7S polyadenylation region. Several other promoters and regulating sequences were tested for efficiency in cassava leaves. Two derivatives of the P35S, one including a partial duplication of the upstream region of the P35S and the other containing a tetramer of the octopine synthase enhancer, were found to be expressed at three times the level of P35S in cassava leaves. The ubiquitin 1 promoter from Arabidopsis thaliana was expressed at the same level as the P35S. No influence on the level of expression was observed when different 3 ends were used. The **biolistic** transient gene expression system in cassava leaves allows rapid analysis of gene constructs and can serve as a preliminary screen for chimeric gene function in the construction of transgenic cassava plants.

TI: Stable transformation of sorghum cell cultures after bombardment with DNA coated microprojectiles.
AU: HAGIO-T; BLOWERS-A-D; EARLE-E-D
CS: Dep. Plant Breeding, Cornell Univ., Ithaca, N.Y. 14853, USA
SO: PLANT CELL REPORTS 10(5): 260-264
PY: 1991
LA: English
AB: Cells from a suspension culture of Sorghum vulgare (sorghum) have been transformed to either hygromycin or kanamycin resistance following uptake of pBC1 or pNGI plasmids, respectively, introduced on DNA-coated high velocity microprojectiles. Hygromycin- and kanamycin-resistant transformants contained hygromycin B phosphotransferase- and neomycin phosphotransferase-hybridizing restriction fragments of the expected size, respectively. A second introduced, but unselected for, reporter uidA gene which encodes beta-glucuronidase activity was also detected by DNA gel blot analysis in these transformants and shown to be expressed at low levels in two of the ten transformants analyzed. Transcripts from the introduced foreign genes accumulated to detectable levels in only these two transformants, both of which had a high copy number of genes integrated into their genome. This report further establishes the **biolistic** method as a useful route for delivery of DNA into the difficult-to-transform monocotyledonous plant species and represents the first stable transformation of this agronomically-important cereal grain.

TI: Transient expression of the beta-glucuronidase gene in embryogenic callus of Picea mariana following microprojection.
AU: DUCHESNE-L-C; CHAREST-P-J
CS: Forest. Can., Petawawa Natl. Forest. Inst., P.O. Box 2000, Chalk River, Ont. K0J 1J0, Can
SO: PLANT CELL REPORTS 10(4): 191-194
PY: 1991
LA: English
AB: A microprojection protocol using the DuPont **Biolistic**-TM **particle** delivery system and the beta-glucuronidase (GUS) reporter gene fused with the 35S promoter of Cauliflower mosaic virus (CaMV) was developed for Picea mariana callus. Comparison of four tungsten **microprojectile** sizes showed the highest transient gene expression with 1.11-mu-m diameter particles. Adsorption of DNA on the microcarriers using calcium chloride led to higher GUS gene activity than using polyethylene glycol. GUS gene activity in P. mariana was the highest when cells were treated 5 and 6 days after subculturing to fresh media. The wheat ABA-inducible Em gene promoter yielded 4.5 times higher GUS gene activity than the 35S CaMV promoter. Comparison of transient GUS gene expression among 10 P. mariana embryogenic cell lines from six different open-pollinated families showed comparable gene activity, with exception of one family showing no GUS gene activity.

TI: Transient expression of beta-glucuronidase in different cellular compartments following biolistic delivery of foreign DNA into wheat leaves and calli.
AU: DANIELL-H; KRISHNAN-M; MCFADDEN-B-F
CS: Dep. Biochem. Biophysics, Washington State Univ., Pullman, Wash. 99164-4660
SO: PLANT CELL REPORTS 9(11): 615-619
PY: 1991
LA: English
AB: Transient expression or beta-glucuronidase (GUS) in different cellular compartments following **biolistic** delivery of chloroplast or nuclear expression vectors into wheat leaves for calli, derived from anther culture or immature embryos, is reported here. When pBI121, the nuclear GUS vector, was used to bombard wheat cells, the beta-glucuronidase product, an insoluble indigo dye, was observed evenly throughout the cytosol. But, when the chloroplast expression vector pHD203-GUS was used for bombardments, the indigo dye (GUS product) was subcellularly localized within the chloroplasts of wheat cells. The observation of GUS expression in albino plastids, when anther culture derived albino leaves were bombarded with the chloroplast expression vector pHD203-GUS, suggests the presence of a functional protein synthetic machinery in these organelles. GUS expression was also observed in regenerable calli derived from wheat immature embryos bombarded with pHD203-GUS. Leaves or calli bombarded with pUC19, as negative controls, did not show any GUS expression. These results constitute the first demonstration of foreign gene expression in chloroplasts of a monocot and that a dicot chloroplast promoter functions in a monocot chloroplast.

TI: Optimization of delivery of foreign DNA into higher-plant chloroplasts.
AU: YE-G-N; DANIELL-H; SANFORD-J-C
CS: Dep. Horticultural Sci., Cornell Univ., Geneva, N.Y. 14456
SO: PLANT MOLECULAR BIOLOGY 15(6): 809-820
PY: 1990
LA: English
AB: We report here an efficient and highly reproducible delivery system, using an improved **biolistic** transformation device, that facilitates transient expression of beta-glucuronidase (GUS) in chloroplasts of cultured tobacco suspension cells. Cultured tobacco cells collected on filter papers were bombarded with tungsten particles coated with pUC118 or pBI101.3 (negative controls), pBI505 (positive nuclear control) or a chloroplast expression vector (pHD203-GUS), and were assayed for GUS activity. No GUS activity was detected in cells bombarded with pUC118 or pBI101.3. Cells bombarded with pBI505 showed high levels of expression with blue color being distributed evenly throughout the whole cytosol of the transformants. pHD203-GUS was expressed exclusively in chloroplasts. We base this conclusion on: (i) the procaryotic nature of the promoter used in the chloroplast expression vector, (ii) delayed GUS staining; (iii) localization of blue color within subcellular compartments corresponding to plastids in both shape and size; and (iv) confirmation of organelle-specific expression of pHD203-GUS using PEG-mediated protoplast transformation. Chloroplast transformation efficiences increased dramatically (about 200-fold) using an improved helium-driven **biolistic** device, as compared to the more commonly used gun powder charge-driven device. Using GUS as a reporter gene and the improved **biolistic** device, optimal **bombardment** conditions were established, consistently producing several hundred transient chloroplast transformants per Petri plate. Chloroplast transformation efficiency was found to be increased further (20-fold) with supplemental osmoticum (0.55 M sorbitol and 0.55 M mannitol) in the **bombardment** and incubation medium. This system provides a highly effective mechanism for introducing and expressing plasmid DNA within higher-plant chloroplasts, and the fact that GUS functions as an effective marker gene now makes many genetic studies possibe which were not possible before.

TI: Transient expression of foreign genes in plant cells and tissues obtained by a simple biolistic device (particle gun).
AU: MORIKAWA-H; IIDA-A; YAMADA-Y
CS: Res. Cent. Cell Tissue Culture, Fac. Agric., Kyoto Univ., Kyoto 606, Jpn
SO: APPLIED MICROBIOLOGY AND BIOTECHNOLOGY 31(3): 320-322
PY: 1989
LA: English
AB: Successful transient expression of genes (luciferase and beta-glucuronidase) in cultured tobacco cells and adventitious shoots of eggplant hypocotyls can be obtained by use of a simple **biolistic** device. This device is driven by controlled gas pressure from a cylinder of nitrogen gas. It has the advantages of being free of explosive heat and of avoiding cell damage caused by expanding gas, due to the "self-sealing" effect of the projectile.

HISTOCHEMICAL SUBSTRATES

Plant Molecular Biology and Genetic Engineering
Expression of the GUS reporter gene in transgenic plants

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50 g	150 --/g	110.--/g

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2 g	720.--/g	535.--/g
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	Sw. Frs.	U.S.-$
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2 g	825.--/g	594.--/g
5 g	638.--/g	483.--/g
10 g	506.--/g	387.--/g

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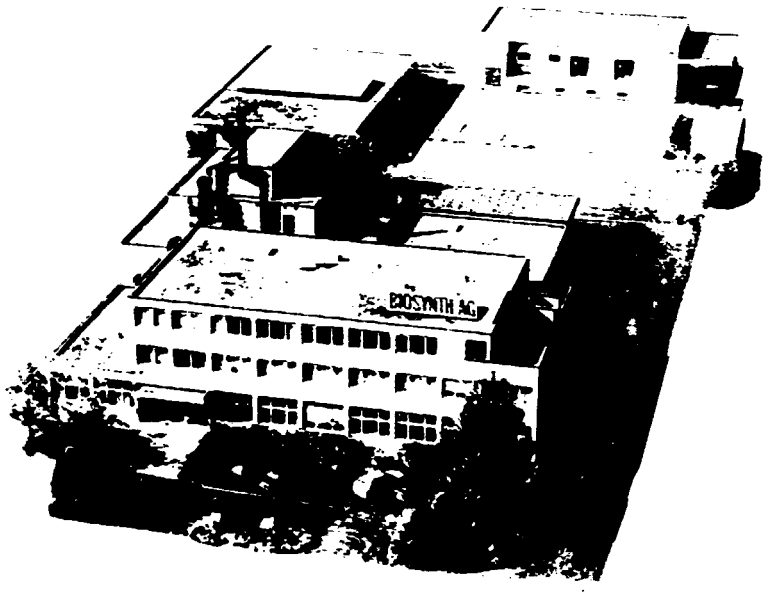

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