OCCASION

This publication has been made available to the public on the occasion of the 50th anniversary of the United Nations Industrial Development Organisation.

DISCLAIMER

FAIR USE POLICY

CONTACT

Please contact publications@unido.org for further information concerning UNIDO publications.

For more information about UNIDO, please visit us at www.unido.org

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

21194

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

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 92/054

ICGEB ref. #: CRP/ HUN91-05

Project initiation: April 1992

Project termination: May 1995

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

Collaborative Research Programme

TERMINAL EVALUATION REPORT

Part 1

Title of Project			
Identification of mitochondrial genes for male sterility in tobacco			
Keywords: cytoplasmic male sterility, mitochondrial DNA recombination, organelle transformation. *Nicotiana*			
UNIDO contract #	92/054	**ICGEB ref. #: CRP/**	HUN91-05
Project initiation:	April 1992	**Project termination:**	May 1995
Principal Investigator's name:	Dr Peter Medgyesy		
Affiliate Centre mail address :	Biological Research Center, Hungarian Academy of Sciences P.O. Box 521, H-6701 Szeged. Hungary		
Telephone no.	36-62-432232	**Telex no.**	82442 bioce h
Fax no.	36-62-433434	**Email**	mpeter@everx.szbk.u-szeged.hu

Abstract:

The aim of the project is the identification and efficient transfer of mitochondrial genes responsible for cytoplasmic male steriiity (CMS) in higher plants. Our approach can facilitate interspecific transfer of CMS without secondary effects frequently deteriorating alloplasmic cultivars. and can elucidate a presently poorly explainable form of nucleus-organelle interaction. Our experimental object is tobacco. which is both a crop and a model species. and in which CMS is important in breeding but poorly analysed at the molecular level. During the three-year-long project period we achieved the following results. In alloplasmic male sterile tobacco plants the appearance of reduced growth characteristics was demonstrated to be exclusively due to the presence of alien mitochondria. Protoplast fusion-mediated repeated mitochondrial DNA (mtDNA) recombinations were shown to result in the formation of stably recombined mitochondrial genomes (chondriomes) possessing a highly reduced amount of foreign mtDNA. This finding, if generally applicable should strongly increase the value of somatic cybrids for breeders. One of the most important results was that mitochondrial DNA recombination proved to be a tool for the separation of agriculturally useful and harmful forms of nucleo-cytoplasmic incompatibility (male sterility and reduced growth). This is a goal not accessible by traditional breeding methods. Stable male sterile plants were produced in which a very few characteristic changes in the mtDNA restriction fragment length polymorphism (RFLP) were located as putative sources of the CMS. The cloning of these unique fragments served a starting material for their functional test by transformation. A vector presumably suitable for mitochondrial transformation in tobacco was also constructed. The cloned tobacco mitochondrial 26S rRNA gene possesses the mutation which is known to cause chloramphenicol resistance in bacteria and yeast mitochondria.

OBJECTIVES/METHODOLOGY
(proposed at the time of the submission of the research proposal)

Proposed objectives

1. Minimalization of the extent of alien cytoplasmic genes in alloplasmic male sterile tobacco plants. Selection of plants with stable and universal male sterile flower phenotype, and with stable and characteristic mtDNA composition

2. Localization and cloning of mtDNA fragments specific for the mitochondrial genomes causing CMS. Construction of a vector with a selectable marker for mitochondrial transformation.

Proposed methodology

Tobacco (*Nicotiana tabacum*) is a crop in which CMS is important in breeding, and also a widely used model species which is especially suitable for a molecular genetic analysis of CMS. The most convenient form of CMS is generated by the introduction of alien mitochondria from an other *Nicotiana* species (alloplasmic male sterility). Transfer of CMS can be most efficiently achieved by protoplast fusion using polyethylene glycol (PEG) treatment. Lethal irradiation of cytoplasm donor protoplasts before fusion inactivated the nuclear genome, and facilitated recovery of cybrid cell lines. Isolation of cybrids with a new nucleus-cytoplasm combination was based on selection for a plastid genetic trait, antibiotic resistance, of the donor protoplasts. This approach results in 95-100% cotransfer of CMS from the cytoplasm donor. For an elucidation of the role of mitochondria in cytoplasmic effects, the alien plastids in alloplasmic plants were exchanged to those of the wild type by pollen-mediated plastid transfer. This sexual cybridization method is the only reliable way to produce new plastid-mitochondrion combinations without any change (recombination) in any of the organelles.

Isolation of mitochondria from leaves of aseptically grown plants was achieved by differential centrifugation in a high ionic strength buffer to remove chromosomal DNA. Isolated mitochondria were DNAse treated to remove plastid DNA contamination. Purification of mtDNA from lysed mitochondria was achieved by standard phenol-chloroform deproteinization. New combinations and refinement of the technical steps aimed at high resolution of mtDNA RFLP patterns suitable for individual cloning of defined fragments.

Restriction enzyme analysis, DNA hybridization, shot-gun cloning and sequencing of mtDNA was performed according to standard protocols published in molecular biology manuals. For a characterization of the chondriome, a comparison can be made between the fusion-derived lines and the fusion parents by an analysis of the mitochondrial RFLP patterns generated by various restriction endonucleases. The selection of the putative CMS specific fragments can also be based on mtDNA hybridization pattern analysis using cloned mitochondrial (eg *atp*) genes supposed to be involved in mtDNA rearrangements causing CMS in other species. The identification of the mtDNA clone carrying the 26S rRNA gene was achieved by the help of the relevant (highly homologous) bacterial 23S rRNA gene probe

The DNA delivery methods considered were the following: DNA coated high velocity metal microprojectiles fired into leaf pieces by a particle gun apparatus; microinjection into cultured cells using a micromanipulator connected to an inverted microscope, transformation of protoplasts by PEG mediated DNA uptake. Analysis of transient gene expression can be performed using a vector carrying the beta-glucuronidase gene (*uidA*) fused to the promoter of the mannopine synthase gene (*mas*). Stable nuclear integration can be followed by a selection for the expression (kanamycin resistance) of the neomycin phosphotransferase gene (*nptII*) fused to the promoter of the nopaline synthase gene (*nos*). Stable plastid integration can be followed by a selection for the expression (streptomycin and/or spectinomycin resistance) of the 16S rRNA gene (*16SrDNA*) of plastid origin

RESULTS
(compare against the set objectives)

1. a. Subsequent cytoplasm transfers (back-fusions to the fertile type) were used for a partial chondriome transfer in isonuclear-isoplastidic tobacco combinations, where CMS marked the alien (donor) mitochondrial genome. Molecular analyses demonstrated that the protoplast fusion mediated mitochondrial DNA recombinations highly reduced the extent of alien mtDNA in the chondriomes. These recombined chondriomes, which became similar to the fertile type, still retained the capacity to cause full male sterility. The most important finding of this part of the experiment was that the repeated mtDNA recombinations resulted in the formation of stably recombined chondriomes. After two rounds of mtDNA recombination (mediated by two successive cytoplasm transfers via protoplast fusion) a stable chondriome was always formed, which remained unchanged after additional back-fusions. In addition to different interspecific *Nicotiana* fusion combinations, the phenomenon was also demonstrated in a taxonomically distant combination (tobacco and potato, from different subfamilies). This finding, if generally applicable, should strongly increase the value of somatic cybrids for breeders.

1. b. A comparative analysis of isonuclear tobacco plants with different plastid-mitochondrion combinations demonstrated that in alloplasmic male sterile plants the appearance of reduced growth characteristics were exclusively due to the presence of alien mitochondria. The interesting finding was that the male sterile plants possessing stably recombined chondriomes showed normal growth characteristics in a comparison with the original alloplasmic line. This result strongly indicated that the mtDNA recombinations during the subsequent fusion steps resulted in an adaptation of the chondriome, directed by the nucleus-mitochondrion incompatibility. In line of this conclusion the analysis of protoplast-derived regenerates (protoclones) also demonstrated the unstable (heterogenous) and stable (homogenous) nature of chondriomes in the first-fusion-derived and the accomodated plants, respectively. The most important conclusion from this part of the experiments was that mitochondrial DNA recombination proved to be a tool for the separation of agriculturally useful and harmful forms of nucleo-cytoplasmic incompatibility (male sterility and reduced growth). This is a goal not accessible by traditional breeding methods.

2. a. The comparative molecular analysis demonstrated that in the stable plants, produced by repeated back-fusions, a very few charactenstic changes of the mtDNA RFLP patterns were located as putative sources of the CMS. The cloning of these unique fragments served a starting material for their functional test by transformation. The consideration that the efficient production of homoplasmic plants after cytoplasmic genetic manipulations needs single cell cloning and extensive cell culture selection motivated us to focus on protoplast transformation techniques to introduce DNA into the cytoplasm. Highly efficient uptake conditions were established using transient gene expression assays, and following the integration of nuclear genetic markers. The main finding of this part of the experiments was the demonstration that for organelle transformation in plants the chemical treatment of protoplasts is an alternative to biolistic delivery systems. The use of plastid genetic markers in experiments employing simple PEG-mediated DNA uptake by protoplasts was suitable to generate stable chloroplast DNA transformants with a frequency comparable with nuclear transformation methods.

2. b. Genetic analysis of chloramphenicol resistant plants, which were selected as supposedly mitochondrial mutants, revealed their instability, although the tests indicated a cytoplasmic location of the trait. The plants lost their resistance in the lack of selection pressure, and the molecular analysis revealed no mutation of the relevant mitochondrial ribosomal RNA gene. No additional mitochondrial mutation was revealed in antibiotic resistant plastid mutants selected in cell culture. A circumvention of the mutant isolation way of the production of a selectable mitochondrial marker was achieved by in vitro mutagenesis. We have now the cloned tobacco mitochondrial 26S rRNA gene possessing the mutation which is known to cause chloramphenicol resistance in yeast mitochondria and in *E. coli*. A preliminary test of this construct for its suitability as a mitochondrial transformation vector in tobacco is in progress.

Work plan and time schedule (originally envisaged)

Proposed work plan

The project was based on the isonuclear-isoplastidic CMS tobacco lines, produced in our laboratory, which showed characteristic flower malformations and possessed individual mtDNA RFLP patterns. Repeated back-fusions to fertile tobacco was aimed at limiting the extent of alien mtDNA in the CMS lines. We planned to choose those CMS lines which were stable, showed the least difference in their RFLP patterns as compared to the fertile type, and which showed the most universal CMS phenotype if transferred to different *Nicotiana* species. For a localization of the CMS specific fragments we planned a comparative analysis of the mtDNA of different CMS lines, the fertile and the CMS parent, and fertile revertants. We planned to facilitate the identification and cloning of these unique fragments by an improvement of the mtDNA isolation and purification methods.

In addition to the cloning of mtDNA fragments carrying putative CMS elements, the other main aim of the project was to establish the technical elements of mitochondrial transformation. A putative mitochondrial mutant expressing chloramphenicol resistance was previously selected in our laboratory, and its genetic and molecular analysis was also initiated in the project. On the basis of the homologous resistance genes in bacteria or in yeast mitochondria, the 26S rRNA gene was to be isolated and analysed from the mitochondrial genome of the resistant *Nicotiana* plant.

Simultaneously, the technical set-up giving efficient transient and stable transformation with test vectors was to be established. Initially the existing direct DNA delivery techniques were to be employed, using the available selectable markers, as model systems. The nuclear transformation technique most efficient for cell culture selection was planned to be developed towards the requirements of organelle transformation.

Proposed time schedule

1. year. Investigation of stability and uniformity of the CMS phenotypes, and the RFLPs of their mtDNA, in sexual (back-cross derived) and somatic (back-fusion or protoclone derived) progenies of the original CMS lines. Selection of CMS lines with the least deviation from the fertile parent detected in RFLP patterns of mtDNA. Analysis of the expression of different CMS sources in the nuclear background of tobacco and other *Nicotiana* species after protoplast fusion mediated cytoplasm transfer. Modification of the standard DNA isolation and purification methods to achieve high mtDNA RFLP pattern resolution in a large number of samples from small amounts of plant material Test of the stability and the mode of inheritance of chloramphenicol resistance in putative mutant plants.

2. year Location of CMS specific mtDNA fragments by a comparative analysis of RFLP and hybridization patterns in the particular mtDNA recombinant CMS lines. Cloning and molecular analysis of the mitochondrial gene supposedly responsible for chloramphenicol resistance. Optimalization of DNA uptake by direct DNA transformation methods following transient and stable expression of appropriate constructs.

3. year Cloning of mtDNA fragments carrying putative CMS determining elements. Characterization of the relation of the clones to the characteristic RFLP patterns. Performance of transformation experiments which aim at direct gene transfer into mitochondria.

Work plan and time schedule (actual)

Actual work plan

The analysis of the first serial of male sterile tobacco plants derived from back-fusions to the fertile type led to unexpected results. In contrast to the expected random and continuous mtDNA recombinations at each fusion step, we revealed a stabilization of the chondriome. The mitochondrial genome, if recombined to a certain extent, showed no rearrangements during further fusion transfers. This surprising results initiated a more extensive study of this particular part of the project. We verified the phenomenon in different *Nicotiana-Nicotiana*, and tobacco-potato, fusion combinations. To get a firm conclusion on the decline of recombination activity we had to investigate the mtDNA RFLP patterns of 15-20 independent plants per fusion, in each case using 5-7 restriction endonucleases.

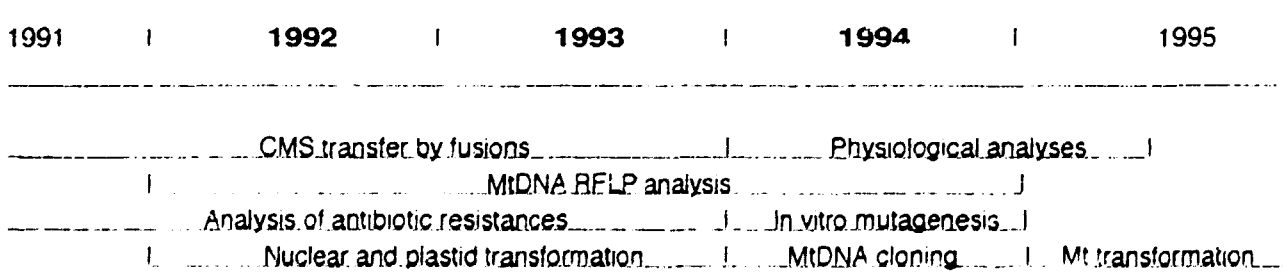
Another new result leading to unscheduled experiments was the separation of the CMS and the reduced growth of alloplasmic tobacco plants by mtDNA recombination. The formation of a stably recombined chondriome in these plants resulted in completely normal growth ability still coupled with the retention of full male sterility. To strengthen the data a number of plants suitable for statistical analysis was grown repeatedly under controlled conditions to measure their growth capacity, growth rate, photosynthesis and respiration capability.

At the time of the submission of the project proposal no organelle transformation method was available in higher plants. Later on the so called Gene Gun proved to be suitable for chloroplast transformation in tobacco. This equipment was not existing in Hungary at that time, therefore we searched for alternative techniques. Apparently the transformation method based on direct DNA uptake by protoplasts seemed to be superior to the Gene Gun from the viewpoint of organelle transformation. After preliminary experiments with transient and stable nuclear gene expression vectors we successfully utilized plastid specific vectors, constructed previously in our laboratory for stable chloroplast transformation in *Nicotiana*. The simplicity of the method highly indiciates its suitability also for mitochondrial transformation.

Most probaly the isolation of stable chloramphenicol resistant mutants in *Nicotiana* in our and other's experiments was hampered by a selection insufficient to achieve a homoplasmic state. The alternative experiments performed together with Dr Agnes Cséplő, collaborative investigator of the project, revealed no additional (mitochondrial) mutation in antibiotic resistant plastid mutants selected in cell culture. Finally, we turned to in vitro mutagenesis methods to introduce that mutation into a cloned *Nicotiana* mitochondrial gene (*26SrDNA*) which causes chloramphenicol resistance in bacteria and yeast mitochondria. We are convinced that by the help of this vector construction, coupled with our DNA delivery technique, the appropriate selection conditions for mitochondrial transformation in tobacco can be efficiently worked out.

Altogether during the project period we have established the elements of a system for efficient CMS transfer in tobacco. In addition, our approach makes amenable defined mtDNA regions to a functional test of their role in the determination of CMS. We would like to make efforts to continue the project because the next period of it, based on mitochondrial transformation, should yield principally new results from both basic and applied research aspects.

Actual time schedule



NETWORKING

Dr Atanas Atanassov. Director. Institute of Genetic Engineering. Agricultural Academy. 2232 Kostinbrod 2. Bulgaria

In the collaborative research programme several protoplast fusion experiments have been performed aimed at transferring CMS and other agriculturally important traits into tobacco. In these experiments Bulgarian tobacco cultivars (Nevrokop 1146 and A24) were used as recipients. Donors involved various wild *Nicotiana* species (*alata. gossei. nesophila. plumbaginifolia and sauderae*). The form of male sterility. the nuclear composition and the general performance of the fusion derived plants was investigated in Kostinbrod. The molecular analysis of the mitochondrial composition of selected lines were performed in Szeged. In addition to mutual short visits. one of the coworkers of Dr Atanassov (Anna Dragoeva) worked in the Szeged laboratory on the project by a long-term ICGEB-UNIDO Fellowship.

Dr Nguyen Duc Thanh. Group Leader. Institute of Biotechnology. National Center for Natural Science and Tecnology. Nghia do. Tu liem. Hanoi. Vietnam.

In the frame of the collaboration cytoplasms causing male sterility have been introduced into Vietnamese tobacco cultivars (NTH. BV1 and BV235) by protoplast fusion. The donor tobacco lines used were those stable mitochondrial recombinant plants. produced in Szeged. which showed full male sterility with no secondary effects on growth ability. Production and field test of the plants were performed in Hanoi. while they were analysed at the molecular level in Szeged. Experiments were also performed to study the mechanism of cotransfer of plastids and mitochondria during protoplast fusion mediated cytoplasm transfer. Dr Thanh spent a training period in Szeged to gain a skill in the isolation and analysis of chloroplast and mitochondrial DNA from CMS lines. The main aim of the training was to facilitate the transfer of molecular technologies to the Hanoi laboratory. Two of the coworkers of Dr Thanh learnt the relevant techniques in the Szeged laboratory in the frame of the International Training Course of BRC

PUBLICATIONS

Medgyesy. P.. Thanh. N.D., Horváth. G.V., Rasochová. L., Rusu. A. (1992) Nucleus-mitochondrion incompatibility drives the DNA recombination-mediated adaptation of the mitochondrial genome (lecture abstract). NATO/ASi Seminar on "Plant Morphogenesis - Molecular Approaches". Heraklio. Greece. p. 97

Horváth. G.V., Thanh. N.D., Rusu. A., Rasochová. L., Medgyesy. P. (1992) Nucleus-mitochondrion incompatibility drives the DNA recombination-mediated adaptation of the mitochondrial genome (poster abstract). DFG Workshop on "Interactions of Three Genomes". Berlin. Germany. p. 115

Csépló. A., Eigel. I., Horváth. G.V., Medgyesy. P., Herrmann. R.G., Koop. H.-U. (1993) Subcellular location of lincomycin resistance in *Nicotiana* mutants. Mol. Gen. Genet. 236: 163-170.

O'Neill. C., Horváth. G.V., Horváth. E., Dix. P.J., Medgyesy. P. (1993) Chloroplast transformation in plants: polyethylene glycol treatment of protoplasts is an alternative to biolistic delivery systems. Plant J. 3: 729-738

Medgyesy. P. (1994) Organelle segregation. recombination and transformation as tools in studying nucleus-organelle incompatibility in Solanaceae (lecture abstract). 8th International Congress on Plant Tissue and Cell Culture. Florence. Italy. p. 161

Medgyesy. P. (1994) Cybrids - Transfer of chloroplast traits through protoplast fusion between sexually incompatible Solanaceae species. In. (Bajaj. Y.P.S. ed.) Somatic hybridization in crop improvement I. Springer-Verlag. Berlin-Heidelberg-New York. pp. 72-85

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Centre	
Budgets as per original proposal		**Summary of expenditures** *	
1) Capital equipment	US$	1) Capital equipment	US$ 16,000
2) consumables	US$	2) consumables	US$ 30,000
3) training	US$	3) training	US$ 7,000
4) literature	US$	4) literature	US$ 3.000
5) miscellaneous	US$	5) miscellaneous	US$ 4.000
TOTAL GRANT	**US$**.....	**TOTAL**	**US$** 60,000

Please itemize the following budget categories (if applicable)

Capital equipment

Lasis DNA Sequencing Power Supply Model 494
SZBK Electrophoresis Power Supply PSE 300
Robbins Hybridization Incubator MOD 1000
New Brunswick Microcentrifuge Model 101 HS
Macintosh Personal Computer PB 520
Macintosh StyleWriter Printer
Privileg Deep Refrigerator
LogoStar Microwave Oven DMR-613
Hansatech Leaf Disc Electrode Unit LD2
Sartorius Laboratory Balance L310
SZBK Water Bath

Training (provide names, duration of training, host laboratory)

Nguyen Duc Thanh (from Instute of Biotechnology, Hanoi), 6 months, BRC (Szeged)
aim: learning techniques of organelle DNA isolation and analysis
Gábor V. Horváth (from BRC, Szeged), 2 months, Max-Planck-Institute (Köln, Germany)
aim: learning techniques of DNA cloning, sequencing and transformation

Literature

A Practical Guide to Molecular Cloning (Wiley)
Cell Culture and Somatic Cell Genetics, Vols 7A and 7B (Academic)
Current Protocols in Molecular Biology (Wiley)
Electrophoresis (Oxford)
Molecular Biology Labfax (BIOS-Blackwell)
Molecular Cloning, Vols 1-3 (CSH)
Plant Molecular Biology Manual (Kluwer)

* Please do not send invoices, receipts etc.; these should be kept by the Affiliated Centre for future reference and sent to ICGEB upon request.