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

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNIDO contract # 91/051

ICGEB ref. #: CRP/ HUN 90-01

Project initiation: 1991

Project termination: 1994

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TERMINAL EVALUATION REPORT

Part 1

Title of Project

Characterization of DNA binding proteins involved in the regulated expression of a wheat chlorophyll a/b binding protein gene

Keywords: trans-acting factors; wheat Cab gene; regulated gene expression

UNIDO contract # 91/051 **ICGEB ref. #: CRP/** HUN90-01
Project initiation: 1991 **Project termination:** 1994

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Abstract:

The expression of the wheat Cab-1 gene encoding the major chlorophyll a/b binding protein is light regulated and tissue specific, it is mediated by phytochrome and further modulated by a circadian clock. The regulation is exerted at the transcription level and by 5' deletion mutant analysis we defined several cis-acting regulatory sequences involved in this complex expression pattern. The analysis of in vitro constructed chimaeric genes in transgenic plants allowed us to localize the cis-regulatory sequences for leaf-specific expression, for light-regulated expression and for circadian clock regulated expression.

1. We have recently defined the exact position and nucleotide sequence of the cis-acting elements by in vitro footprinting,
2. We characterized by gel retardation assays and mutation analysis those DNA-binding proteins that specifically bind to the Cab-1 promoter regions thus defined,
3. We are presently engaged in isolating genes encoding the above described DNA-binding proteins (transcription factors);
4. In the near future we intend to determine the expression pattern of the genes encoding these various trans-acting proteins and to characterize them in vivo under various physiological conditions (light vs. dark, leaf vs. root, etc.).

These studies will help to understand the molecular mechanism of nuclear gene transcription in higher plants and will provide information about the terminal steps of various signal transduction chains affecting the expression of the Cab-1 gene. Judicious overexpression or inhibition of the genes encoding the trans-acting proteins involved can lead to the formation of novel regulatory circuits for gene expression in higher plants.

Part 2

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

1. Characterization of cis-acting elements interacting specifically with DNA-binding proteins.

A. DNase I footprinting experiments

In order to precisely define the position and sequence of the cis-acting elements of the wheat Cab-1 promoter involved in DNA-protein interactions, DNase I footprinting experiments will be performed. We intend to carry out *in vitro* DNase I footprinting using light vs. dark grown tobacco and wheat leaf nuclear extracts and leaf vs. root nuclear extracts. These experiments will allow us to determine whether the low level expression of the Cab-1 gene in roots and dark grown leaves and its high level expression in light grown leaves can be correlated with differential footprints. To characterize cis-acting elements of the wheat Cab-1 promoter for circadian clock responsive gene expression, *in vitro* footprinting experiments using nuclear extracts prepared at 3 hour intervals throughout the day will be carried out.

B. Gel retardation assays, mutagenesis experiments

In order to identify protein factors present in nuclear extracts isolated from various sources, gel retardation assays will be performed. Synthetic oligonucleotides identical with the footprinted regions of the Cab-1 promoter will be radiolabelled and used as binding targets. The sequence specificity of binding will be assessed by competition with unlabelled specific competitor DNAs. Cross competition experiments using various cis-regulatory sequences as competitors will reveal the exact number of the various nuclear factors that bind to specific regions of the Cab-1 promoter. In order to characterize the relative importance of the individual nucleotides whithin the foot-printed region, synthetic oligonucleotides will be tested for their ability to cross-compete with wild type binding sequences.

2. Isolation and characterization of genes encoding DNA-binding proteins involved in the regulated expression of the wheat Cab-1 gene.

A. Construction of gt11 expression libraries

Gel retardarion assays combined with foot-printing experiments will reveal the number of cis-acting elements and the number of putative DNA-binding proteins interacting with the various probes representing the upstream region of the Cab-1 gene. PolyA$^+$ mRNA from various tobacco and wheat tissues will be purified and cDNA libraries in gt11 expression vectors will be constructed.

B. Cloning of cDNAs encoding DNA-binding proteins using recognition site probes

To isolate cDNA clones encoding DNA-binding proteins specifically interacting with unique cis-acting regulatory sequences of the Cab-1 gene, gt11 expression libraries will be screened with Cab-1 promoter sequences identified as recognition sites. This strategy will hopefully obviate the necessity of purifying these proteins prior to isolating their genes.

C. Characterization of isolated genes coding for transcriptional activators specific for the Cab-1 promoter

Proteins from selected cDNA clones will be purified and antisera will be generated. Parallel with this work, tobacco and wheat genomic libraries will be constructed in EMBL 4 cloning vector and screened with the appropriate cDNA clones. Selected genomic clones will be transformed into tobacco and their expression patterns will be analyzed.

RESULTS
(compare against the set objectives)

1. Characterization of cis-acting elements interacting specifically with DNA-binding proteins

A. DNase I footprinting experiments

We prepared nuclear extracts at different time points from various tissues of tobacco and wheat plants grown under different light/dark conditions and performed the planned DNase I footprinting experiments. We concluded that promoter regions of the Cab-1 gene, designated as CabA (-244-220), CabB (-216-196), CabC (-188-170), CabD (-154-130) and CabE (-120-90) are the most likely candidates for binding transcription factors necessary for regulated expression. For the exact sequence of these motifs see Table 1.

B. Gel retardation assays, mutagenesis experiments

We showed that each identified region of the Cab-1 promoter is able to bind nuclear proteins present in nuclear extracts isolated from various sources. Furthermore we concluded, based on cross competition experiments, that these sequence motifs interact with five different, unique transcription factors. The relative importance of the individual nucleotides whithin the footprinted region for interaction with these nuclear proteins was also determined. The results of these experiments are shown in Table 1.

Table 1.

CabA: AAGCAGCGGTCTCTTTCGACTTG
CabB: AGCTGGTCCAGTCACGAGCC
CabC: AGCCCTAACCATAGCCACAA
CabD: TACATCTCATCCATTTAA
CabE: TTCTTTTCACCACCGTCTCTC

Sequences of cis-regulatory elements of the wheat Cab-1 promoter determined *in vitro* by footprinting and gel retardation assays. Binding of nuclear proteins was completely eliminated by mutations at the positions underlined.

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2. Isolation and characterization of genes encoding DNA binding proteins involved in the regulated expression of the wheat Cab-1 gene

A. Construction of gt11 expression libraries

We isolated poly(A)$^+$ mRNAs from leaf tissue of dark or light grown tobacco and wheat plants and constructed four cDNA libraries as planned.

B. Cloning of cDNAs encoding DNA binding proteins using recognition site probes

Several cDNA clones coding for proteins specifically interacting with the CabA and CabE sequence motifs of the Cab-1 promoter have been isolated. Sequence analysis of the isolated cDNA clones revealed that the deduced amino acid sequence of these putative transcriptional factors displayed i./ (CabA) high homology to histone H1 and ii./ (CabE) high homology to the recently identified GT-1 transcriptional activator shown to be involved in the light and tissue specific expression of the pea rbcsA gene.

C. Characterization of isolated genes coding for transcriptional activators specific for the Cab-1 promoter

Genomic clones corresponding to the identified cDNAs have been isolated and sequenced. Expression levels and patterns of genes encoding these putative transcriptional activators have been identified. Regeneration of transgenic tobacco plants expressing these cDNAs under the control of various tissue specific and inducible promoters is in progress.

Part 3

Work plan and time schedule (originally envisaged)

1990-1991

1. Characterization of cis-acting elements interacting specifically with DNA-binding proteins.

In the first year we plan to complete the characterization of cis-acting elements involved in the regulated expression of the Cab-1 gene. To identify DNA sequences responsible for the phytochrome responsiveness and for the circadian clock regulated gene expression, analysis of transgenic tobacco plants expressing a series of 5'-deletion mutants as well as chimeric gene constructs comprising various regions of the Cab-1 promoter will be completed. These experiments and *in vitro* footprinting assays will, hopefully, lead to the identification of the exact position of regulatory elements required for maximal, regulated expression in various tissues and under various environmental conditions. Simultaneously the first gel retardation assays will be performed in order to determine the number of proteins interacting with the wheat Cab-1 promoter. Based on the results, the first cDNA libraries will be constructed.

1991-1992

2. Isolation and characterization of genes encoding DNA binding proteins involved in the regulated expression of the wheat Cab-1 gene.

We plan to continue the construction of various cDNA libraries and will attempt the isolation of the first cDNA clones encoding transcriptional factors specific for the Cab-1 promoter. Cis-regulatory elements shown to be necessary for the regulated expression of the Cab-1 gene will be mutagenized and the effect of mutations will be determined *in vivo* (i.e in transgenic plants). During this period we hope to isolate the first positive cDNA clones and, based on the deduced amino acid sequences, to obtain the first information about the molecular properties of these trans-acting factors. Parallel with this experiments the first genomic libraries will be constructed and cDNA clones coding for putative DNA-binding proteins will be used as probes to screen these genomic libraries.

1992-1994

3. Isolation and characterization of genes encoding DNA binding proteins involved in the regulated expression of the wheat Cab-1 gene.

Isolated genomic clones coding for DNA-binding proteins will be sequenced. The characterized genes will be expressed in transgenic plants using different plant promoters with various regulatory features. We will attempt to express these genes in a novel pattern and study the biological consequences of the newly created regulatory circuits. The data compiled in this three-year period will hopefully allow us to determine the *in vivo* function of these transcriptional factors in various signal transduction pathways mediating the expression of the wheat Cab-1 gene.

Work plan and time schedule

(actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

1990-1993

1. Characterization of cis-acting elements interacting specifically with DNA binding proteins

The biological relevance of the putative cis-regulatory elements, identified by footprinting and gel retardation assays, was characterized in transgenic plants *in vivo*. Analysis of transgenic plants expressing chimeric genes whose promoter contained the various tetramerized derivatives of the putative regulatory motifs linked to the TATA-box region of the the cauliflower mosaic virus 35S promoter revealed that four out of the five footprinted sequence motifs are involved in regulating the leaf specific expression of the wheat Cab-1 gene, but none of them is sufficient to confer responsiveness to phytochrome and the circadian clock in this promoter context. Detailed analysis of the expression pattern and level of the wheat Cab-1 gene indicated that transcription of this gene is, most likely, regulated by multiple, overlapping cis-acting regulatory elements and/or by multiple interacting transcription factors. We assume that footprinting and gel retardation assays failed to determine the exact sequences of these regulatory elements, most likely, because of the low abundance of these transcription factors in the isolated leaf nuclear extracts. To define the localization and number of cis-regulatory elements required for phytochrome and circadian regulated expression we generated a series of site-specific mutations (linker scanning) in the promoter region of the Cab-1 gene (between -211 and -90) and introduced these mutants in transgenic tobacco plants. Characterization of the expression pattern of these newly generated Cab-1 promoter mutants is in progress. Preliminary data indicate, in good agreement with our hypothesis, that the footprinted cis-regulatory elements are necessary but not sufficient as independent units to confer circadian and phytochrome regulated gene expression of the Cab-1 gene.

1992-93

2. Isolation and characterization of genes encoding DNA binding proteins involved in the regulated expression of the wheat Cab-1 gene

Parallel with the analysis of transgenic plants we constructed several cDNA and genomic libraries from tobacco and wheat seedlings. We screened (using the so-called South-Western technique) our expression libraries with the octamerized and radiolabelled synthetic oligonucleotides representing the various footprinted sequence motifs of the Cab-1 promoter. We identified several cDNA clones and to date we have characterized two types. The deduced amino acid sequences of these proteins displayed high homology to transcription factors or to known proteins. The isolated proteins specifically interacted with the CabA (histone H1) or CabE (GT-1 transcription factor) cis-regulatory elements which were shown to be necessary and sufficient for leaf specific expression in transgenic tobacco plants. These results show that these cis-regulatory elements (CabA, CabE) and the interacting transcription factors are components of the signal transduction pathway mediating the leaf specific expression of the Cab-1 gene.

Furthermore we showed that the mRNA level of the histone H1 gene exhibited a characteristic diurnal oscillation. This observation, together with the fact that H1 histones are able to bind to a well-defined sequence whithin the Cab promoter (CabA) indicate a role for plant histones in the activation of Cab-1 transcription by the biological clock. Experiments designed to prove the direct involvement of H1 histones in the regulation of the Cab-1 promoter (i.e. analysis of transgenic plants expressing mutant Cab genes containing mutated CabA motifs) are in progress.

NETWORKING

We are in continuous although informal contact and collaboration with the following laboratories:

1. T. Börner, Humboldt University, Berlin
2. N.-H. Chua, Rockefeller University, New York
3. E. Schäfer, Albert Ludwigs University, Freiburg

PUBLICATIONS

Please see attached sheet.

List of publications supported by ICGEB grant CRP/HUN90-01

1. **Ádám, É., Széll, M., Páy, A., Fejes E., Nagy, F.** (1991) Cirdacian clock and light regulated transcription of the wheat Cab-1 gene in wheat and in transgenic plants. Proceedings of the NATO Advanced Reserach Workshop "Phytochrome Properties and Biological Action", B. Thomas and C.B.Johnson (eds.), NATO ASI Cell Biology Series, Springer-Verlag, Berlin/Heidelberg, Vol. H 50, pp.191-200.

2. **Páy, A., Fejes, E., Széll, M., Ádám, É., Nagy, F.** (1991) Cis-regulatory elements for the circadian clock regulated transcription of the wheat Cab-1 gene. In: Plant Molecular Biology 2, NATO ASI Molecular Biology Series, Plenum Publishing Company, Oxford, pp. 519-525.

3. **Széll, M., Szekeres, M., Ádám, É., Fejes, E., Nagy, F.** (1992) Cis-regulatory elements responsible for the tissue-specific expression of the wheat Cab-1 gene. In: Regulation of Chloroplast Biogenesis, NATO ASI Series A, Life Sciences Vol. 226, pp. 57-61.

4. **Ádám, É.,** Deák, M., Kay, S., Chua, N.-H., **Nagy, F.** (1993) Sequence of a tobacco *(Nicotiana tacabum)* gene coding for type A phytochrome. Plant Physiol. 101, 1407-1408.

5. **Nagy, F., Fejes, E.,** Wehmeyer, B., Dallmann, G., Schäfer, E. (1993) The circadian oscillator is regulated by a very low fluence response of phytochrome in wheat. Proc. Natl. Acad. Sci. USA 90, 6290-6294.

6. Batschauer, A., Gilmartin, P.M., **Nagy, F.,** Schäfer, E. (1993) The molecular biology of photoregulated genes. In: Photomorphogenesis in Plants, R.E. Kendrick and G.H.M. Kronenberg (eds.), Kluwer, pp. 559-599.

7. Hess, W., Müller, A., **Nagy, F.,** Börner, T. (1994) Ribosome-deficient plastids affect transcription of light-induced nuclear genes: genetic evidence for a plastid-derived signal. MGG 242, 305-312.

8. **Ádám, É., Széll, M., Szekeres, M.,** Schäfer, E. **Nagy, F.** (1994) The developmental and tissue-specific expression of tobacco phytochrome-A genes. The Plant Journal 6, 283-293.

9. Harter, K., Kircher, S., Frohmeyer, H., Krenz, M., **Nagy, F.,** Schäfer, E. (1994) Light-regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. The Plant Cell 6, 545-559.

10. **Szekeres, M.,** Haizel, T., **Ádám, É., Nagy, F.** (1995) Molecular characterization and expression of a tobacco histone H1 cDNA. Plant Mol. Biol. 27, 597-605.

11. Kolar, C., **Ádám, É.,** Schäfer, E., **Nagy, F.** (1995) Expression of tobacco Cab genes is controlled by two circadian oscillators in a developmentally regulated fashion. Proc. Natl. Acad. Sci. USA 92, 2174-2178.

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$ 12.500	1) Capital equipment	US$ 18.675
2) consumables	US$ 47.000	2) consumables	US$ 44.125
3) training	US$ 9.000	3) training	US$ 7.200
4) literature	US$ 1.500	4) literature	US$ -
5) miscellaneous	US$	5) miscellaneous	US$
TOTAL GRANT	US$ 70.000	**TOTAL**	US$ 70.000

Please itemize the following budget categories (if applicable)

Capital equipment

Sorvall centrifuga
Mini Cycler
Mettler balance
Incubator
Balance (universal)
Electrophoresis + power supply

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

"Photomorphogenesis in plants", 1991.szept.22-27, University of Maryland, College Park, MD, USA. Ádám Éva
4th International Congress of Plant Molecular Biology, 1994. jún. 19-24. Amsterdam. Ádám Éva
20th International Conference on Chronobiology, 1991. jún. 18-21. Tel Aviv. Szekeres Miklós
21st FEBS Meeting, 1992. aug. 9-14. Dublin. Szekeres Miklós
The Rockefeller University, Laboratory of Plant Molecular Biology, Prof. Nam-Hai Chua, New York, 1991. júl. 4-aug.4. Szekeres Miklós

Literature

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