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PRESENCE OF A NEGATIVE LIGHT REGULATORY FACTOR BINDING TO THE *cab*3 PROMOTER OF *Arabidopsis thaliana*

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Abstract – Expression of light-harvesting chlorophyll a/b-binding protein gene (*cab*) is repressed in the dark and activated by light. However, the detail of its regulatory mechanism is not characterized so far. To identify the interactions of *cis*-acting elements and *trans*-acting factors involved in this regulation, nuclear extracts from the light-grown and dark-adapted *Arabidopsis thaliana* leaves were analyzed for mobility shift assay against 134 bp (-376/-243), 146 bp (-244/-99) and 74 bp (-103/-30) fragments of *cab*3 promoter. The 74 bp and 134 bp fragments had two retarded bands and one retardation band, respectively, both in light-grown and dark-adapted samples. On the other hand, the 146 bp fragment had one band in the light-grown tissues but two retardation bands in the dark-adapted tissues. A new retardation band appeared only in the dark-adapted leaves may function as a negative regulatory factor to repress the *cab*3 expression in the dark. Several light-regulatory motifs are scattered in the 146 bp region of *cab*3 promoter. One of the light-regulatory motifs could be the binding site for the negative regulatory factor.

INTRODUCTION

Light-regulated gene expression in plants plays important roles in plant photomorphogenesis. Transcription of light-harvesting chlorophyll a/b-binding protein gene (*cab*), whose gene product is associated with photosystem II,[1] is regulated by both light quality and quantity.[2] The *cab* mRNA levels are elevated by red light (660 nm) and the increased levels are reversed by far-red light (730nm), indicating that the transcription of *cab* gene is regulated by phytochrome.[2,3] The light signal perceived by phytochrome that absorbs red and far-red lights is transmitted to nucleus to control the *cab* gene expression *via* unknown pathway of a signal transduction. The light signal regulates the *cab* transcription by interacting DNA motifs (*cis*-acting elements) of the promoters with cognate DNA binding proteins (*trans*-acting factors). The DNA motifs could be classified into two groups; one is common sequence elements necessary for the activation of transcription which are present in all promoters such as TATA, CCAAT and G-box, and the other is specific sequence elements necessary for regulating expression of particular genes.[4] The *cis*-acting elements regulated by light are called 'light regulatory element' (LRE) and the *trans*-acting factors are called 'light regulatory factor' (LRF).

An's group[5,6] studied *A. thaliana cab* promoters to identify the LRE by transforming 5', 3' and internal deletion mutants of the *cab* promoter in tobacco. They reported that LRE I (-164/-131) and LRE II (-209/-164) on the *cab*3 promoter are essential to confer light-dependent tissue-specific expression of the *cab*3. In our previous report, we tried to identify LRF that interacted with the LREs of the *A. thaliana cab*1 promoter by a mobility shift assay with leaf nuclear extracts.[7] The results suggested that dark repression of the *cab*1 may be regulated by a negative regulatory factor (NRF) interacting with 65 bp fragment (-318/-254) of the *cab*1 promoter. The NRF binding site seemed to be present within the LREs identified by An's group. In the present study, we are trying to examine the existence of any LRF involved in the dark repression of *A. thaliana cab*3 using small fragments of the *cab*3 LREs identified by Mirta *et al.* (1989).[6]

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† *Abbreviations*: LRE, light-responsive element; NRF, negative regulatory factor; LRF, light regulatory factor.

MATERIALS AND METHODS

Plant materials. The seeds of *Arabidopsis thaliana* cv. Landsberg were sown and grown at 26°C for 3 weeks on vermiculite under light intensity of 50 – 80μmol/m^2/s. Plants were exposed to 18 h light and 6 h dark every day. For dark adaptation, the light-grown plants were transferred to a dark room at 26°C and kept for 2 days before sampling. The samples were immediately frozen in liquid nitrogen and stored at -70°C until use.

Chemicals. Acrylamide, bis-acrylamide, Klenow fragment and restriction enzymes were obtained from Boehringer Mannheim (Mannhein, Germany). Tris was purchased from United States Biochemical (Cleveland, USA). [α-^{32}P]-dATP was from Amersham International plc (Amersham, UK). Phenylmethylsulfonylfluoride (PMSF), Benzamidine, Poly

(dIdC)ploy(dIdC) and all other chemicals were obtaind from Sigma Chemical Co. (St. Louis, USA).

Preparation of probe DNA fragments. *A. thaliana cab*3 promoter was cloned into pUC18 to construct pAB180, which was transformed into *E. coli.* The plasmid was extracted and purified by the alkaline lysis method.[8] The purified plasmid was digested with *Bam*HI and *Eco*RI, electrophoresed in 1% agarose gels and electroeluted from the gel to isolate a 1,115 bp (-956/+159) fragment of *cab*3 promoter. The promoter was digested with *Hae*III and *Taq*I, and separated on 3% agarose to yield a 248 bp (-244/+4) fragment. Two fragments of 146 bp (-244/-99) and 74 bp (-103/-30) were isolated by digestion of the 248 bp fragment with *Mae*III, and subsequent electrophoresis on 6% non-denaturing polyacrylamide gel followed by electroelution. One microgram of the purified DNA fragments were end-labeled with [α-^{32}P]-dATP using Klenow fragment as described previously.[9] The labeled probes were purified by chromatography on a Sephadex G-50 column.

Preparation of nuclear extracts. Leaves of *Arabidopsis thaliana* were homogenized with a mortar and pestle in a 25 m*M* Tris-HCl buffer (pH 7.8) containing 10 m*M* $MgCl_2$, 6 m*M* β-mercaptoethanol, 0.5 m*M* PMSF, 0.5 m*M* benzamidine and 0.8 *M* sucrose. The homogenates were filtered through two layers of miracloth and centrifuged at 6,000 g for 10 min to precipitate nuclei.[10] The nuclear pellet was carefully suspended in a suspension buffer (25 m*M* Tris-HCl, pH 7.8, 10 m*M* $MgCl_2$, 6 m*M* β-mercaptoethanol, 0.5 m*M* PMSF, 0.5 m*M* benzamidine, 0.46 *M* sucrose, 0.5% Triton X-100) and centrifuged on a 25% Percoll/2 *M* sucrose gradient to partially purify the nuclei.[11] The nuclei were washed and resuspended with a washing buffer (20 m*M* Tris-HCl, pH 7.8, 1 m*M* EDTA, 0.5 m*M* PMSF, 0.5 m*M* benzamidine, 1 m*M* DTT, 25% glycerol). A mixture of the nuclei suspension and 3x volume of 2 *M* ammonium sulfate was gently rocked on a rotary shaker at 4°C for 30 min and centrifuged at 30,000 g for 60 min to obtain nuclear extract. The extract was concentrated with UF-membrane (LOT AD 05848B, MW cutoff 30,000) to 1-2 mg protein per mL of the solution.

Mobility shift assay. The reaction mixture contained 1-1.5 ng of DNA probe, 0.1-0.5 μg of carrier DNA [poly(dIdC) poly(dIdC)], 1-2 μg of nuclear proteins, 16.7 m*M* Tris-HCl (pH 7.8), 1 m*M* EDTA, 8% glycerol, 0.67 m*M* DTT, 66.7 m*M* KCl, 0.33 m*M* PMSF and 0.33 m*M* benzamidine in a total volume of 26 μL. The mixture was incubated at 25°C for 15 min and immediately electrophoresed on 6% non-denaturing polyacrylamide gel which was pre-run with a 0.125 m*M* Tris-HCl buffer (pH 7.9) containing 20% glycerol and 2% β-mercaptoethanol at 3 V/cm for 1hr. Electrophoresis was carried out in a low ionic strength buffer (20m*M* Tris-borate and 1m*M* EDTA, pH 7.8) at 5 V/cm. The gel was dried onto a filter paper and subjected to autoradiography on Kodak X-Omat AR film with a DuPont Cronex intensifying screen.

Assay of protein concentration. The protein concentration was determined by the method of Bradford.[12]

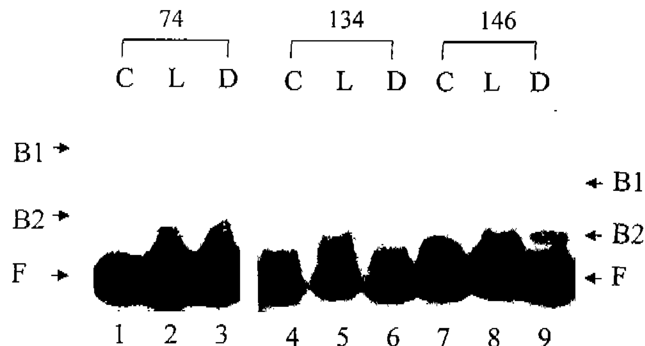

Figure 1. Mobility shift assay of the 74 bp (-103/-30), the 134 bp (-376/-243) and the 146 bp (-244/-99) of the *cab*3 promoter with nuclear protein extracts of the light-grown and the dark-adapted samples. For the light-grown tissues (L), seedlings were grown for 21 days under white light. For the dark-adapted tissues (D), seedlings grown for 19 days under white light were transferred and grown in the darkness for 48 hr. After harvesting the leaves at the end of light treatments, the nuclei were isolated and used immediately for the assay. The binding reaction mixture (26 μL) contained 1 ng of DNA, 1.4 μg of proteins and 0.5 μg of carrier DNA. The mixture was incubated in low ionic strength medium (μ= 0.075) of pH 7.8 at room temperature for 15 min and electrophoresed on a 6% nondenaturing polyacrylamide gel. Lane 1-3; 74 bp fragment, Lane 4-6; 134 bp fragment, Lane 7-9; 146 bp fragment, C; probe DNA only, L; nuclear proteins from the light-grown tissue, D; nuclear proteins from the dark-adapted tissue, F; free probe, B1 and B2; retardation bands.

RESULTS AND DISCUSSION

In an attempt to identify the interactions of LRE and LRF that are involved in the dark repression of *cab* expression, leaf nucleus protein extracts were used for mobility shift assays with three *cab*3 promoter fragments of 134 bp (-376/-243), 146 bp (-244/-99) and 74 bp (-103/-30). As reported previously, the 146 bp and 134 bp fragments had one retardation band and the 74 bp fragment had two retardation bands with the nuclear extracts from the light-grown sample.[7] The dark-adapted sample had the same retardation bands with the 74 bp and 134 bp fragment as the light-grown sample did, as seen in Fig. 1. However, the 146 bp fragment formed one retardation band with the nuclear extracts from the light-grown sample but two retardation bands with the nuclear extracts from the dark-adapted sample. To confirm the binding difference of the 146 bp fragment between the light-grown and dark-adapted tissues, the binding ability of nuclear proteins to the fragment was re-examined at different concentrations of carrier DNA (Fig. 2). Retardation band B1 was always showed both in the light-grown and dark-adapted samples and retardation band B2 was shown only in the dark-adapted

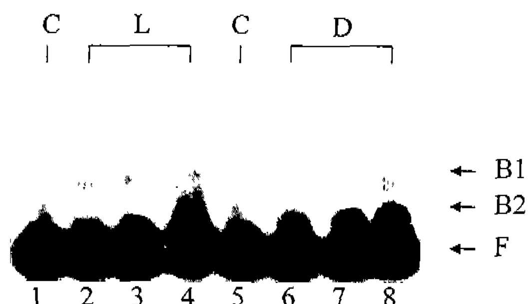

Figure 2. Mobility shift assay of the 146 bp (-244/-99) of the *cab*3 promoter with nuclear protein extracts at different concentrations of carrier DNA. The assay conditions were the same with those described in Fig. 1 except the concentrations of the reactants. The binding reactions (26 μL) contained 1.9 ng of probe DNA, 1.4 μg of nuclear proteins and different amounts of the carrier DNA. C; probe DNA only, L; light-grown sample, D; dark-adapted sample, Lane 1 and 5; probe DNA only, Lane 2 and 6; probe DNA + nuclear proteins + 0.5 μg carrier DNA, Lane 3 and 7; probe DNA + nuclear proteins + 0.25 μg carrier DNA, Lane 4 and 8; probe DNA + nuclear proteins + 0.125 μg carrier DNA, F; free probe, B1 and B2; retardation bands.

sample, regardless of the amounts of the carrier DNA added (No. 6, 7 and 8 in Fig. 2). Although the band B2 was very close to the free probe in the mobility shift assay, the B2 formed a distinct band separated from the free probe and high intensity. Since B2 band was shown only in the dark-adapted sample which represses the *cab* gene expression, it could be regarded as a negative regulatory factor (NRF).

The 146 bp region of *cab*3 promoter includes several light regulatory *cis*-acting elements such as LRE I and II, CCAAT box, G-box, GATA motif as indicated in Fig. 3. The binding site of the B2 band is apparently in the region of the LRE I and II, which were identified as major light regulatory sites for gene expression.[5] In our previous report, it has been shown that a NRF binding to the 65 bp (-318/-254) region of *A. thaliana cab*1 promoter may function to repress the *cab*1 gene expression in the dark.[7] In responses to dark adaptation, the retardation band pattern of the 146 bp fragment of *cab*3 promoter was very similar to that of the 65 bp fragment of *cab*1 promoter.

As shown in Fig. 3, both fragments include CCAAT box which functions mainly as a positive promoter element in a large number of eukaryotic promoters. Many different CCAAT-binding factors were discovered in animal but not yet reported in plant.[13-15] Recently, it has been reported that a negative response element (NRE) within the human vitamin K-dependent matrix Gla protein (hMGP) gene promoter contains a CCAAT box.[16] Although the NRF (B2 band) involved in the dark repression of *cab* bound to the 65 bp fragment of the *cab*1 promoter and the 146 bp fragment of the *cab*3 promoter that contain CCAAT box (Fig. 3), the interaction between the NRF and CCAAT box is not known. Gene expression could be regulated by combinatorial interactions of LREs.[17]

The 146 bp region of *cab*3 promoter also includes several other light regulatory *cis*-acting elements such as GATA motif and GT-4 binding site as seen in Fig. 3. The GATA motifs are found in many light regulated promoters and known to play important roles in the light responsive transcription. The GATA motif in the *Lemna gibba cab*AB19 promoter is reported to be required for phytochrome regulation of this gene.[18] Since three GATA motifs are present in the 146 bp region of *cab*3 promoter[19] as shown in Fig. 3, they could be a possible candidate for a binding site for the B2 retardation band. Another feasible binding site for the B2 retardation band is the sequence of GTTAAA, which is contained in the 146 bp fragment (-244/-99). The GTTAAA core sequence in *Nicotiana plumbaginifolia cab-E* promoter

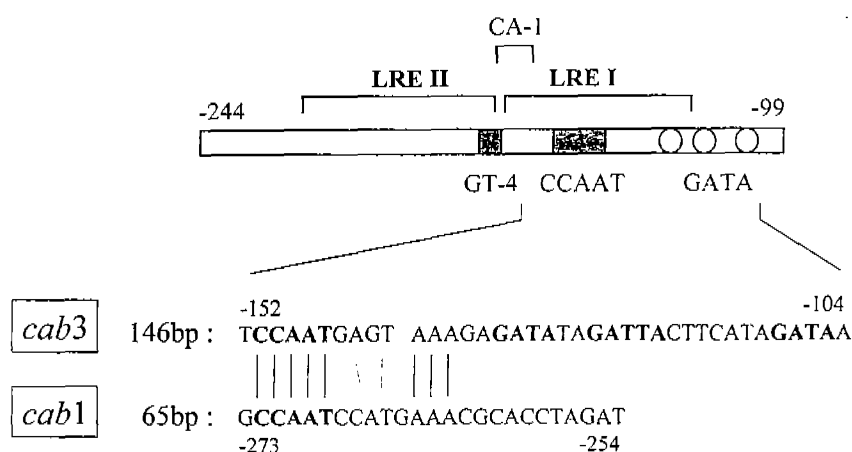

Figure 3. Functional map of the 146 bp fragment (-244/-99) of the *cab*3 promoter and its sequence comparision with the -318 to -254 region (65 bp) of the *cab*1 promoter of *A. thaliana*. LRE I and II indicate loci of the putative light regulatory elements identified by Mitra *et al.* (1989)[6]. The positions of three GATA motifs (-131/-110), CCAAT box (-145/-141), GT-4 binding site (GTTAAA) and CA-1 binding site (-165/-156) have been indicated.

is a binding site for GT-4 factor, which is a well known light regulatory factor.[20] As indicated in Fig. 3, the -165 to -156 region of *cab*3 promoter has similar sequence to the CA-1 binding site, which is essential to high level expression and phytochrome regulation in the *A. thaliana cab*1 promoter.[21]

The region of -200 to -92 of the *A. thaliana cab*2 promoter has binding sites for several factors such as CUF-1, Toe, CUF-2/3, Tac, Tic, CGF-1/GT-1 that were reported to increase transcription levels or to contribute to the light-induced circadian oscillation in *cab*2 expression.[22,23] The sequence homology of the *cab*2 and 3 promoters of *A. thaliana* from the translation start site to -256 of *cab*3 is 87%.[6] These results may suggest a possibility that the 146 bp region of the *cab*3 promoter may have binding sites for such light regulatory factors.

The B1 and B2 may have different binding sites each other in the 146 bp region of *cab*3 promoter. However, the *cab*3 gene expression in the dark could be repressed by the interaction of the factors B1 and B2 and the existence of a lot of LREs in the 146 bp region of the *cab*3 promoter may suggest a very complicated regulation of *cab*3 gene expression in responses to light and dark.

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REFERENCES

1. Jansson, S. (1994) The light-harvesting chlorophyll a/b-binding proteins. *Biochim. Biophysic. Acta.* **1184**, 1-19.
2. Nagy, F., S. A. Kay, M. Boutry, M.-Y. Hsu and N.-H. Chua (1986) Phytochrome-controlled expression of a wheat *cab* gene in transgenic tobacco seedlings. *EMBO J.* **5**, 1119-1124.
3. Adamska, I. (1995) Regulation of early light-inducible protein gene expression by blue and red light in etiolated seedlings involves nuclear and plastid factors. *Plant Physiol.* **107**, 1167-1175.
4. Latchman, D. S. (1990) *Gene regulation: A eukaryotic perspective*, Unwin Hyman, London, p.138.
5. Ha, S. B. and G. An (1988) Identification of upstream regulatory element involved in the developmental expression of the *Arabidopsis thaliana cab*1 gene. *Proc. Natl. Acad. Sci. USA* **85**, 8017-8021.
6. Mitra, A., H. K. Choi and G. An (1989) Structural and functional analyses of *Arabidopsis thaliana* chlorophyll a/b-binding protein (*cab*) promoters. *Plant Mol. Biol.* **12**, 169-179.
7. Choi, D.-K., H.-J. Park and I.-S. Kim (1996) A negative regulatory factor for the dark repression of *Arabidopsis thaliana cab*1 gene. *Photochem. Photobiol.* **64**, 870-875.
8. Maniatis, T., E. F. Fritch and J. Sambrook (1989) Molecular cloning: A laboratory mannual, Cold Spring Harbor Lab., Cold spring Harbor, NY.
9. Lee H.-H., H.-J. Park and I.-S. Kim (1996) Binding of leaf nuclear protein extracts to light-responsive elements of *cab* promoters of *Arabidopsis thaliana. J. Photoscience* **3**, 85-90.
10. Giuliano, G., E. Pichersky, V. S. Malik, M. P. Timko, P. A. Scolnik and A. R. Cashmore (1988) An evolutionary conserved protein binding sequence upstream of a plant light-regulated gene. *Proc. Natl. Acad. Sci. USA* **85**, 7089-7093.
11. Luthe, D. S. and R. S. Quatrano (1980) Transcription in isolated wheat nuclei. *Plant Physiol.* **65**, 305-308.
12. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
13. Johnson, P. F. and S. L. McKnight (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.* **58**, 799-839.
14. Maity, S. N and B. D. Crombregghe (1996) Purification, characterization, and role of CCAAT-binding factor in transcription. *Methods Enzymol.* **273**, 217-232.
15. Wu, G. D., E. L. Lai, N. Huang and X. Wen (1997) Oct-1 and CCAAT/enhancer-binding protein (C/EBP) bind to overlapping elements within the interleukin-8 promoter. *J. Biol. Chem.* **272**, 2396-2403.
16. Kirfel, J., M. Kelter, L. M. Cancela, P. A. Price and R. Schule (1997) Identification of a novel negative retinoic acid responsive element in the promoter of the human matrix Gla protein gene. *Proc. Natl. Acad. Sci. USA* **94**, 2227-2232.
17. Puente, P., N. Wei and X. W. Deng (1996) Combinatorial interplay of promoter elements constitutes the minimal determinants for light and developmental control of gene expression in *Arabidopsis. EMBO J.* **15**, 3732-3743.
18. Kehoe, D. M., J. Degenhart, I. Winicov and E. M. Tobin (1994) Two 10-bp regions are critical for phytochrome regulation of a *Lemna gibba Lhcb* gene promoter. *Plant Cell.* **6**, 1123-1134.
19. Terzaghi, W. B. and A. R. Cashmore (1995) Light-regulated transcription. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 445-474.
20. Schindler, U. and A. R. Cashmore (1990) Photoregulated gene expression mayinvolve ubiquitous DNA binding proteins. *EMBO J.* **9**, 3415-3427.
21. Kenigsbuch, D. and E. M. Tobin (1995) A region of the *Arabidopsis Lhcb*1*3 promoter that binds to CA-1 activity is essential for high expression and phytochrome regualation. *Plant Physiol.* **108**, 1023-1027.
22. Anderson, S.L. and S. A. Kay (1995) Functional dissection of circadian clock- and phytochrome-regulated transcription of the *Arabidopsis CAB*2 gene. *Proc. Natl. Acad. Sci. USA* **92**, 1500-1504.
23. Carre, I. A. and S. A. Kay (1995) Multiple DNA-protein complexes at a circadian-regulated promoter element. *Plant Cell.* **1**, 2039-2051.