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BINDING OF LEAF NUCLEAR PROTEIN EXTRACTS TO LIGHT-RESPONSIVE ELEMENTS OF *cab* PROMOTERS OF *Arabidopsis thaliana*

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Abstract — The binding ability of leaf nuclear extracts to the light-responsive elements (LREs)[§] of *cab* promoters of *Arabidopsis thaliana* has been investigated. The *cab* promoters were fragmented with restr ction endonucleases into LRE that were identified by Mitra *et al.* [*Plant Mol. Biol.*12, 169-179 (1989)] and other small fragments. After end labeling with Klenow fragment, the fragments were assayed for binding with the leaf nuclear proteins that were prepared by solubilizing the purified nuclei with 0.5 *M* ammonium sulfate. The binding ability was assayed by mobility shift assay. To perform successful mobility shift assay, several factors affecting the interaction of protein with DNA were optimized before performing the assay. The LREs had several retardation bands. However, the other promoter fragments from the transcription start site to the far upstream region of the promoters had also retardation bands. No particular relationships could be found between the retardation band distributions and the loci of LRE. It is likely that the light-regulation of *cab* gene expression may be controlled by the multiple interactions of the regulatory protein factors with DNA motifs.

INTRODUCTION

Light is essential for normal plant growth and development not only as a source of energy for photosynthesis but also as a signal for conducting plant morphogenesis. If germinated in the dark, the seedlings become etiolated and have different morphology from the plant grown under light.[1-3] One of the most drastic difference is the expression level of photosynthetic genes. Upon illumination of the etiolated plants, it becomes greening mainly by activating the photosynthetic genes.[4,5] In general, gene expression is regulated by interacting a specific DNA motif (*cis*-acting element) of promoter with protein factor (*trans*-acting factor). To understand the effects of light on gene expression, a photosynthetic gene, *rbcS* (the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase gene), has been extensively studied.[6,7] As a consequence, several DNA motifs and its binding partners have been identified and characterized. On the other hand, those of *cab* (light-harvesting chlorophyll a/b-binding protein gene) have been poorly understood so far, even though *cab* promoters from several plants such as wheat, *Nicotiana plumbaginifolia*, pea, tomato and *Arabidopsis thaliana* have been studied.

The *cab* gene of *A. thaliana*[8] is a multigene family comprising 5 genes. Although the *cab*1, 2 and 3 genes code for an identical mature protein for photosystem II, they have different promoters.[9,10] The promoter activities were controlled differentially at certain developmental stages, and with tissue-specific and photosynthetic manner.[11] An's group[9,10] extensively studied the *cab* promoters of *A. thaliana* by deletion mutants and identified several loci of light-responsive elements (LREs). They have shown that the regulatory elements necessary for the light-dependent and tissue-specific activities reside in approximately 300 bp 5' upstream region of the promoters.[9] However, the specific DNA motifs and their binding factors that are light-dependent have not been reported yet.

In the present study, we have investigated the binding ability of the leaf nuclear extracts to the *cab*2 and 3 promoters of *A. thaliana*. The binding study was focussed on the regions of the LRE that were identified by Mitra *et al.*[9]. The results could provide a clue to identify the DNA motifs and their protein

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§ *Abbreviations* : ASF-1, activation sequence factor 1; *cab*, light-harvesting chlorophyll a/b-binding protein gene; GBF,G-box binding factor; PMSF, phenylmethyl-sulfonylfluoride; *rbcS*, the small subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase gene; LRE, light responsive element; DTT, dithiothreitol.

binding factors involved in light-regulated *cab* gene expression.

MATERIALS AND METHODS

Plant materials. The wild type seeds of *A. thaliana* cv. Landsberg were grown at 26°C for 3 weeks on vermiculite which was soaked in 0.3% hyponex. The light intensity was maintained 7000-8000 lux 18 h and cycled 6 h dark every day. The harvested leaves were immediately frozen in liquid nitrogen and stored at -70°C until use.

Probe DNA preparation and endlabeling. *A. thaliana* *cab*2 and 3 genes were cloned into pUC8 and pGA569 to construct pAB165 and pAB180, respectively.[11,12] The plasmids transformed into *E. coli* were extracted and purified by the alkaline lysis method.[13] Each purified plasmid was digested with *Bam*HI and *Eco*RI. The fragmented plasmids were electrophoresed in 1% agarose gels and electroeluted to isolate 947 bp (-788/+159) of *cab*2 promoter and 1115 bp (-956/+159) of *cab*3 promoter. The resulting *cab* 2 promoter (947 bp) was fragmented with *Hae*III and *Hin*fI into 123 bp (-484/-362) and 368 bp (-364/+4). The *cab* 3 promoter (1115 bp) was also digested with *Hae*III and *Taq*I, and separated on 3% agarose gels to yield 647 bp (-891/-244) and 248 bp (-244/+4) fragments. Both fragments were further digested with *Mae*III to yield 134 bp(-367/-243) from 647 bp fragment, and 146 bp (-244/-99) and 74 bp (-103/-30) from the 248 bp fragment. The digested fragments were separated on 7% polyacrylamide gels and isolated by subsequent electroelution. For the end labeling with Klenow fragment, a reaction mixture of [^{32}P]-dATP, 0.1 m*M* dNTP (dCTP, dGTP and dTTP), one unit of Klenow fragment and 5x labeling buffer (0.25 *M* Tris-HCl, pH 7.5, 5 m*M* $MgCl_2$, 5 m*M* dithiothreitol[DTT], 0.25 mg ml bovine serum albumin was added to 1 μg of the purified DNA fragments in a total volume of 50 μL.[14] The reaction was carried out for 30 min at room temperature and terminated by adding 0.5 *M* EDTA. The labeled probes were clarified by Sephadex G-50 column.

Preparation of nuclear extracts. Leaves grown for 3 weeks under light were extracted in a homogenization buffer (25 m*M* Tris-HCl, pH 7.8, 10 m*M* $MgCl_2$, 6 m*M* β-mercaptoethanol, 0.5m*M* phenymethylsulfonylfluoride [PMSF], 0.5 m*M* benzamidine and 0.8 *M* sucrose). The extracts were filtered through two layers of miracloth and centrifuged at 6000 g for 10 min to precipitate nuclei.[14,15] The nuclear pellet was carefully suspended in a 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 purified by 25% Percoll/2 M sucrose gradient centrifugation.[7] To remove residual Percoll and Triton, the nuclear preparation was then washed twice by centrifuging at 6000 g 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). The nuclear pellet was resuspended in a ice-cold washing buffer and mixed with one third volume of 2 *M* ammonium sulfate. The mixture was gently rocked on a rotary shaker at 4°C for 30 min and centrifuged at 30000 g for 60 min to remove insoluble materials.[7] The nuclear extract was concentrated with UF-membrane (lot AD 05848B, MW cutoff 30000) to a concentration of 1-2 mg protein per mL of solution.

Mobility shift assay. The reaction mixture contained 1-1.5 ng of DNA probe, 4 μg of carrier DNA [poly(dIdC) poly(dIdC)], 8-10 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 4-6% polyacrylamide gels depending on the size of the probes. The gel was pre-run with sample buffer (0.125 m*M* Tris-HCl, pH 7.9, 20% glycerol, 2% β-mercaptoethanol, 0.25% bromophenol blue) at 3 V/cm for 1 h. Electrophoresis was carried out in a low ionic strength buffer (20 m*M* Tris-borate and 1 m*M* EDTA, pH 7.8) at 5 V/cm.[14] 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.[18]

RESULTS AND DISCUSSION

The regulatory protein factors control gene expression in response to the environmental stimuli and a single stimulus triggers the action of one or more regulatory factors. To understand the regulatory mechanism of *cab* gene expression, it is important to search such protein factors that bind to the *cab* promoters.

Mobility shift assay is a useful tool to identify the interacting protein factors to DNA motifs. A successful performance of mobility shift assay depends on the properties of the interacting protein and DNA molecules both in solution and during electrophoresis.[19] The stable complex formation during the processes is a most important factor to obtain quantitative data. The stability is affected mainly by the composition of reaction mixture and the electrophoretic condition. It is necessary to optimize such factors to perform successful results. Employing 123 bp (-484/-362) DNA fragment of *cab*2 promoter, several conditions were optimized by trial and error as summerized in Table 1. Both the harvest time and freshness of the sample for extract preparation were important factors to obtain reproducible results. The 3 week-grown tissues gave better reproducibility than the 2 or 5 week-grown tissues. It was also important that electrophoresis was carried out in a low ionic strength buffer. Twenty m*M* Tris-borate buffer (pH 7.8) containing 1 m*M* EDTA gave better result than 50 m*M* Tris-glycine (pH 8.3) buffer containing 2 m*M* EDTA.

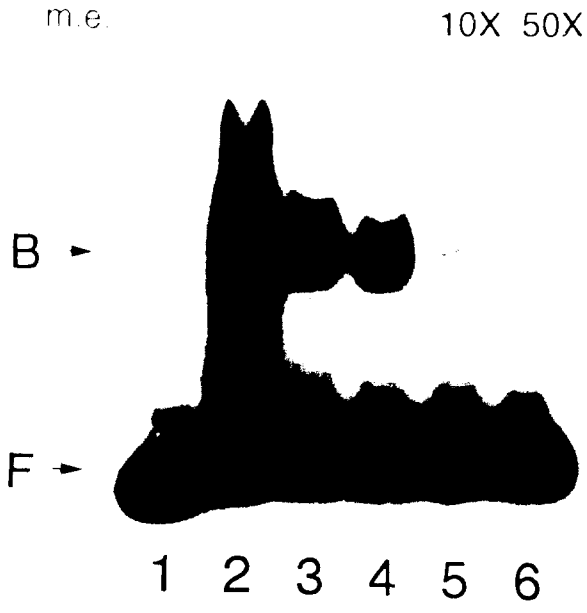

Figure 1. Mobility shift assay of a 123 bp (-484/-362) fragment of the *cab*2 promoter with *Arabidopsis* leaf nuclear proteins. In a total reaction volume of 26 μL contained 1.6 ng of probe, 4 μL of carrier DNA (lanes 3-6) and a 10- or 50-fold molar excess (m.e.) of the unlabeled fragment (lanes 5 and 6). The mixture was incubated in a low ionic strength medium (μ=0.08) at pH 7.8 for 15 min at room temperature. The incubation mixture was electrophoresed on a 4.5% nondenaturing polyacrylamide gel. Lane 1; labeled probe only, lane 2; 10 μg of nuclear protein with probe but without carrier DNA, lane 3; 20 μg of nuclear protein incubated with probe and carrier DNA, lane 4; 10 μg of nuclear protein incubated with probe and carrier DNA, lanes 5 and 6; reaction mixture 4 plus 10- and 50-fold m.e. of the unlabeled probe, respectively. B; bound probe, F; free probe.

Figure 1 shows the mobility shift assay of the 123 bp (-484/-362) fragment of the *cab*2 promoter that has one retardation band under the conditions described in Table 1. The ratio of protein to probe DNA was one of the most important factors. Incubation of 10 μg of nuclear protein with 1.5 ng of probe DNA gave much better retardation band than 5 μg of protein did (data not included). Binding specificity of retardation band was determined by a binding competition with an excess unlabeled promoter fragments. As seen in lanes 5 and 6 of Fig. 1, 10- or 50-fold molar excess (m.e.) of unlabeled fragment drastically reduced the intensity of the retardation band. However, *cab*3 structural gene (700 bp) digested with *Hind* III failed to compete for the binding. The competitive binding assays indicate that the retardation band comes from a specific interaction between the probe DNA and nuclear proteins.

Table 1. An optimum condition for the mobility shift assay of *A. thaliana cab* promoter fragments with the leaf nuclear protein extracts.*

Factor	Optimum Conc.	Tested ranges
Probe DNA	1.5 ng	0.5-2 ng
Nuclear protein	8-10 μg	5-30 μg
Carrier DNA	4 μg	1-8 μg
Ionic strength	0.07-0.08	0.05-0.15

*Ionic strength of the incubation (16.7 m*M* Tris-HCl, 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, pH 7.8) adjusted with KCl. The total volume of the reaction mixture was 26 μL.

Several promoter fragments of *cab* genes were subjected to the mobility shift assay with the nuclear proteins under the same conditions described for the 123 bp (-484/-362) fragment of *cab*2 promoter. The binding assay was mainly focussed on the regions of LRE as identified by Mirta *et al.*[9]. They reported that LRE I was located at the 45 bp (-209/-164) region

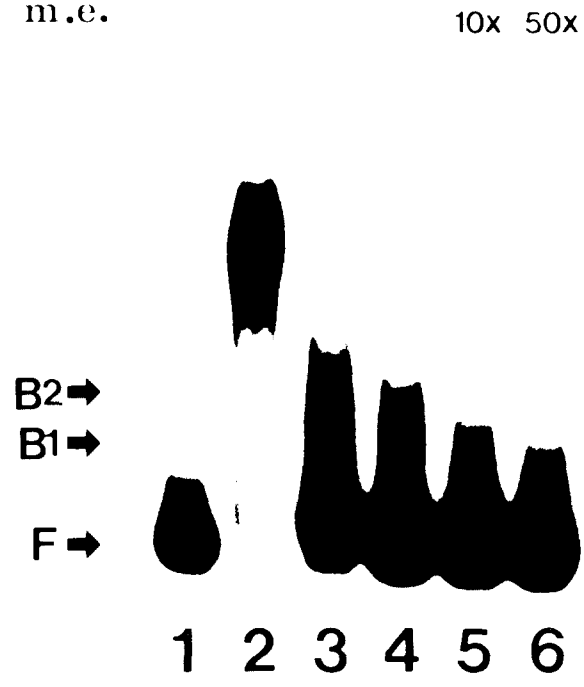

Figure 2. Mobility shift assay of a 368 bp (-364/+4) fragment of the *cab*2 promoter with *Arabidopsis* nuclear proteins. The experimental conditions were the same as described in Fig. 1. The reaction mixture was electrophoresed on a 4% nondenaturing acrylamide gel. Lane 1; labeled probe only, lane 2; assay mixture without carrier DNA, lanes 3 and 4; whole reaction mixture containing probe DNA, nuclear protein extract (10 μg protein) and carrier DNA, lanes 5 and 6; whole reaction mixture with 10- and 50-fold m.e. of unlabeled probe, respectively. B; bound probe, F; free probe.

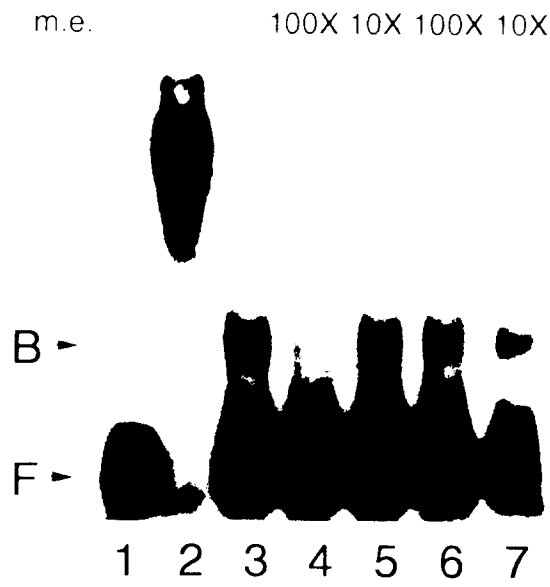

Figure 3. Mobility shift assay of a 146 bp (-244/-99) fragment of the *cab*3 promoter with nuclear protein extracts. The experimental conditions were the same as described in Fig. 1. Lane 1; labeled probe only, lane 2; reaction mixture without carrier DNA, lane 3; whole reaction mixture containing with nuclear protein extract (10 μg protein), probe and carrier DNA, lanes 4 and 5;whole reaction mixture with a 100- and 10-fold molar excess (m.e.) of unlabeled *cab*3 promoter DNA, respectively, lanes 6 and 7; whole reaction mixture with a 100- and 10-fold m.e. of unrelated DNA (*cab*3 structural gene, 700 bp), respectively. B; bound probe, F; free probe.

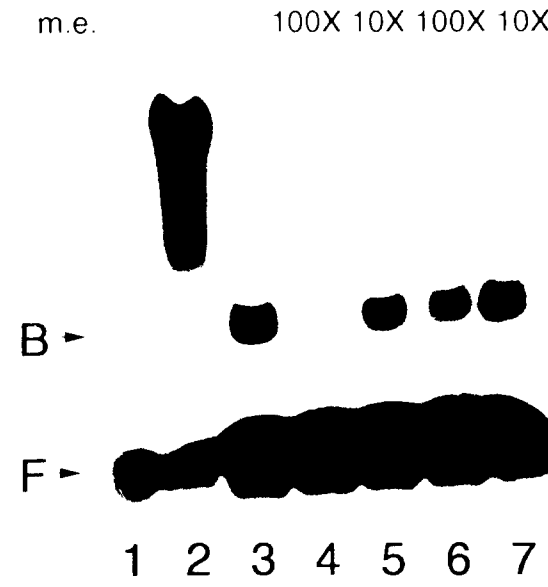

Figure 4. Mobility shift assay of a 74 bp (-103/-30) fragment of the *cab*3 promoter with nuclear protein extracts. The experimental conditions were the same as described in Fig. 1. Lane 1; labeled probe only, lane 2; reaction mixture without carrier DNA, lane 3; whole reaction mixture containing with nuclear protein extract (10 μg protein), probe and carrier DNA, lanes 4 and 5; whole reaction mixture with a 100- and 10-fold molar excess (m.e.) of unlabeled *cab*3 promoter DNA, respectively, lanes 6 and 7; whole reaction mixture with a 100- and 10-fold m.e. of unrelated DNA (*cab*3 structural gene, 700 bp), respectively. B; bound probe, F; free probe.

and LRE II at the 90 bp (-164/-74) region of the *cab*3 promoter. LRE I (-204/-158) and LRE II (-158/-74) regions of the *cab*2 promoter were deduced from the LRE regions of *cab*3 on the basis of their promoter sequence homology. The G-box, G-box like sequence,[20,21] GT box[20] and GATA motifs[22] were known to be an essential elements for the light-responsive gene expression of *rbcS* and *cab*.[23,24] For the *cab* promoters of *A. thaliana*, the homologous sequences to such boxes /motifs cluster near the regions of the LRE. The 368 bp fragment (-364/+4) of *cab*2 that includes LRE I and II, G-box, CCAAT and GATA box formed two retardation bands with the nuclear extracts (Fig. 2). Since several *cis*-acting elements present in that region, the two retardation bands may indicate that there are two different binding factors. The binding specificity was also confirmed by competitive binding with the unlabeled 248 bp (-244/+4) fragmet[9,25,26]. The 239 bp(-720/-482) fragment includes DNA sequence ($^{-498}$TGACG^{-494}) that are similar to ASF-1 factor binding site in the CaMV 35S promoter[25] and also contained two DNA sequences ($^{-608}$GTTAAA^{-603}, $^{-513}$GGTTAT^{-508}) which are similar with GT-1 binding site found in *N. plumbaginifolia* *cab*-E promoter.[26] Both ASF-1 and GT-1 binding sites are known to be light-dependent promoter elements. This fragment had only one retardation band (data not shown). As shown in Fig. 1, the 123 bp (-484/-362) fragment had one retardation band although no LRE was known in this region.[9]

The 1115 bp (-956/+159) fragment of the *cab*3 promoter was digested with *Sau*3AI and *Hae*III to prepare a 320 bp (-316/+4) fragment which contains the LRE[9] and several important regulatory elements such as G box, CCAAT, TATA and GATA. Since the 320 bp fragment had several retardation bands in the preliminary assays, the *cab*3 promoter was further cleaved into smaller fragments and assayed for the binding to the nuclear proteins. The 146 bp (-244/-99) fragment contains G-box, CCAAT and GATA box, and LRE I and II identified by Mirta *et al.*[9]. This fragment has also the same DNA sequences (^{66}GTTAAA^{61} to GT-1, ^{116}TCATAGATAACAAACGTT^{-99} to 3AF-1) with those of GT-1 binding site in *N. plumbaginifolia* *cab*-E promoter and 3AF-1 binding site in pea *rbcS*-3A promoter. This fragment had only one retardation band as shown in Fig. 3. A small fragment of 74 bp (-103/-30) which contains TATA box and some part of LRE I also formed one retardation band (Fig. 4), but

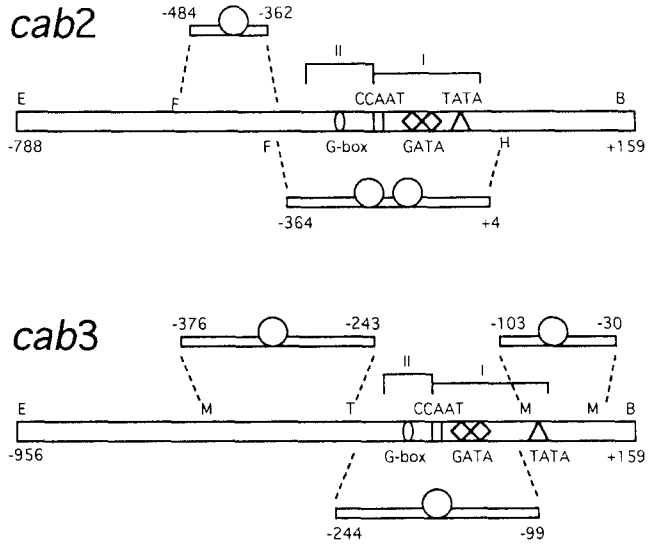

Figure 5. Occurrance of the mobility retarded bands in *cab*2 and 3 promoters of *A. thaliana* assayed with its leaf nuclear protein extracts. The assay conditions were described in Fig. 1. The open circles indicate the numbers of the retardation bands. I and II indicate the loci of the putative light regulatory elements (LREs) reported by Mitra *et al.*[9]. The sites of the important regulatory elements (GATA, TATA, G-box and CCAAT) are also indicated. The numbers at the edges of bars indicate the position of base pairs. Endonucleases used: B; *Bam*HI, E; *Eco*RI, F; *Hinf*I, H; *Hae*III, M; *Mae*III and T; *Taq*I.

no retardation band was observed with 38 bp (-34/+4) fragment of *cab*3(date not shown). A 134 bp fragment (-376/-243) in which no LRE was known had one retardation band. Distributions of the retardation bands on *cab* promoters are summarized in Fig. 5.

The essential sequences of LRE identified by Mitra *et al.* correspond to the 368 bp (-364/+4) fragment of *cab*2 and 146 bp (-244/-99) and 74 bp (-103/-30) fragments of *cab*3[9]. Thus, it seems likely that the 368 bp (-364/+4) fragment of *cab*2 and 146 bp (-244/-99) fragment of *cab*3 are important elements for light responsive expression of the *A. thaliana cab*. Although these fragments were considered to have more retardation bands than the other promoter fragments that do not contain LRE, our results were not the case. It seems likely that light controls *cab* gene expression by multiple interactions of the DNA motifs with regulatory protein factors. Since the 368 bp, 239 bp and 123 bp fragments of *cab*2 contain several known *cis*-acting elements, it is likely that the protein binding to these fragments might be different each other. However, the factors binding to *cab*2 and *cab*3 promoters may not share in common since both genes are regulated at the same patterns and their promoters have high sequence homology. To identify *trans*-acting factors that bind to LRE, further studies of comparative mobility shift assay need to be carried out with the samples received different light/dark treatments.

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