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A Region of the Arabidopsis *Lhcb1*3* Promoter That Binds to CA-1 Activity Is Essential for High Expression and Phytochrome Regulation[1]

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We have previously characterized a protein from *Arabidopsis thaliana*, called CA-1, that bound to a specific region of the *Lhcb1*3* promoter. This binding activity was of interest because the sequence to which it bound is included in a portion of the promoter that is sufficient for phytochrome regulation and because the activity was absent in photomorphogenic mutant *det1* seedlings (L. Sun, R.A. Doxsee, E. Harel, E.M. Tobin [1993] Plant Cell 5: 109–121). We have now directly tested whether the nucleotide sequence to which CA-1 binds is required for regulation of the transcription of this gene by phytochrome. A mutation that abolished CA-1 binding in vitro was introduced into a 1.15-kb segment of the *Lhcb1*3* promoter, and both the wild-type and mutant promoter fragments were fused to a *uidA* reporter gene and used to stably transform *A. thaliana*. Ten different homozygous lines were examined for phytochrome responsiveness for each of the two constructs by assaying β-glucuronidase activity. The wild-type construct showed normal phytochrome responsiveness. The mutant construct showed no phytochrome response, and the overall level of β-glucuronidase activity in etiolated seedlings was decreased by about 2 orders of magnitude. We did not detect a response to a B photoreceptor other than phytochrome itself for either the wild-type or mutant construct. We conclude that information essential for both a high level of expression and phytochrome responsiveness is contained in a 27-bp region to which the CA-1 activity binds.

The regulation of plant genes by light has been an area of intense investigation in recent years. Of particular interest has been the way in which the phytochrome family of photoreceptors can regulate the expression of specific genes (reviewed by Tobin and Kehoe, 1994). However, very little is known either about the specific nucleotide sequences that make a gene phytochrome responsive or about the transcription factors with which such sequences interact. Two of the gene families that can be regulated by phytochrome and that have been the subject of extensive studies are those encoding the light-harvesting Chl *a*/*b*-proteins of PSII (*Lhcb* genes, formerly called *cab* genes) and the small subunit of Rubisco. Research in this area has been sometimes difficult to interpret, in part because of the multiplicity of signals to which these genes respond and in part because proteins that interact with the promoters of these genes have often been found to be members of a family of related genes. Phytochrome regulation of *Lhcb* genes has been studied in many different species, and relatively large promoter regions sufficient for such regulation have been identified for four *Lhcb* genes in three species (Nagy et al., 1987; Sun et al., 1993; Anderson et al., 1994; Kehoe et al., 1994). We have recently shown that for one of these genes, an *Lhcb2* gene of *Lemna gibba*, two closely spaced 10-bp regions containing conserved sequence motifs are each necessary for phytochrome responsiveness (Kehoe et al., 1994). Thus, continuing to refine our understanding of what promoter sequences play an essential and specific role in phytochrome regulation should lead to the direct identification of the components of the final steps in the transduction pathway.

We have characterized a DNA-binding activity in *Arabidopsis thaliana* that can interact with the *Lhcb1*3* promoter (originally called *cabAB140*, Leutwiler et al., 1986; for new terminology, see Jansson et al., 1992). The activity was found to footprint a region of this promoter extending from −111 to −73 relative to the start of transcription, and it was designated as CA-1. It is of particular interest in terms of light-regulated gene expression because the activity was not detected in *det1* seedlings, a mutant line that develops the morphology of a light-grown plant and expresses relatively high levels of *Lhcb* RNA when grown in complete D (Chory et al., 1989; Sun et al., 1993). The region of the promoter identified by footprinting occurs within a larger region (−183 to +648) of a construct (Ha and An, 1988) shown to be sufficient for conferring phytochrome regulation on a reporter gene in transgenic tobacco (Sun et al., 1993). It was also shown that a mutation (mut 1) in the −126 to −100 region identified by footprinting could substantially abolish binding to the CA-1 activity (Sun et al., 1993). However, the possible functional role of this region in conferring phytochrome regulation was not previously determined.

We have now tested the functional role of the −126 to −100 region of the *Lhcb1*3* promoter in vivo by using

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Abbreviations: B, blue light; D, darkness; FR, far-red light; R, red light; WS, Wassilevskija; WT, wild type.

transgenic Arabidopsis plants transformed with promoter:: reporter gene fusions.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Light Sources

Arabidopsis thaliana ecotype WS was used for transformations and later as the nontransformed control for GUS background activity. Seeds were sterilized in 50% bleach for 10 min, washed twice with sterilized water, and sown on Whatman No. 1 filter paper placed on GM medium (Murashige-Skoog basal medium [Sigma], 3 mM Mes, 2% Suc, 0.7% phytagar, pH 5.7). The imbibing seeds were given 2 d of cold (4°C) treatment in D, followed by 20 min of white light (given after warming to room temperature) to ensure uniform germination. The plates were returned to D at 25°C for 5 d and then illuminated by R and FR as described by Tobin (1981) or with 2 min of B (300 μE m^{-2} s^{-1}) using a blue filter (No. 851, "daylight blue"; Edmund Scientific, Barrington, NJ). After these treatments, seedlings were returned to the dark for 14 to 18 h before harvesting and assaying. In the experiment with the white-light-grown plants, plates were placed in a growth chamber (55 μE m^{-2} s^{-1}) for 6 d after the 2 d of imbibition at 4°C as described before.

Constructs

Both WT and mutant constructs contained 1150 bp of the *Lhcb1*3* promoter. The mutant contained the same changes as the m1 mutant fragment described by Sun et al. (1993) and was derived from a construct in which the altered nucleotides were introduced by site-directed PCR mutagenesis (Sun, 1993). Both constructs were made as shown in Figure 1 by using PCR to create *Eco*RI and *Bam*HI sites for cloning. The oligonucleotides used were 5′-GGGAATTCCAATCACTAGACCTCACAGGC-3′ (for the *Eco*RI site) and 5′-GGGGATCCTGGTATTGATGCGATTTTCGC-3′ (for the *Bam*HI site).

The co-integrative plasmid used was PGV1501CGUS (Koltunow et al., 1990), which contains the *uidA* gene with 50 bp of the cauliflower mosaic virus 35S promoter plus 8 bp of transcript and the neophosphotransferase II 3′ untranslated region (Fig. 1). This plasmid was linearized with *Eco*RI and *Bam*HI and then ligated with either the WT or the mutant *Lhcb1*3* promoter. The PCR products were completely sequenced after cloning using a dideoxy nucleotide sequencing kit (United States Biochemicals).

Plant Transformation and Selection

The plant transformation procedures were done as described by Valvekenes et al. (1988). Transgenic plants with one site of insertion of the T-DNA were identified by segregation of T_2 progeny in a 3:1 ratio (resistant to susceptible) on GM medium containing 50 mg/L kanamycin. Only lines that showed no difference in the level of resistance to kanamycin between the homozygous and the heterozygous plants were chosen for later work.

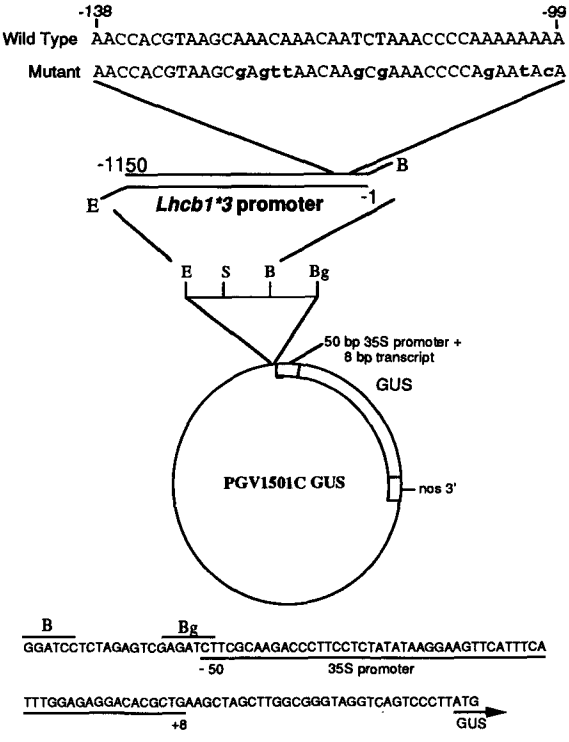

Figure 1. Diagram of WT and mutant constructs used for transformations. The sequences of the *Lhcb1*3* promoter regions between −138 and −99 are shown at the top for both WT and mutant constructs. The nucleotides altered in the mutant construct are shown in lowercase bold letters. The insertion of the 1150-bp promoter region as an *Eco*RI/*Bam*HI fragment into the PGV1501C GUS vector is diagrammed in the middle. At the bottom, the sequence that includes the 35S minimal promoter (50 bp) from the *Bam*HI site to the start of translation of GUS (arrow) is shown. The numbers below refer to the positions relative to the transcription start of the 35S promoter. E, *Eco*RI; S, *Sma*I; B, *Bam*HI; Bg, *Bgl*II; nos, nopaline synthase.

Biochemical Assays

Plants were homogenized as described by Okubara et al. (1993) with some modifications. The grinding buffer contained 0.2 M phosphate buffer, pH 7.5, and 0.5 μg/mL leupeptin (Sigma) was added. About 100 seedlings were analyzed per sample and homogenized in 500 μL of the grinding buffer. One hundred microliters of the mutant plant extracts and 10 μL of the WT plant extracts were analyzed for GUS activity using 4-methylumbelliferyl glucuronide (Sigma) as substrate as described by Jefferson (1987). GUS enzyme amounts in the extracts were calculated by comparison to the activity of purified GUS enzyme (Sigma). Total protein was quantified with Bio-Rad Bradford protein assay dye reagent using BSA as a standard. Background activities were measured in extracts of nontransformed WS ecotype plants and were subtracted from the measured GUS values in each experiment.

RNA Isolation and Detection of GUS RNA

RNA was isolated as described by Brusslan and Tobin (1992) and assayed by electrophoresis, blotting, and hy-

bridization to probe for the *uidA* gene labeled by random priming. The probe consisted of a 600-bp fragment of the *uidA* gene isolated by electrophoresis from PBI221 (Clontech, Palo Alto, CA) after *Eco*RV and *Bam*HI digestion.

RESULTS

Isolation of Homozygous Transformed Lines

Multiple lines transformed with either the WT or mutant construct were selected. The primary transformants were selfed, individual T_2 progeny were grown, and seeds from each of the T_2 plants were tested for segregation of kanamycin resistance to identify plants that were homozygous at a single insertion site for the introduced construct. Seeds from homozygous T_2 plants derived from 10 different primary transformants for each construct were used to test the expression and regulation of the GUS reporter activity. The homozygous lines selected and used for further experiments were those that also showed a high level of resistance to kanamycin as heterozygotes, and all of the lines selected in this way were also found to be expressing the introduced construct at substantial levels.

The CA-1-Binding Site Has a Role in the Enhancement of *Lhcb1*3* Expression

The relative expression levels of the WT and mutant constructs were compared in both etiolated and green seedlings. Figure 2 shows the GUS activity of seedlings of 10 different WT and 10 different mutant lines grown in the dark for 6 d. There was an approximately 200-fold difference in the GUS activity/μg protein that accumulated during this 6 d of growth, suggesting that the mutation affected a positive quantitative element in the promoter. The lower level of expression seen in the mutant lines was substantially above the background level (ranging from 1.5- to 5.5-fold higher) in plants of the nontransformed parental line (WS); thus, the mutation did not entirely abolish promoter activity. A comparison of GUS accumulation was also made with five WT and five mutant lines (WT lines 10, 11, 12, 13, and 14 and mutant lines 18, 20, 21, 22, and 24) for 6-d-old seedlings grown in continuous white light. In this case, the amount of total protein/seedling was about 4 times greater than in the etiolated seedlings. The accumulation of GUS activity/seedling was in the same range as in the etiolated seedlings for the mutant construct and about 900-fold higher in the WT construct (data not shown).

The Mutant Construct Fails to Respond to Phytochrome Induction

We next tested the phytochrome responsiveness of the introduced constructs. The results of such an experiment are shown in Figure 3 for 10 individual lines for each construct. Figure 3A shows that each line with the WT construct was responsive to phytochrome action, with a single R illumination of 5-d-old etiolated seedlings causing an increase in GUS activity and an FR treatment given immediately after R substantially reversing the effect of R.

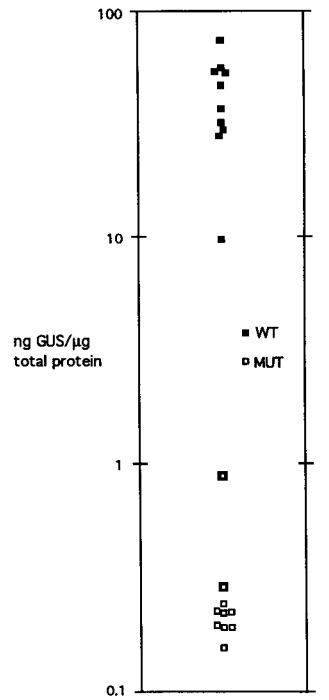

Figure 2. Relative GUS expression levels in plants transformed with WT and mutant (MUT) promoter constructs. GUS activity was assayed in plants that had been germinated and grown for 6 d in continuous D.

However, as shown in Figure 3B, the transgenic lines with the mutant construct showed no increase in GUS activity after the R treatment. These results are combined and shown in Figure 4 as averages for the 10 lines for each construct, normalized to the D level for each line. We conclude that, in addition to reducing the expression level, the 9-bp mutation abolished the ability of this promoter to confer responsiveness to phytochrome to the reporter gene, and these results suggest that the CA-1 binding region is essential for the normal phytochrome regulation of this gene.

We also tested the extent of the phytochrome induction of GUS RNA in one of the lines (line 10) transformed with the WT construct. Although the level of this RNA was much lower than the *Lhcb* RNA, presumably because the 1.15-kb promoter segment does not completely duplicate the function of the endogenous gene, we could detect it by RNA blot analysis. Quantitation on a phosphorimager (Molecular Dynamics, Sunnyvale, CA) showed that the GUS RNA was induced 4-fold after 4 h by a 2-min R treatment given to 6-d-old etiolated seedlings (data not shown).

The WT Construct Can Respond to B Acting on the Phytochrome System

To test whether B responsiveness of the *Lhcb1*3* gene might involve an entirely separate promoter region from the CA-1-binding region, we tested the GUS activities of transgenic plants with both the WT and mutant constructs

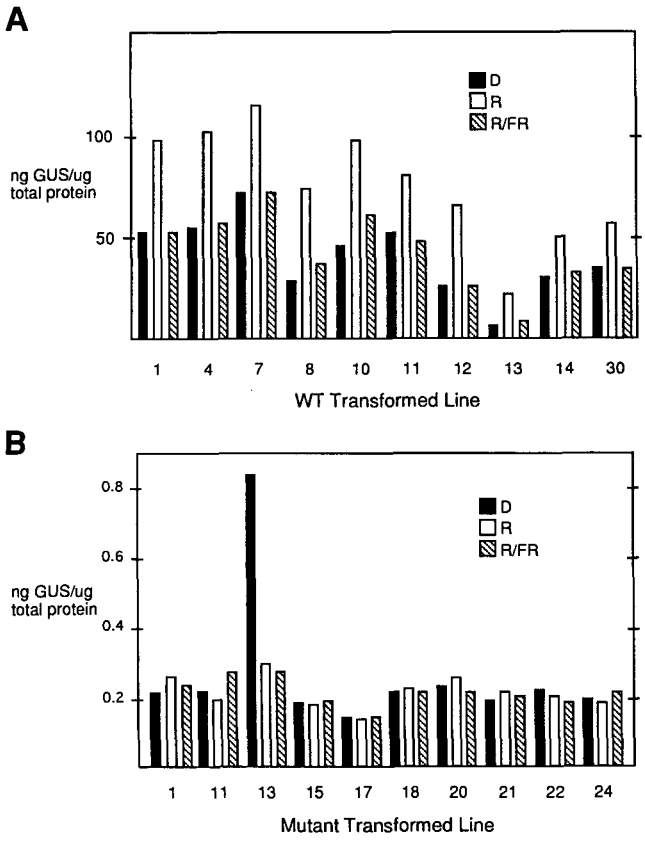

Figure 3. Phytochrome responsiveness of individual transformed lines. Individual lines (indicated by numbers at the bottom) containing either the WT (Fig. 3A) or mutant (Fig. 3B) promoter construct were grown in complete D for 5 d and then given 2 min of R or 2 min of R plus 10 min of FR (R/FR) or no light treatment (D). Plants were harvested and GUS activity levels assayed 18 h later.

after B treatment of etiolated seedlings. Figure 5 shows the results of these experiments. Treatment with B induced a significant increase in GUS activity in the plants transformed with the WT promoter construct, but the extent of the increase was less than that induced by R. Furthermore, following the B treatment immediately with FR reversed the effect of B, suggesting that the effect of the B treatment could be entirely accounted for by phytochrome. Thus, it was not surprising that the lines with the mutant promoter construct showed no increase in GUS activity in response to a B treatment.

DISCUSSION

We have found that a 27-bp region of the Arabidopsis *Lhcb1*3* promoter, extending from −126 through −100 relative to the start of transcription, is responsible for greatly increasing the activity of the promoter, and it is necessary for phytochrome responsiveness in vivo. The fact that the mutant promoter construct was expressed at a much lower level than the WT construct would not preclude the detection of a phytochrome response, because the GUS activity was high enough to be easily measured even in etiolated seedlings of the transformants containing the mutant construct. We have previously shown that this region is involved in binding CA-1 activity (Sun et al., 1993), and we conclude from the results presented here that CA-1 is likely to be an essential component for phytochrome regulation of this gene. It is possible that the identified region may interact with another region or regions of the promoter in conferring phytochrome responsiveness, but the testing of this hypothesis awaits further experiments.

The fact that the relative difference in GUS expression between D and R samples was less than the relative difference in *Lhcb1* RNA levels between such samples (Karlin-Neumann et al., 1988) is probably due to a combination of factors, including the developmental pattern of expression of *Lhcb1* genes observed during growth in D (Brusslan and

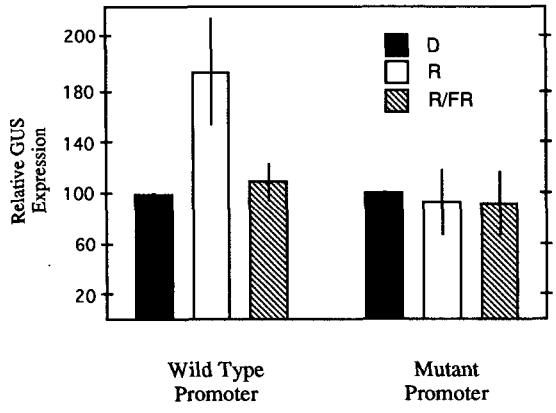

Figure 4. Average phytochrome responsiveness of 10 different WT and 10 different mutant lines. Values from the experiment shown in Figure 3 were normalized for each line to the level of GUS expression in the D samples. The averages of these normalized values for the 10 lines of each construct are shown, with the SD indicated as vertical lines for the R and R/FR values.

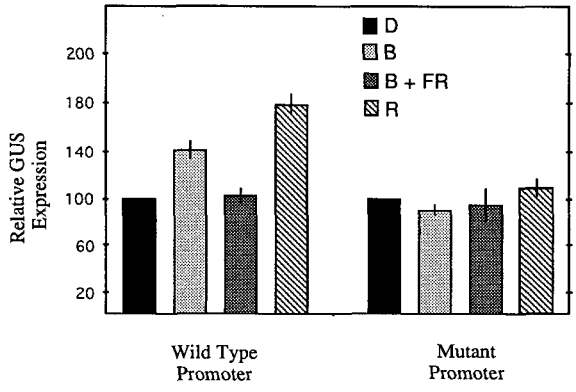

Figure 5. Average B responsiveness of five different WT and five different mutant lines. Normalized average values for the indicated treatments of five WT and five mutant lines were calculated as described for Figure 4. The SD is shown as vertical lines. Plants were grown in complete D for 5 d and then given 2 min of B, 2 min of R, 2 min of B plus 5 min of FR (B/FR), or no light (D) treatment. Plants were harvested and GUS activity levels assayed 14 h later.

Tobin, 1992) and the stability of GUS protein. We did detect a 4-fold R induction of the GUS RNA in 6-d-old etiolated plants; however, in similar plants assayed for GUS activity, the enzyme has accumulated to a substantial level during growth in D, and so the increase in response to R led to a lesser fold increase than seen for the RNA. Such a difference between GUS protein and RNA levels has also been seen by others (Wei et al., 1994).

The region affected by the mutation includes some sequence identity (7 of 10 nucleotides) with one of the regions (LS5) identified in a *Lemna Lhcb2* gene as necessary for phytochrome regulation (Kehoe et al., 1994). However, it seems to function differently than the LS5 region in that it has a strong enhancing function, whereas the LS5 region apparently contained sequences involved in repressing transcription in D. Therefore, it is possible that the two species use similar sequence elements in different ways. Alternatively, multiple regulatory elements may be affected in mut 1, and the region could include both an enhancing element and an element involved in repressing activity in D.

Because it is possible that B and phytochrome responses may share some elements of signal transduction (Liscum and Hangarter, 1994), it was of interest to test whether the mutant construct had also lost B responsiveness. However, we were not able to observe a B receptor-mediated response of the WT promoter construct at the level of GUS activity. Gao and Kaufman (1994) reported a B response for RNA transcribed from this gene. It is possible either that the region responsible for B responsiveness lies upstream of −1150 or that the effect of light absorbed by a B receptor (other than by phytochrome itself) is too small to be detected by changes in GUS activity.

Because the mutation that abolished phytochrome responsiveness also abolished CA-1 binding (Sun et al., 1993), our results suggest the possibility that the CA-1 activity is a component of the phytochrome signal transduction pathway leading to increased transcription of this gene. The results presented here do not support the idea that the CA-1 activity has a repressing function, as proposed previously by Sun et al. (1993) and based on the finding that the *det1* mutant lacked CA-1 activity. However, we recently examined CA-1 activity in a null allele of *det1* and found that it does have CA-1 activity (Z.-Y. Wang and E.M. Tobin, unpublished data). The *det1*-1 allele used in the earlier work may differ in this activity because it may make a truncated version of the WT DET1 protein (Pepper et al., 1994). Our full understanding of these disparate observations awaits further experiments.

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