DE1, a 12-Base Pair cis-Regulatory Element Sufficient to Confer Dark-inducible and Light Down-regulated Expression to a Minimal Promoter in Pea*

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We found a cis-regulatory element of 12 base pairs (bp) (GGATTTTACAGT) capable of conferring light responsiveness to a minimal promoter, CaMV 35S46, in pea. The 12-bp sequence is located in the 5′ upstream region of the light down-regulated gene pra2, which encodes a small GTPase belonging to the YPT/rab family. Here we examined gain-of-function analyses using synthetic promoter-luciferase constructs in a transient assay and found that the 12-bp element alone was sufficient to confer dark induction, as well as light down-regulation on the minimal promoter. We named this dark inducible element DE1. Effects of various light conditions on the reporter gene activity showed that DE1 received signals from phytochrome A, phytochrome B, and blue light photoreceptors. Using phytochrome-deficient mutants, we showed that the pra2 protein level in seedlings was indeed regulated by various light signals. Previously we had reported that a small GTPase gene in pea, pra2, which belongs to YPT/rab family (11), is one of the genes whose expression is down-regulated by phytochrome (12). The pra2 gene is mainly expressed in the growing zone of etiolated epicotyls, and its expression is repressed when the plant is illuminated (10, 13). Because small GTPases are molecular switches that are turned on by GTP and off by the hydrolysis of GTP to GDP, and members of YPT/rab family play important roles during intracellular transport, we propose that pra2 protein may participate in vesicle transport during stem elongation of etiolated seedlings (13). We were interested in the down-regulation of the pra2 gene, and we surveyed cis-elements located in the 5′ upstream region using a reporter gene in a transient assay (10). We found that a 12-bp sequence in the 5′ upstream region of this gene was important for the light down-regulation of the reporter gene and that this response was mediated by phytochrome B. However, we do not know whether the 12-bp element alone confers light down-regulation to a minimal promoter and whether the 12-bp element receives signals from photoreceptors other than phytochrome B. To answer these questions, we have examined the role of the 12-bp element by gain-of-function analysis in a transient assay to determine the effect of light conditions on the reporter gene. We found that the 12-bp element was sufficient to confer light down-regulation to a minimal promoter and capable of receiving signals from various photoreceptors. We named this dark inducible element DE1.

Because the DE1 has a capacity to respond to various light signals, there is a possibility that expression of the pra2 gene is indeed regulated by various light signals. Previously we had only shown the involvement of phytochrome B (10). Here we used mutants to examine the involvement of other photoreceptors in the regulation of pra2 expression. In Arabidopsis, 5 phytochrome genes (PHYA–PHYE) have been isolated, and it has been revealed that each phytochrome has distinct but at times overlapping functions (14). Many photomorphogenic mutants have been isolated (15, 16) and shown to be powerful tools for investigating light-regulated expression (17, 18). In pea, have been reported, there is no report of a cis-element that is sufficient to confer light responsiveness by itself to a minimal promoter (3). Compared with up-regulation, the mechanism for light down-regulation of transcription is even less clear. Some genes down-regulated by light have been identified such as AS1 (4), ATHB-2 (5), NPRs (6), TUB1 (7), and PHYA (8), but only a few cis-elements for down-regulation have been reported (8–10), and the details remain unclear.

Previously we have reported that a small GTPase gene in pea, pra2, which belongs to YPT/rab family (11), is one of the genes whose expression is down-regulated by phytochrome (12). The pra2 gene is mainly expressed in the growing zone of etiolated epicotyls, and its expression is repressed when the plant is illuminated (10, 13). Because small GTPases are molecular switches that are turned on by GTP and off by the hydrolysis of GTP to GDP, and members of YPT/rab family play important roles during intracellular transport, we propose that pra2 protein may participate in vesicle transport during stem elongation of etiolated seedlings (13). We were interested in the down-regulation of the pra2 gene, and we surveyed cis-elements located in the 5′ upstream region using a reporter gene in a transient assay (10). We found that a 12-bp sequence in the 5′ upstream region of this gene was important for the light down-regulation of the reporter gene and that this response was mediated by phytochrome B. However, we do not know whether the 12-bp element alone confers light down-regulation to a minimal promoter and whether the 12-bp element receives signals from photoreceptors other than phytochrome B. To answer these questions, we have examined the role of the 12-bp element by gain-of-function analysis in a transient assay to determine the effect of light conditions on the reporter gene. We found that the 12-bp element was sufficient to confer light down-regulation to a minimal promoter and capable of receiving signals from various photoreceptors. We named this dark inducible element DE1.

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several phytochrome mutants has been isolated. The *pcd1* and *pcd2* mutants block steps leading to the formation of the phytochrome chromophore, phytochromobilin (19, 20), whereas the *fun1* and *lv* mutants are deficient in phytochrome A and phytochrome B apoproteins, respectively (21, 22). We have used these mutants to investigate whether photoreceptors, in addition to phytochrome B, are involved in the expression of the *prap2* protein and whether DE1 confers light down-regulation as expected.

In this paper, we show that the DE1 is indeed a single light-responsive element and actually received signals from various photoreceptors.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Growth Conditions**—For the protein level measurements, pea seeds (*Pisum sativum* L.) were soaked in water and sown on irrigated rock wool. Seedlings were grown in the dark for 5 days at 25 °C. Before light treatment, the epicotyl between 0 and 1 cm from the apical hook was marked by pen under a dim green safe light. Plants were then irradiated with brief red (2 min) or far-red (5 min) light, returned to darkness, and kept for 12 h. In the experiments to examine the high irradiance response (HIR), plants were continuously irradiated with the appropriate light for 12 h. After irradiation, the stem length was measured. Proteins were extracted from the upper 1 cm portion of the epicotyl.

For the transient expression assay, pea seeds were imbibed and sown individually in plastic pots. Seedlings were grown in the dark for 5 or 6 days at 25 °C. Seedlings were then placed horizontally in the bombardment device (Model GIE-III, Tanaka Co. Ltd.). The bombardment procedure was as described by Inaba et al. (10). The gold particles were coated with a mixture of two kinds of plasmids, a plasmid containing one of the synthetic promoter-luciferase (LUC) constructs and a plasmid containing a 35 S-GUS construct as the internal standard. All steps during bombardment were performed in a darkroom under a dim green safe light. After bombardment, the seedlings were illuminated by the appropriate light conditions.

**Construction of Synthetic Promoter**—The 35S46-LUC vector was created by inserting the 35S46 promoter into the promoterless pBI221-LUC vector. The 35S46 promoter was created by PCR. The oligonucleotide, 5'–TGAAGGAATTTTACAGTGAATTTGAGG-3', was deleted from the 12-bp sequence, GGATTTTACAGT, involved in phytochrome *cis*-regulatory element, a 12-bp element. The length of exposure to red light was 2 min at 30.5 °C. After irradiation, the stem length was measured. Proteins were extracted from the upper 1 cm portion of the epicotyl.

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**Light Sources**—We used light-emitting diodes (LEDs) as the light sources for continuous irradiation. LEDs used in the experiments were as follows: Red, STICK-mR LED (λ<sub>max</sub> = 660 nm at 30 μmol m<sup>−2</sup> s<sup>−1</sup>; TOKYO RIKI Co. Ltd., Tokyo, Japan); Far-red, STICK-mFR (λ<sub>max</sub> = 735 nm at 25 μmol m<sup>−2</sup> s<sup>−1</sup>); Blue, STICK-mLED (λ<sub>max</sub> = 470 nm at 25 μmol m<sup>−2</sup> s<sup>−1</sup>). Equipment for the red/far-red reversible response was the same as described previously (10, 12). The length of exposure to red was 2 min at 30.5 μmol m<sup>−2</sup> s<sup>−1</sup>, and far-red was 5 min at 36.5 μmol m<sup>−2</sup> s<sup>−1</sup>.

**Extraction of Protein and Immunoblotting**—Total protein for immunoblotting was extracted by grinding tissue in a mortar and pestle on ice with sand together with 0.3 ml of buffer and 3 stem pieces (between 0 and 1 cm below the apical hook). The buffer contained 125 mM Tris-HCl (pH 6.8), 6% SDS, and 20% glycerol. The mixture was heated at 100 °C for 3 min and centrifuged. The supernatant protein was separated by SDS-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, probed with monoclonal IgG against *prap2* protein (13) and goat anti-mouse IgG conjugated to peroxidase (Bio-Rad, and developed with an ECL kit (Amersham Pharmacia Biotech).

**Measurement of Enzyme Activity**—The stem of the bombarded pea seedling (between 0 and 1 cm below the apical hook) was ground in liquid nitrogen using a chilled mortar and pestle. The powder was dispensed into a microcentrifuge tube and mixed with 300 μl of the buffer, which consists of 100 mM potassium phosphate (pH 7.8), 1 mM dithiothreitol, 1% Triton X-100, and 1 mM EDTA and then centrifuged at 15,000 g at 4 °C for 5 min. The supernatant was frozen at −80 °C until the enzyme assay was conducted. Luciferase assays were performed as described by Miller et al. (23) using the Pica Gene luciferase assay kit (Wako, Osaka, Japan). Photon emission derived from LUC activity was counted by AUTO LUMAT LB953 (Berthold, Bad Wildbad, Germany). GUS assays were conducted using the method of Jefferson et al. (24), with some modification (25). 4-Methylumbelliferone solutions (2.5 mM in 0.2 M Na2CO3) were used as standards. All LUC values were normalized to the corresponding GUS values. Samples of at least 6 bombardments were independently assayed for each construct.

**RESULTS**

**The 12-bp Element, DE1, Was Sufficient to Confer Light Down-regulation**—Previously, we identified a cis-element, a 12-bp sequence, GGATT TTACAGT, involved in phytochrome down-regulated expression of the *prap2* gene of pea (10). Gain-of-function analysis showed that the 93-bp sequence containing the 12-bp element could confer light down-regulation when fused to a heterologous, CaMV 35S90 promoter (califlower mosaic virus 35 S promoter of 90 bp from the transcriptional start site). The CaMV 35S90 promoter has the *as*-1 element, which interacts with the fused element and complicates interpretation of the results (3). It is necessary to confirm the role of the 12-bp element in light responsiveness using a minimal promoter, CaMV 35S46, that has 46 bp from the transcriptional start site and is a smaller version of CaMV 35S90. We constructed the reporter plasmid, pGF9, containing a nine-tandem repeat of the cis-element, CaMV 35S46, and the luciferase gene (Fig. 1). Using the plasmid pGF9 in a transient assay, we tested whether the 12-bp element was sufficient to confer light down-regulation of the minimal promoter in a red/far-red reversible manner. After bombardment, we placed the plants under a range of light conditions, before incubating them for 12 h in darkness, prior to measurement of their luciferase activity (Fig. 2A, pGF9). The expression of the reporter gene was highest in darkness (lane D). The brief red light treatment repressed the expression (lane R), and subsequent far-red light immediately after red light treatment reversed the effect (lane R/F). The 3 single-base pair mutated construct, pGF9M, in which the adenines in the 12-bp element were changed to cytosines, decreased to 20% of the level observed in the case of pGF9 in darkness and abolished the red/far-red reversible response. These results indicate that the 12-bp element is dark-inducible and sufficient to confer red/far-red reversible light down-regulation to the minimal promoter. When the nine tandem copies of the cis-element were fused to CaMV 35S46 promoter in the reverse direction, we could also observe red/far-red reversible response, indicating that the function of this element is independent of its direction (data not shown). We named this dark inducible element DE1.

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mophore biosynthesis (19, 20). The \textit{fun1–1} and \textit{lv-5} mutants are deficient in phytochromes A and B, respectively (21, 22). We exposed these mutants to various light conditions and examined the \textit{pra2} protein level by immunoblotting (Fig. 3). When grown in complete darkness, all plants expressed the \textit{pra2} protein (lane \textit{D}). Red/far-red light reversible down-regulation was observed in wild type and \textit{fun1–1} plants, but not in the \textit{lv-5} and \textit{pcd1pcd2} plants (Fig. 3, upper row). These results indicate that this LFR is not mediated by phytochrome A but is mediated by phytochrome B. The results from \textit{pcd1pcd2} plants show that chromophore biosynthesis is required for functional phytochrome B activity. This result is in agreement with expectations from our previous results (12, 13).

To examine whether photoreceptors other than phytochrome B are involved in \textit{pra2} expression, the effects of continuous irradiation were also examined in these mutants (Fig. 3, lower row). The effect of continuous red irradiation on \textit{pcd1pcd2} plants was different from that of wild type (lane \textit{Rc}), indicating that phytochromes mediate this red-HIR as expected. Red-HIR is usually mediated by phytochrome B but not by phytochrome A (14), and the result from the phytochrome A-deficient \textit{fun1–1} plants was in agreement with this suggestion. However, there was some down-regulation in the phytochrome B-deficient \textit{lv-5} mutant, suggesting some other phytochrome(s) may also play a role in the red-HIR. The effect of continuous far-red on mutant \textit{lv5} was similar to that on wild type. The expression by \textit{fun1–1} or \textit{pcd1pcd2} plants was reduced in comparison to wild type plants implicating phytochrome A in the far-red-HIR. The effect of continuous blue light on all three mutants was similar to that of wild type (lane \textit{Bc}), indicating that phytochromes are not involved in this response. Presumably blue light photoreceptors are involved in this response. Thus, the mutant studies indicate that light received by phytochrome A, phytochrome B, and blue-light receptors affected the expression of the \textit{pra2} protein.

Previously we observed that the \textit{pra2} protein level was correlated with stem elongation in etiolated pea seedlings (13). To test this observation for the mutants, we measured the epicotyl elongation (Fig. 4) and compared it with the \textit{pra2} protein level represented in Fig. 3 (lower row). Changes in stem elongation were correlated with the \textit{pra2} expression pattern, consistent with the proposition that the \textit{pra2} protein may be involved in stem elongation.

\textbf{Specific Response Mediated by the DE1 Was Diminished in Phytochrome Mutants—}It is important to demonstrate that the DE1 is capable of receiving signals from photoreceptors using the above-characterized mutants. We examined the effect of light on the reporter gene expression by the transient assay using the plasmid pGF9. If the DE1 actually receives signals from a photoreceptor, deficiency of the photoreceptor should reduce the specific response mediated by it. In Fig. 3, we showed that a phytochrome A-deficient mutant, \textit{fun1–1}, is deficient in the far-red-HIR, and a phytochrome B-deficient mutant, \textit{lv-5}, is deficient in the LFR for \textit{pra2} expression. Here, we tested the effect of continuous far-red light or brief red light on the reporter gene expression of \textit{fun1–1} and \textit{lv-5} plants. We used the activities in darkness as the induction control and activities in continuous blue light as the repression control, because both activities in the two mutants were similar to those of wild type plants (Fig. 3). In both mutants, the activities of the reporter gene were highest in darkness and repressed by blue light (Fig. 5, lanes \textit{D} and \textit{Bc}). In the phytochrome A-deficient mutant \textit{fun1–1}, response to continuous far-red light (lane \textit{Fc}) was somewhat attenuated, whereas a significant response to brief red light (lane \textit{R}) was retained. In the phytochrome B-deficient mutant \textit{lv-5}, the response to brief red light (lane \textit{R}) was atten-

\begin{figure}
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\caption{Effect of light on reporter enzyme activity in wild type plants. Synthetic promoter-luciferase constructs (pGF9 or pGF9M) were introduced into the growing region of etiolated pea epicotyl by particle bombardment with the 35S-GUS construct as the internal standard. The reporter enzyme activity was measured 12 h after the start of light irradiation. Relative activity was defined under “Experimental Procedures,” and the average of pGF9 in darkness was taken as 100. Values are the means of at least six independently bombarded samples, with error bars representing S.E.}
\end{figure}
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Fig. 3. Immunoblot analysis of the pra2 protein after irradiation with various wavelengths of light. Upper panel, six-day-old seedlings grown in darkness were irradiated with various light conditions. D, dark; Rc, continuous red; Fc, continuous far-red; Bc, continuous blue; R, brief red; R/F, brief far-red immediately after brief red. After 12 h total proteins from the upper 1 cm of the epicotyl were extracted, separated by SDS-polyacrylamide gel electrophoresis (12.5% gel), and probed with anti-pra2 protein IgG. 30 μg of protein were loaded in each lane. Lower panel, to confirm the equal loading of proteins, the gel was stained with Coomassie Brilliant Blue after the SDS-polyacrylamide gel electrophoresis (12.5% gel). WT, wild type.

Fig. 4. Stem elongation during light irradiation. The stem of 6-day-old seedlings was marked 1 cm below the apical hook before irradiation for 12 h. After irradiation elongation was measured. WT, wild type. D, dark; Rc, continuous red; Fc, continuous far-red; Bc, continuous blue.

Fig. 5. Effect of light on the reporter enzyme activity in mutant plants. The pGF9 construct was introduced into the growing region of etiolated pea shoots by particle bombardment with the 35 S-GUS construct as the internal standard. Plants were irradiated with various lights, and the reporter enzyme activity was measured after a 12-h incubation. D, dark; R, brief red; Fc, continuous far-red; Bc, continuous blue. Relative activity was defined in the legend to Fig. 2. Values are the means of at least six independently bombarded samples, with error bars representing S.E.

DISCUSSION

In this report, we have shown that a 12-bp element, DE1, could confer light responsiveness to a minimal promoter, CaMV 35S46, using a reporter gene in a transient assay and that the element received the signal from phytochrome A, phytochrome B, and blue light receptors (Fig. 2). These findings were confirmed by the use of phytochrome-deficient mutants, because the DE1 lost the ability to confer light responsiveness only for the specific light conditions relevant to that photoreceptor (Fig. 5). It seems probable that various photoreceptors transduce the light signals to the DE1. To date there are some reports for gain-of-function analysis of various cis-elements. Most experiments have been done using the CaMV 35S90 promoter that has the as-1 element. The GT-1 tetramer can confer light responsiveness to the CaMV 35S90 promoter, but not to the CaMV 35S46 promoter, probably because the as-1 element is needed to build a light-responsive module with the GT-1-binding site (3). In case of light down-regulation, the RE3 could confer light responsiveness to the modified CaMV 35S46 promoter, probably interacting with the B domain of the 35 S promoter (9). Thus, combinatorial interaction of light-respon-
sive elements determines the response characteristics of light-regulated promoters (18, 26). The smallest sequence unit sufficient to confer light responsiveness to the CaMV 35S46 promoter has been a 52-bp element of the CHS unit I (3, 27). This 52-bp element is long compared with the DE1. To our knowledge, DE1 is the first small cis-element reported that, by itself, confers light responsiveness to a minimal promoter, 35S46.

We propose that the cis-elements for light down-regulation should be divided into two groups. One is the repressor-binding site under light, and the other is the activator-binding site in darkness. Previously, we proposed that the 12-bp element regulates induction in darkness rather than repression by light (10). The present data show that introduction of the pGF9 element into the minimal promoter has been a 52-bp element of the CaMV 35S46, which itself, confers light responsiveness to a minimal promoter, 35S46.

Moreover, the possibility that DE1 is an activator-binding site in darkness. Previous gel mobility shift assays also suggested the hypothesis that the DE1 is an activator-binding site in darkness. Previously, we proposed that the 12-bp element regulates induction in darkness rather than repression by light (10). The present data show that introduction of the pGF9 element into the minimal promoter has been a 52-bp element of the CaMV 35S46, which itself, confers light responsiveness to a minimal promoter, 35S46.

In conclusion, we have shown that a 12-bp cis-regulatory element, DE1, confers dark induction to a minimal promoter. Introduction of the DE1 into other species should reveal whether this element is functionally conserved between species.

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