Cis-Acting Elements Essential for Light Regulation of the Nuclear Gene Encoding the A Subunit of Chloroplast Glyceraldehyde 3-Phosphate Dehydrogenase in Arabidopsis thaliana

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We report the characterization of cis-acting elements involved in light regulation of the nuclear gene (GapA) that encodes the A subunit of glyceraldehyde 3-phosphate dehydrogenase in Arabidopsis thaliana. Our previous deletion analyses indicate that the -277 to -195 upstream region of GapA is essential for light induction of the β-glucuronidase reporter gene in transgenic tobacco (Nicotiana tabacum) plants. This region contains three direct repeats with the consensus sequence 5'-CAAATGAA(A/C)A-3' (Gap boxes). Our results show that 2-bp substitutions of the last four nucleotides (AA or GA) of the Gap boxes by CC abolish light induction of the β-glucuronidase reporter gene in vivo and affect binding of the Gap box binding factor in vitro. We have also identified an additional cis-acting element, AE (Activation Element) box, that is involved in regulation of GapA. A combination of a Gap box trimer and an AE box dimer can confer light responsiveness on the cauliflower mosaic virus 35S promoter containing the -92 to +6 upstream sequence, whereas oligomers of Gap boxes or AE boxes alone cannot confer light responsiveness on the same promoter. These results suggest that Gap boxes and AE boxes function together as the light-responsive element of GapA.

Transcription is one of the primary steps at which light regulates gene expression in plants (Giuliano et al., 1988; Gidoni et al., 1989; Gilmartin et al., 1990; Manzara et al., 1991; Quail, 1991). The most extensively studied light-regulated genes are the nuclear genes RbcS and Lhc from several plant species. Multiple cis-acting regulatory elements and trans-acting factors are known to be involved in light regulation of these genes (Kuhlemeier et al., 1987; Gilmartin et al., 1990, 1992; Thompson and White, 1991; Kehoe et al., 1994; Terzaghi and Cashmore, 1995). The cis-acting regulatory elements can be divided into two functional groups (Gilmartin et al., 1990): the LRE, which is absolutely required for light induction, and modulation elements, which modulate only the level of expression conferred by the LRE. The sequence located between -166 and -50 of the pea rbcS3A gene contains the best-characterized LRE (Green et al., 1987, 1988; Lam and Chua, 1990).

Two sequence motifs located within this region, box II and box III, are required for light responsiveness (Gilmartin et al., 1990; Lam and Chua, 1990). When a box II tetramer was fused to the promoter fragment 35S(-92), which contains -92 to +6 sequences of the 35S gene from cauliflower mosaic virus, the resulting chimeric promoter was able to confer light responsiveness on a reporter gene in transgenic tobacco (Nicotiana tabacum) plants (Lam and Chua, 1990). However, the box II tetramer was not able to confer light response on the 35S(-46) basal promoter, indicating that additional elements are required to interact with GT-1 to constitute a fully functional LRE. In addition, it has been shown that an rbcS3A promoter fragment with the sequences between -166 and +1 can confer light induction in mature plants but not in seedlings (Kuhlemeier et al., 1988), implying that different regulatory elements are utilized at different developmental stages, or that other factors are required to interact with GT-1 for full regulatory functions. Furthermore, it has been shown that elements different from box II are critical for light regulation of the Lhcb2 gene from Lemma gibba (Kehoe et al., 1994). Therefore, it is important to characterize how other photosynthetic genes are regulated by light and to determine regulatory elements for each gene.

We have been studying light regulation of two nuclear genes, GapA and GapB, that encode chloroplast GAPDH from Arabidopsis thaliana. In higher plants there are two chloroplast GAPDH isozymes, with subunit structures of A₄ and A₃B₂, which are key enzymes in the photosynthetic carbon fixation cycle (Cerff, 1982). In previous studies we have shown that the expression of these two genes is coordinately regulated by light at the transcriptional level in tobacco and A. thaliana (Shih and Goodman, 1988;...
Dewdney et al., 1993). In dark-treated, mature A. thaliana plants, continuous light is required to activate transcription of GapA and GapB genes (Dewdney et al., 1993; Conley and Shih, 1995). Our results show that this effect is mediated by a combination of phytochromes and the blue-light photoreceptor encoded by the HY4 gene (Conley and Shih, 1995). Deletion analysis indicated that the −277- to −195-bp region of the GapA gene and the −261- to −173-bp region of the GapB gene are necessary for light responsiveness (Conley et al., 1994; Kwon et al., 1994; Park, 1995). Analysis of the GapA upstream sequence revealed that three copies of a direct repeat with the consensus sequence 5'-CAATGAA(G/A)A-3' are located within this region. Deletion of one copy of this repeat from the GapA promoter reduced light induction by 6-fold, whereas deletion of all three copies of the direct repeats abolished light induction completely (Conley et al., 1994). Analysis of the GapB gene revealed that four copies of similar repeats with a consensus sequence of 5'-ATGAA(G/A)A-3' exist in the GapB promoter region (−261 to −173). Deletion of these repeats from the GapB promoter also abolished light induction completely (Kwon et al., 1994). These results strongly suggest that the repeat elements of 5'-ATGAA(G/A)A-3', designated as the Gap box, are essential for light induction of GapA and GapB genes of A. thaliana. However, we cannot rule out the possibility that, within these regions, sequences other than Gap boxes are also regulatory elements.

Here we report direct evidence that Gap boxes are essential for light regulation of the GapA gene. In addition, we have identified an additional cis-acting regulatory element, the AE box, which interacts with the Gap box to confer light responsiveness. Our data show that a combination of a Gap box trimer and an AE box dimer is sufficient to confer light responsiveness on the 35S(-92) promoter, which is normally not regulated by light.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Tobacco plants (Nicotiana tabacum SR1) were used for leaf disc transformation. Transgenic tobacco plants were cultivated in tissue culture boxes (Magenta GA-7, Sigma) or 3-inch-square plastic pots in an environmental chamber at 25°C under a 16-h light/8-h dark cycle. For enzyme assays, plants grown in tissue culture boxes were used. For light-regulation studies and gel-mobility shift assays, soil-grown plants, continuous light is required to activate transcription of GapA and GapB genes (Dewdney et al., 1993; Conley and Shih, 1995). Our results show that this effect is mediated by a combination of phytochromes and the blue-light photoreceptor encoded by the HY4 gene (Conley and Shih, 1995). Deletion analysis indicated that the −277- to −195-bp region of the GapA gene and the −261- to −173-bp region of the GapB gene are necessary for light responsiveness (Conley et al., 1994; Kwon et al., 1994; Park, 1995). Analysis of the GapA upstream sequence revealed that three copies of a direct repeat with the consensus sequence 5'-CAATGAA(G/A)A-3' are located within this region. Deletion of one copy of this repeat from the GapA promoter reduced light induction by 6-fold, whereas deletion of all three copies of the direct repeats abolished light induction completely (Conley et al., 1994). Analysis of the GapB gene revealed that four copies of similar repeats with a consensus sequence of 5'-ATGAA(G/A)A-3' exist in the GapB promoter region (−261 to −173). Deletion of these repeats from the GapB promoter also abolished light induction completely (Kwon et al., 1994). These results strongly suggest that the repeat elements of 5'-ATGAA(G/A)A-3', designated as the Gap box, are essential for light induction of GapA and GapB genes of A. thaliana. However, we cannot rule out the possibility that, within these regions, sequences other than Gap boxes are also regulatory elements.

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Construction of Promoter Deletion-Gus Fusions and Transgenic Tobacco Plants

Promoter deletions m18 and m19 were generated by PCR with appropriate primers and fused to the Gus coding region as described (Conley et al., 1994). Nucleotide sequences of the promoter regions of the resulting constructs were verified by the method of Sanger et al. (1977) using the Sequenase enzyme and reagents from United States Biochemical. Construction of various Gap box and AE box oligomers to m19 was as described by Park (1995).

The binary vectors containing the GapA promoter-Gus constructs were mobilized into Agrobacterium tumefaciens LBA 4404 by triparental mating (Bevan, 1984). The resulting A. tumefaciens strains harboring the binary vectors were used in leaf disc transformation of tobacco (Nicotiana tabacum) SR1. These transformed leaf discs were grown in a culture box containing Murashige and Skoog medium (Murashige and Skoog, 1962) plus 3% Suc, 0.5 μg/mL BA, 200 μg/mL carbenicillin, and 100 μg/mL kanamycin to select for and regenerate transgenic plants. These transgenic plants were transferred into a soil mixture and grown in environmental chambers under a 16-h light/8-h dark cycle at 25°C. The conditions for light and dark treatment were described in detail by Conley et al. (1994).

Gus Assays

Gus activities of 15 independent transgenic tobacco plants for each construct were assayed using the fluorometric assay according to the method of Jefferson et al. (1987). Gus activities were determined by quantifying the enzymatic conversion of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone using a spectrofluorimeter (TK100, Hoefer Scientific Instruments, San Francisco, CA).

Quantitative RT-PCR

Total RNA from leaves of transgenic tobacco plants was isolated by a modification of the procedures of Sharrick and Quail (1989) and is described in detail by Conley et al. (1994). RNA samples were treated twice with DNase I to deplete contaminating genomic DNA (Simpson et al., 1992).

First-strand cDNA was synthesized in a 20-μL reaction mixture containing 2 μg of RNA, 0.08 μg of synthetic primer, 5'-ACAGTCTTGCGCGACATGCG-3' (primer 2), which corresponds to nucleotide sequences of the Gus antisense strand, 40 μM of each of the four deoxynucleotides, 200 units of RT, and 20 units of RNase. The reaction was carried out at 37°C for 30 min and stopped by heating to 65°C for 10 min. The resulting cDNAs were then amplified using PCR in a 100-μL reaction mixture containing an additional 0.25 μg of primer 2, 0.33 μg of primer 1, 5'-ACGTCCCTG AGAAACCCCAA-3' (which corresponds to Gus sense strand) 3 × 10⁶ cpm 32P-end-labeled sense primer, 10 μL of 10X Taq DNA polymerase buffer, 10 μL of 2.5 mM MgCl₂, 250 μM of each of the four deoxynucleotides, variable amounts of competitor DNA, and 2.5 units of Taq DNA polymerase. A 173-bp internally deleted Gus DNA fragment was used as the competitor DNA. The PCR reaction was performed in a DNA Thermal Cycler (Perkin Elmer/Cetus) for 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. After the reaction, 66.6 μL of DNA sequencing gel-loading buffer (Sambrook et al., 1989) was added and the mixture was denatured by heating at 94°C for 10 min. Twenty microliters of the denatured sample was separated by electrophoresis on a denaturing gel con-
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Results
The Gap Boxes Are Essential for Light Regulation of the GapA Gene

We chose the consensus 5'-CAAATGAAGA-3' Gap box core sequence, designated as A1 (Conley et al., 1994), as the basis for further studies. Synthetic A1 trimer (A1T), A1 dimer (A1D), and A1 monomer (A1M), were linked to promoter fragment m19. The m19 fragment lacks the Gap boxes of the GapA promoter, but has an intact promoter proximal sequence (see Fig. 1). These fragments were fused to the Gus coding region of the binary vector pBl101 and the resulting constructs were used to generate transgenic tobacco plants (Bevan, 1984). Fifteen independent transgenic plants from each of these constructs were randomly selected to measure Gus activity in the leaves. Consistent with our previous results (Conley et al., 1994; Kwon et al., 1994), between 15 and 50% of the transgenic lines from each construct have no Gus activities. Among those that exhibit Gus activities, northern blot analysis indicates that relative Gus mRNA levels between light- and dark-treated plants from transgenic lines bearing the same construct are very similar (data not shown). Therefore, one transgenic line from each construct that expresses near-average Gus activity was chosen and propagated vegetatively to generate enough plants for quantitative RT-PCR analysis.

Total RNA from dark- or light-treated plants was isolated and analyzed by the competitive RT-PCR method (Siebert and Larrick, 1992; Simpson et al., 1992; Conley et al., 1994). The plasmid clone pAGus/Bs+, which contains an internally deleted Gus structural gene fragment, was used as the competitive template in PCR. The chosen primer pair (see "Materials and Methods") will produce 585- and 422-bp PCR products from Gus mRNA and competitor templates, respectively. In the first set of experiments, RNAs from dark- and light-treated transgenic plants from each of the constructs were amplified by RT-PCR with equal amounts of competitor included in each reaction (Fig. 2a). The amount of Gus mRNA present in each reaction can be calculated by comparing the relative intensity of the Gus cDNA band with the competitor band for each reaction and calibrating against a titration curve described by Conley et al. (1994) and Kwon et al. (1994). To confirm this quantitation, a second set of RT-PCRs was performed, in which the reactions shown in Figure 2a were repeated, except that competitor templates corresponding to the amounts of Gus mRNA estimated from Figure 2a for each reaction were used. In these experiments (Fig. 2b), the Gus cDNA band and the competitor band in each reaction exhibited about equal intensity, indicating that our calculations were fairly accurate. Combining results from these two experiments should allow us to accurately quantify levels of light induction in transgenic tobacco plants.

The relative Gus mRNA levels between light- and dark-treated plants shown in Figure 2c are the average of three independent experiments. We estimated that there is a <10% SE for the ratios of Gus mRNA levels between light- and dark-treated plants determined for each transgenic construct. Figure 2 shows that in transgenic plants containing the GapA promoter construct m18 (-277), in which the three Gap boxes are retained (Fig. 1), there is a 15-fold light induction on the Gus reporter gene expression. In contrast, in transgenic plants containing the promoter construct m19, which contains no Gap box (Fig. 1), the Gus mRNA levels in dark- and light-treated plants are very similar. Figure 2 also shows that there is a 7-fold light induction of Gus mRNA in A1T-m19 transgenic plants. In addition,
Figure 2. Measurement of light induction by RT-PCR. Transgenic tobacco plants containing various GapA promoter-Gus fusions (Fig. 1) were grown under different light conditions and subjected to RT-PCR analysis. a, Total RNAs (2 µg) from light-treated (L) and dark-treated (D) plants of each transgenic line were used for RT-PCR reactions with 0.006 pg of competitor DNA included in each reaction. b, The same as in a, except that different amounts of competitor DNA are used for each reaction. For reactions shown in b, amounts of competitor DNA used are shown at the bottom of each lane. The arrows indicate the positions of PCR products for Gus mRNA (mRNA) and competitor templates (AGUS). c, Gus mRNA levels were quantified and plotted as the ratio between L and D Gus mRNA levels, as determined by RT-PCR reactions in a and b, from light- and dark-treated plants of each transgenic line.

There are 5- and 4-fold light induction in Al-D-m19 and Al-M-m19 plants, respectively. These results confirm the involvement of Gap boxes in light induction of GapA and the correlation of the copy number of Gap boxes with the extent of light induction.

Mutations in Gap Boxes Affect Light Induction In Vivo and Binding of GAPF In Vitro

To examine the effects of mutations on the function of Gap boxes, four modified synthetic GapA trimers, A1 to A4 (Fig. 3), in which two nucleotides in each Al box are changed, were generated in vitro. These DNA fragments were then linked to m19 (Fig. 1). Binary vectors linking these chimeric promoter fragments to the Gus coding region were constructed and used to generate transgenic tobacco plants. Gus mRNA levels of these transgenic plants grown under different light conditions were measured by RT-PCR (Fig. 3, b and c). The data show that replacement of the AA (A4) or GA (A5) dinucleotide within the core sequence of the Gap box with CC abolishes light induction completely. In addition, replacement of AT (A2) or TG (A3) with CC reduces light induction slightly (from 7- to 4-fold). These results provide direct evidence that the Gap boxes are essential for light regulation of Gap genes in vivo.

We previously identified a nuclear factor, GAPF, that binds specifically to the Gap boxes within the upstream regions of GapA and GapB (Kwon et al., 1994). Gel-mobility shift assays were performed to determine whether mutations affecting light induction in vivo also affect binding of GAPF in vitro. Figure 4 shows that a DNA fragment containing the synthetic Al trimer (A1) binds as efficiently to
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Figure 4. Effects of Gap box mutations on binding of GAPF. ALRE (lanes 1 and 2) or A1 to A5 trimers (lanes 3–12) were used as probes in gel-mobility shift assays. Specific activities for all six probes are between 2.5 to 3 x 10^6 cpm/µg DNA. The odd-numbered lanes are reactions with no cell extracts added, and the even-numbered lanes are reactions with 5 µg of total cell extracts prepared from Arabidopsis included as the sources of DNA binding proteins. The positions of DNA fragments with or without bound proteins are indicated by B (bound) or F (free), respectively.

GAPF (lane 4) as the ALRE fragment that contains sequences between -277 and -195 of the Gap A promoter (lane 2). Similarly, binding of GAPF to A2T (lane 6) and A3T (lane 8), which affect light induction slightly in vivo (Fig. 3), was not affected (A2T) or only slightly reduced (A3T). In contrast, when a trimer containing the mutation A4T, which abolishes light induction in vivo, was used as a template, there was no binding of GAPF (lane 10). However, when the mutant trimer A5T (lane 12), which also affects light induction drastically in vivo, was used as a template, binding of GAPF was only slightly reduced. These results indicate that the effects of Gap box mutations on the binding of GAPF in vitro is not completely correlated with their effects on the promoter function in vivo. However, there is an additional, slower-migrating complex formed in reactions with ALRE and A1T as binding probes (lanes 2 and 4). The significance of this weak binding complex will be discussed later (see “Discussion”). Figure 4 also shows that in reactions with A3T or A5T as binding probes, in addition to the GAPF band, an additional, faster-migrating band appeared in each reaction (lanes 8 and 12). These binding activities cannot be abolished by the addition of an excess of pBluescript plasmid DNA in the reaction mixtures (data not shown). Because whole-cell extracts were used as the sources of binding proteins, we speculate that the dinucleotide replacement in A3T and A5T fortuitously created binding sites for proteins other than GAPF.

A Combination of Gap Box Trimer-AE Dimer Is Required to Confer Light Responsiveness on the 35S(-92) Promoter

The BLRE fragment that contains sequences between -263 to -152 of the Gap B promoter (Fig. 1), when fused to the 35S(-92) promoter fragment in either orientation, was able to confer light responsiveness on the Gus reporter gene in transgenic tobacco plants (Kwon et al., 1994). Surprisingly, we have found that a similar ALRE fragment (-277 to -195) that contains all three Gap boxes of the Gap A promoter (Fig. 1) cannot confer light inducibility on the 35S(-92) promoter (Fig. 5, a and b).

The observation that BLRE but not ALRE can function as a light-dependent, enhancer-like element was unexpected, since both fragments have been shown to contain elements that are essential for light regulation. However, we have found that a synthetic Gap box trimer is sufficient to replace the sequence between -263 and -173 of Gap B upstream sequences (Park, 1995), suggesting that the Gap boxes plus the sequences between -172 and -152 of Gap B (Fig. 1) are responsible for light responsiveness and that the element located between -172 and -152 of Gap B is missing from the ALRE. Comparison of nucleotide sequences suggested that a sequence motif 5'-AGAAACCT-3', designated as the AE (Activation Element) boxes, located between -164 and -158 of Gap B, is a candidate for such an element. There are two AE boxes located downstream from the last Gap boxes of the Gap A and Gap B promoters (Fig. 1). The two AE boxes of Gap A are located between -141 and -134 (AGAAAACT) and between -100 and -93 (AGAAAAA), whereas the second AE box (AGAAAACTT) of Gap B

Figure 5. Effects of Gap boxes on heterologous promoters. a, Gus mRNA levels from light-treated (L) or dark-treated (D) transgenic tobacco plants bearing various constructs were determined by RT-PCR. The reaction conditions were identical to those described in Figure 3. The constructs used are shown at the top of each lane. b, Quantitation of RT-PCR reactions from a. Shown are the relative Gus mRNA levels from light- and dark-treated plants for each transgenic
is located between −139 and −132. None of the AE boxes from GapA is included in the ALRE fragment.

To test whether the Gap box and the AE box are components of an LRE, transgenic plants containing various combinations of Gap boxes and AE boxes were made. RT-PCR analyses (Fig. 5) show that a construct linking three Gap boxes and two AE boxes to the 35S(−92) promoter, A1T–AE35S(−92), is able to confer a 4-fold light induction on the Gus reporter gene in transgenic plants. In contrast, in leaves from transgenic plants containing 35S(−92), there is no difference in Gus mRNA levels from light- or dark-treated plants. Figure 5 also shows that in transgenic plants containing construct linking A1 or a Gap box hexamer (A11) to the 35S(−92) promoter, there are no apparent differences between Gus mRNA levels in leaves of light- and dark-treated plants (Fig. 5). We have found that an AE box dimer alone is not sufficient to confer light responsiveness on the 35S(−92) promoter (Park, 1995). Taken together, these results show that a combination of the Gap box trimer and the AE box dimer is required to confer light responsiveness on the 35S(−92) promoter, which is not normally regulated by light. However, there is no detectable Gus mRNA in transgenic plants containing 35S(−46)–Gus or A1T–AE35S(−46)–Gus, regardless of the light condition (data not shown). We have screened more than 20 independent transgenic lines for each construct. This suggests that an element between −92 and −46 of the 35S promoter, which contains the binding site (as-1 site) for nuclear factor ASF-1 (Lam et al., 1989), is required for the Gap box and AE box to function. It has been shown that an as-1 tetramer can confer constitutive but not light-responsive activation to the 35S(−92) promoter (Lam and Chua, 1990). However, the as-1 site is also required to interact with a box II tetramer to confer light responsiveness (Lam and Chua, 1990).

Identification of the AE Box Binding Protein

To identify a nuclear protein that interacts with AE boxes, two DNA fragments, AE35 (a GapB upstream DNA fragment (−172 to −123)) that contains two AE boxes (−164 to −158, AGAAACCTT, and −138 to −132, AGAATCTT), and AE35, a synthetic AE dimer (AGAAACCTTGTAAAGAAACCTT) that has only three nucleotides between the two AE boxes, were used as probes in gel-mobility shift assays (Fig. 6). Each probe gave a prominent retarded band (lanes 2 and 7) when Arabidopsis whole-cell extracts were used as sources of DNA binding proteins. These binding activities disappeared when a 50-fold molar excess of unlabeled AE35 DNA fragment was included in the binding reactions (lanes 3 and 8). Similarly, when a 50-fold molar excess of unlabeled AE35 DNA fragment was included in the binding reactions, binding activities to AE35 (lanes 4) and AE35 (lane 9) were reduced substantially. In contrast, when excessive amounts of pBluescript plasmid DNA (4000× molar excess) were included in the binding reactions, these binding activities were reduced slightly (lane 5) or not affected (lane 10). These results suggest that the observed band shift represents specific DNA-protein interactions between AE boxes and a protein factor, designated as AEF, present in Arabidopsis whole-cell extracts. The data shown in Figure 6 further suggest that AEF binds to AE35 (lane 7) more efficiently than to AE35 (lane 2).

Gel-mobility shift assays were also used to investigate whether binding of AEF to AE boxes would be affected if AE boxes were included as the
competitor (Fig. 7). The results show that, when \( \text{AE}_5 \) was used as a probe for the binding of \( \text{AEF} \), a 50-fold molar excess of unlabeled \( \text{Al}_5 \) completely abolished the binding of \( \text{AEF} \) to \( \text{Al}_5 \) (lanes 2 and 3). Figure 7 also shows that, when used as a probe, the Gap box trimer \( \text{Al}_5 \) gave a major GAPF binding complex and a minor, slower-migrating complex (lane 5). However, when a 50-fold molar excess of unlabeled \( \text{AE}_5 \) was included in the reaction mixtures, the formation of the major GAPF binding complex was reduced only slightly, whereas the formation of the slower-migrating complex was abolished (lane 6).

**DISCUSSION**

We have provided direct evidence that Gap box elements are essential for light regulation of GapA gene expression. The GapA promoter construct \( \text{m18} \) (−277), in which the region upstream from the Gap boxes is deleted, confers a 15-fold light induction on the Gus reporter gene expression in transgenic tobacco plants. However, another promoter construct, \( \text{m19} \) (−195), in which only the sequence proximal to the Gap boxes remains, lacks the ability to confer light inducibility. Addition of a synthetic Gap box trimer (\( \text{Al}_5 \)), dimer (\( \text{Al}_2 \)), or monomer (\( \text{Al}_1 \)) to \( \text{m19} \) results in a 7-, 5-, or 4-fold light induction of the Gus reporter gene (Fig. 2). Although these results confirm the importance of Gap boxes in light regulation of GapA, they indicate that the addition of Gap box oligomers to \( \text{m19} \) is not sufficient to restore the 15-fold light induction observed in plants containing the promoter construct \( \text{m18} \) (Fig. 1). There are two possible explanations for these results. First, in addition to the Gap boxes, additional elements located between −277 and −195 of the GapA promoter may be needed for full induction. Second, the three Gap boxes are the only elements within this region required for light induction, but the spacing between Gap boxes in the synthetic oligomer is not optimal. We favor the first possibility, since we have observed that the addition of \( \text{Al}_5 \) to the GapB promoter construct \( \text{B19} \) (−175), which contains a deletion that removes all of the Gap boxes of the GapB promoter (Fig. 1), can fully restore light inducibility (Park, 1995).

We have previously shown that BLRE, a 111-bp GapB promoter fragment containing four Gap boxes, is sufficient to confer light inducibility on the 3S5(−92) promoter (Kwon et al., 1994). However, a similar GapA promoter fragment, ALRE (−277 to −195), containing the three Gap boxes, fails to confer light responsiveness on the 3S5(−92) promoter (Fig. 5). We found that an additional \( \text{cis} \) element, AE box, which is contained in BLRE but not ALRE, is required to interact with Gap boxes for light responsiveness. The presence of the AE box and light inducibility of the promoters are correlated. The BLRE fragment containing one copy of the AE box can confer light regulation in the 3S5(−92)/Gus construct, whereas the ALRE fragment, which does not have the AE box, does not function (Fig. 5). Furthermore, we have shown that a combination of a synthetic Gap box trimer and an AE box dimer (\( \text{Al}_5 \)-\( \text{AE}_2 \)) can confer light responsiveness on the 3S5(−92) basal promoter in transgenic tobacco plants, whereas oligomers of either Gap boxes or AE boxes alone are not sufficient to confer light responsiveness on the same construct (Fig. 5). This gain-of-function result provides strong evidence that both Gap boxes and AE boxes are essential components of LRE in light regulation of the GapA gene. However, the fact that a combination of the Gap box and AE box is not sufficient to confer light responsiveness on the 3S5(−46) promoter suggests that an additional \( \text{as}-1 \)-like element (Lam et al., 1989) is needed to constitute a fully functional LRE.

Site-specific mutagenesis analysis of the consensus Gap box sequences (5′-CAATGAAAGA-3′) revealed that the last four nucleotides, AAGA, are critical for Gap box function in vivo. The mutated Gap box trimers in which two of the last four nucleotides, either AA or GA, were substituted by CC (\( \text{A4}_5 \) and \( \text{A5}_5 \)) do not restore light inducibility to the m19-Gus construct. In contrast, \( \text{A2}_5 \) and \( \text{A3}_5 \), in which two of the first four nucleotides of the Gap boxes, either AA or TG, were changed to CC, can restore light inducibility to 50% of levels for the \( \text{Al}_5 \) trimer (Fig. 3). Gel-mobility shift assays show that, in general, mutations in Gap boxes affecting light induction in vivo also affect binding of GAPF to Gap boxes in vitro (Fig. 4), with the exception of the mutation in the last two nucleotides (\( \text{A5}_5 \)) of the Gap box. When \( \text{A5}_5 \) was used as a template, binding of GAPF was only slightly affected, although it abolishes light induction completely in vivo. Two possibilities can be offered to interpret these results. First, binding of GAPF to Gap boxes in vitro is not relevant to the promoter function of the GapA gene, and other nuclear factors are responsible for interacting with Gap boxes in vivo to confer light regulation. Second, binding activities of GAPF to Gap boxes are functionally related to the GapA promoter function in vivo, and additional protein factors are required to interact with GAPF to confer light responsiveness. In this view the mutation in \( \text{A5}_5 \), although not affecting the binding of GAPF to Gap boxes drastically, will not allow proper interaction between GAPF and the additional protein factors that are essential for light responsiveness. Experiments designed to distinguish these two possibilities are currently underway.

In our previous work, we have shown that the protein factor GAPF binds specifically to Gap boxes (Kwon et al., 1994). In this report we have identified another protein factor, AEF, that interacts specifically with AE boxes (Fig. 6). Two observations suggest that GAPF and AEF are two distinct proteins. First, the core sequence for Gap boxes and AE boxes are completely different; second, and more importantly, only a combination of a Gap box trimer and an AE box dimer, but not an oligomer of Gap boxes or AE boxes, can confer light responsiveness on the 3S5(−92) promoter. However, experiments presented in Figure 7 raise the question of whether GAPF and AEF are indeed distinct factors. The results show that when \( \text{AE}_2 \) was used as a probe for the binding of AEF, a 50-fold molar excess of unlabeled \( \text{Al}_5 \) completely abolished the binding of AEF to \( \text{AE}_2 \) (Fig. 7, lane 3). In contrast, when an excess of unlabeled \( \text{AE}_2 \) was included in the reaction mixtures, binding of the GAPF to \( \text{Al}_5 \) was only slightly affected (Fig. 7, lane 6). An obvious explanation would be that the same protein factor can bind to both \( \text{Al}_5 \) and \( \text{AE}_2 \) in vitro, with much
better affinity to A1p. Alternatively, these results can be explained by proposing that there is a direct interaction between GAPF and AEF and that the affinity for the interaction between GAPF and AEF is stronger than the affinity for the binding of AEF to A2p. We noticed that, in addition to the GAPF complex, A1p gave an additional minor, slower-migrating complex (Figs. 4 and 7). An excess of unlabelled A2p in the binding reactions can compete only for the formation of the slower-migrating complex but not the GAPF complex (Fig. 7, lane 6). These data are consistent with the possibility that the interaction between GAPF and AEF does occur and that the slower-migrating complex may contain both GAPF and AEF. These possibilities can be distinguished when purified GAPF and AEF become available for determination of the primary binding site for each factor.

We have shown previously that the GAPF is distinct from the GT-1 factor (Kwon et al., 1994), which binds specifically to box II within the LRE of the rbcS3A gene from pea (Green et al., 1987, 1988; Lam and Chua, 1990). We showed that excessive amounts of the box II tetramer did not compete with the binding of ALRE or BLRE to the GAPF. Conversely, the binding of GT-1 to the box II tetramer is dominated by an excess of unlabelled ALRE or BLRE (Kwon et al., 1994). There are two major differences between LREs of GapA and rbcS3A. First, in addition to the as-1-like element, the LRE of GapA requires two additional elements in vivo, whereas the LRE of rbcS3A requires only one element, box II (or GT-1 binding site) (Gilmartin et al., 1990). Second, we have found that m18, the construct with Gap boxes and AE boxes intact (Fig. 1), is capable of conferring light responsiveness and organ-specificity on the Gus reporter gene in both seedlings and mature plants. GapA and AE boxes intact (Fig. 1), is capable of conferring light responsiveness and organ-specificity on the Gus reporter gene in both seedlings and mature plants. These results can be explained by proposing that there is a direct interaction between GAPF and AEF and that the affinity for the interaction between GAPF and AEF is stronger than the affinity for the binding of AEF to A2p. We noticed that, in addition to the GAPF complex, A1p gave an additional minor, slower-migrating complex (Figs. 4 and 7). An excess of unlabelled A2p in the binding reactions can compete only for the formation of the slower-migrating complex but not the GAPF complex (Fig. 7, lane 6). These data are consistent with the possibility that the interaction between GAPF and AEF does occur and that the slower-migrating complex may contain both GAPF and AEF. These possibilities can be distinguished when purified GAPF and AEF become available for determination of the primary binding site for each factor.

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LITERATURE CITED


Light-Responsive Elements of Arabidopsis Glyceraldehyde 3-Phosphate Dehydrogenase Genes


