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# Identification of a Light-Responsive Region of the Nuclear Gene Encoding the B Subunit of Chloroplast Glyceraldehyde 3-Phosphate Dehydrogenase from *Arabidopsis thaliana*[1]

**Hawk-Bin Kwon, Soo-Chul Park, Hsiao-Ping Peng, Howard M. Goodman, Julia Dewdney, and Ming-Che Shih***

Department of Biological Sciences, University of Iowa, Iowa City, Iowa 52242 (H.-B.K., S.-C.P, H.-P.P., M.-C. S.); and Department of Genetics, Harvard Medical School and Department of Molecular Biology, Massachusetts General Hospital, Boston, Massachusetts 02114 (H.M.G., J.D.)

**We report here the identification of a *cis*-acting region involved in light regulation of the nuclear gene (*GapB*) encoding the B subunit of chloroplast glyceraldehyde 3-phosphate dehydrogenase from *Arabidopsis thaliana*. Our results show that a 664-bp *GapB* promoter fragment is sufficient to confer light induction and organ-specific expression of the *Escherichia coli* β-glucuronidase reporter gene (*Gus*) in transgenic tobacco (*Nicotiana tabacum*) plants. Deletion analysis indicates that the −261 to −173 upstream region of the *GapB* gene is essential for light induction. This region contains four direct repeats with the consensus sequence 5′-ATGAA(A/G)A-3′ (Gap boxes). Deletion of all four repeats abolishes light induction completely. In addition, we have linked a 109-bp (−263 to −152) *GapB* upstream fragment containing the four direct repeats in two orientations to the −92 to +6 upstream sequence of the cauliflower mosaic virus 35S basal promoter. The resulting chimeric promoters are able to confer light induction and to enhance leaf-specific expression of the *Gus* reporter gene in transgenic tobacco plants. Based on these results we conclude that Gap boxes are essential for light regulation and organ-specific expression of the *GapB* gene in *A. thaliana*. Using gel mobility shift assays we have also identified a nuclear factor from tobacco that interacts with *GapA* and *GapB* DNA fragments containing these Gap boxes. Competition assays indicate that Gap boxes are the binding sites for this factor. Although this binding activity is present in nuclear extracts from leaves and roots of light-grown or dark-treated tobacco plants, the activity is less abundant in nuclear extracts prepared from leaves of dark-treated plants or from roots of greenhouse-grown plants. In addition, our data show that this binding factor is distinct from the GT-1 factor, which binds to Box II and Box III within the light-responsive element of the *RbcS*-3A gene of pea.**

In addition to being the energy source for photosynthesis, light is required as a signal for transcription of many photosynthetic genes in higher plants (for reviews, see Tobin and Silverthorne, 1985; Gilmartin et al., 1990). Among these light-regulated genes, *RbcS* and *Lhc* genes have been studied the most (Dean et al., 1985; Giuliano et al., 1988; Gidoni et al., 1989; Gilmartin et al., 1990; Manzara et al., 1991; Quail, 1991). Results from these studies indicate that specific interactions between *cis*-acting elements and nuclear factors are required to confer light responsiveness (Gilmartin et al., 1990; Schindler and Cashmore, 1990; Manzara et al., 1991). These studies also showed that *cis*-acting regulatory elements for light responsiveness are located within the 5′ upstream regions of many light-regulated genes (Giuliano et al., 1988; Donald and Cashmore, 1990; Gilmartin et al., 1990). In most cases, multiple DNA elements are required for efficient light control, implying that multiple protein factors interact to achieve transcriptional activation. However, even among members of the *RbcS* gene family from the same species, not all of these *cis*-acting elements are conserved (Manzara et al., 1991). In addition, studies of the pea *RbcS*-3A gene indicated that different *cis*-acting regulatory elements may be involved in light regulation during different developmental stages (Kuhlemeier et al., 1988). The studies of nuclear factors for light responsiveness suggest that two types of DNA-binding proteins are involved: those that interact with the LRE in the upstream region of photosynthetic genes and those that modulate the level of expression conferred by the LRE (Gilmartin et al., 1990). The GT-1 factor (Green et al., 1987, 1988; Lam and Chua, 1990) that binds to Box II and Box III of the *RbcS*-3A gene promoter of pea appears to belong to the former class, whereas ASF-2 (Lam and Chua, 1989) and 3AF1 (Lam et al., 1990) may belong to the latter. These observations raise the question of whether genes that are similarly regulated by light share common *cis*- and *trans*-acting regulatory elements.

We have previously shown that *GapA* and *GapB* from *Arabidopsis thaliana* are regulated by light (Dewdney et al., 1993). Kinetic analysis of mRNA accumulation during light induction suggested that these two genes and the *RbcS* genes are coordinately regulated by light in both tobacco (*Nicotiana tabacum*) and *A. thaliana* (Shih and Goodman, 1988; T.R.

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* Corresponding author; fax 1–319–335–3620.

Abbreviations: CaMV, cauliflower mosaic virus; *GapA* and *GapB*, nuclear genes encoding A and B subunits, respectively, of chloroplast glyceraldehyde 3-phosphate dehydrogenase; GAPF, Gap box binding factor; *Gus*, gene that encodes the *Escherichia coli* β-glucuronidase; GUS, *E. coli* β-glucuronidase; *Lhc*, gene that encodes the light-harvesting chlorophyll *a*/*b*-binding protein; LRE, light-responsive element; *RbcS*, gene that encodes the small subunit of ribulose 1,5-bisphosphate carboxylase; RT, reverse transcriptase.

Conley and M.-C. Shih, unpublished data). We report here the characterization of *cis*-acting elements involved in light regulation of the *GapB* gene from *A. thaliana*. Our results show that a 0.66-kb *GapB* promoter fragment is sufficient to confer light inducibility and organ specificity in transgenic tobacco plants when *Gus* was used as the gene fusion marker. Sequence analysis indicates that there are four direct repeats with the consensus sequence 5′-ATGAA(A/G)A-3′ located between −236 and −180 in the *GapB* gene. Deletion of two copies of these repeats had no apparent effect on light induction of the *Gus* reporter gene in transgenic tobacco plants, but deletion of all four copies abolished light induction completely. In addition, we have fused a *GapB* promoter fragment (−263 to −152) that includes these four repeated elements to the DNA fragment containing the −92 to +6 sequence of the 35S promoter of the CaMV. The results show that this DNA fragment is able to confer an orientation-independent light responsiveness and to enhance leaf-specific expression of the −92(35S) basal promoter, which by itself is not regulated by light and can confer only root-specific expression. Also we report the identification of a nuclear factor from tobacco that binds specifically to the repeated elements within regions that are essential for light regulation of *GapA* and *GapB* genes of *A. thaliana*. This binding activity is present in nuclear extracts from leaves and roots of light-grown and dark-treated tobacco plants, although nuclear extracts prepared from leaves of dark-treated plants or from roots of light-grown plants show weaker binding activity. In addition, our data show that this binding factor is distinct from the GT-1 factor, which binds to the LRE of the *RbcS*-3A gene of pea.

## 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 (Percivall) 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, plants were grown in pots in soil mix. Light-treated samples were harvested in the middle of the light period. Dark-treated samples were harvested after 5 d in continuous darkness. All plants were harvested at similar developmental stages.

### Recombinant DNA Techniques and Sequencing

Standard enzymic manipulations and purification procedures were performed essentially as described by Sambrook et al. (1989). For DNA sequencing, a 2.5-kb DNA fragment including 1.8 kb of sequence 5′ to the *GapB* transcription start site was subcloned into pBS$^+$ (Stratagene) to generate pBS$^+$/GapBH1, and serial 5′ deletion mutants were generated by exonuclease III-S1 nuclease digestion. DNA sequencing was performed by the dideoxy sequencing method using a Sequenase kit (United States Biochemical).

### Deletion Mutants of the *GapB* Promoter Region

A *GapB* promoter (−664)/*Gus* fusion was constructed in pUC12 (pUC12/GapBH2-Gus). Site-directed mutagenesis was used to create an *Nco*I site in the translation initiation codon of *GapB* in pBS$^+$. A 700-bp *Hin*dIII-*Nco*I fragment from pBS$^+$/GapBH1 that contains the 664-bp promoter sequence and the 5′ untranslated region of the *GapB* gene was inserted into the *Nco*I site of pUC12/Gus, which has also had an *Nco*I site introduced into the translation initiation codon by site-directed mutagenesis (Yang et al., 1993), to create pUC12/GapBH2-Gus. To generate serial deletion mutants of the *GapB* promoter, pUC12/BH2-Gus was double digested with *Kpn*I and *Cla*I, and unidirectional deletion was performed with exonuclease III and S1 nuclease to create serial 5′ *GapB* promoter deletion mutants.

### Transgenic Tobacco Plants

The 2.3- to 3-kb *Hin*dIII-*Eco*RI fragments that included each of the *GapB* promoter deletion-*Gus* fusions were used to replace the *Hin*dIII-*Eco*RI *Gus* fragment of pBI101 (Jefferson et al., 1987). The resulting binary vectors, pBI101/GapB-Gus, were mobilized into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Bevan, 1984). Leaf discs of *N. tabacum* SR1 were transformed by *A. tumefaciens* harboring the pBI101/GapB-GUS vectors. Transformants and regenerated plants were selected on solid Murashige and Skoog medium containing 0.5 μg/mL 6-benzylaminopurine, 3% Suc, 100 μg/mL kanamycin, and 200 μg/mL carbenicillin. Regenerated plants were grown in tissue culture boxes or transferred to soil mix and grown in an environmental chamber at 25°C under a 16-h light/8-h dark cycle.

### RT-PCR Procedures

Total RNA was isolated by a modification of procedures of Sharrock and Quail (1989) and described in detail by Dewdney et al. (1993). For RT-PCR reactions, RNA samples were treated with DNase I twice to deplete contaminating genomic DNA (Simpson et al., 1992). Quantitation of the RNA blots was done by scanning autoradiograms with a Bio-Rad model 620 densitometer.

Two primers, 5′-ACGTCCTGTAGAAACCCCAA-3′ (primer 1) and 5′-ACAGTCTTGCGCGACATGCG-3′ (primer 2), which correspond to the nucleotide sequences of the *Gus*-coding strand and antisense strand, respectively, were synthesized for RT-PCR. First-strand cDNA synthesis was performed in a 20-μL reaction mixture containing 2 μg of RNA, 0.08 μg of primer 2, 40 μM each of the four deoxynucleotides, 200 units of Moloney murine leukemia virus RT, and 20 units of ribonuclease inhibitor. The reaction mixture was incubated at 42°C for 30 min and stopped by heating to 65°C for 10 min. For PCR, 20 μL of the RT reaction mixture was combined with an additional 0.25 μg of primer 2, 0.33 μg of primer 1, $1 \times 10^6$ cpm $^{32}$P-labeled sense primer, 10 μL of 10 × DNA polymerase buffer (500 mM Tris [pH 9.0], 500 mM NaCl), 10 mL of 25 mM $MgCl_2$, deoxynucleotides to final concentrations of 250 μM each, variable amounts of competitor DNA, and 2.5 units of Taq DNA polymerase. Final reaction volumes were brought up to 100 μL by addition

of $H_2O$. The PCR was performed in a Perkin-Elmer Cetus DNA thermal cycler for 24 cycles of 1 min at 94°C, 1 min at 55°C, and then 2 min at 72°C. Reaction samples were combined with 66.6 μL of DNA-sequencing gel-loading buffer (Sambrook et al., 1989). After the samples were mixed, they were denatured by heating at 94°C for 5 min, followed by separation by electrophoresis on a 4% polyacrylamide/8 M urea-denaturing gel. After electrophoresis, the gels were exposed to XAR-5 x-ray films (Kodak) for varying periods with intensifying screens at −70°C.

### Construction of pΔGus/BS$^+$

To construct a *Gus* plasmid clone with an internal deletion for use as a competitor template in RT-PCR, a 610-bp *Bam*HI-*Hin*cII fragment of the plasmid pUC19/Gus, which contains the N-terminal end of the *Gus*-coding sequence, was subcloned into the *Bam*HI-*Hin*cII site of pBS$^+$ (Stratagene) to create pGusH/BS$^+$. A 173-bp *Sna*BI-*Eco*RV fragment was then removed from pGusH/BS$^+$ to create pΔGus/BS$^+$.

### Fluorometric GUS Assay

Leaf tissues from 15 independent transformants for each construct were powdered in liquid nitrogen and suspended in 500 μL of lysis buffer (Jefferson et al., 1987). Samples were centrifuged and the supernatants collected in microcentrifuge tubes. Protein concentrations were determined by the method of Bradford. Fluorogenic reactions were carried out for 5-, 15-, and 30-min intervals at 37°C in 200 μL of lysis buffer with 1 mM 4 methylumbelliferyl-β-D-glucuronide. Reactions typically contained 25 or 50 μg of protein extract. After reactions were stopped with 1.8 mL of 0.2 M sodium carbonate, fluorescence of the 4-methylumbelliferone product was determined in a Hoefer TKO-100 minifluorometer (Jefferson et al., 1987).

### Preparation of Nuclei and Nuclear Extracts

Frozen tissues were powdered in liquid nitrogen with a mortar and pestle and resuspended in nuclear isolation buffer (10 mM Hepes-KOH [pH 7.6], 5 mM $MgCl_2$, 0.8 M Suc, 10 mM 2-mercaptoethanol, 2 mM PMSF, and 2 mg/mL antipain). The homogenate was filtered through 1-mm and 70-μm Nitex mesh (Tetko, Inc., Briarcliff, NY). The nuclei were pelleted from the homogenate at 3000*g* for 10 min, carefully resuspended in nuclear isolation buffer with 0.3% Triton X-100, and purified on Percoll gradients. The nuclei were washed twice to remove residual Percoll and Triton X-100 with nuclear isolation buffer. Nuclear extracts were prepared by a slight modification of the method described by Green et al. (1987). Nuclei were pelleted at 3000*g* for 10 min. The pellet was gently resuspended in nuclear lysis buffer (110 mM KCl, 15 mM Hepes-KOH [pH 7.6], 5 mM $MgCl_2$, 5 mM DTT, PMSF, and antipain) and transferred to Beckman SW41 centrifuge tubes. Ammonium sulfate was added to a final concentration of 0.4 M, and the tubes were rocked gently on ice for 30 min. After centrifugation at 40,000 rpm for 1 h at 4°C, proteins were precipitated by the addition of 0.35 g/mL freshly ground ammonium sulfate with shaking for 1 h on ice. After the sample was centrifuged at 10,000*g* for 15 min, the pellet was resuspended in nuclear extraction buffer (40 mM KCl, 25 mM Hepes-KOH [pH 7.6], 0.1 mM EDTA, 10% glycerol, and 1 mM DTT) and dialyzed against nuclear extraction buffer for 3 h in three changes of buffer. Insoluble material was removed by centrifugation at 12,000*g* for 10 min at 4°C. Extracts were then aliquoted, frozen in liquid nitrogen, and stored at −70°C.

### DNA Probes and Competitors

Standard enzymic and purification procedures involving DNA manipulation were performed essentially as described by Sambrook et al. (1989). ALRE (three Gap boxes, −276 to −195) was purified as a *Hin*dIII-*Eco*RI fragment from pBS$^+$/GapALRE-2. BLRE (four Gap boxes, −263 to −152) was isolated as a *Hin*dIII-*Eco*RI fragment from pUC12/LREb-1. To make monomers and dimers of a synthetic consensus Gap box (A1), two oligonucleotides, 5′-AATTCCAAATGAAGAG-3′ (oligonucleotide a1) and its complementary oligonucleotide, were synthesized and annealed to generate a double-stranded DNA fragment. To facilitate subcloning, 5′-AATTC-3′ was added to the 5′ end of each oligomer to generate an *Eco*RI site. By subcloning one and two of each synthetic box into the *Eco*RI site in pBS$^+$, two plasmid clones containing the A1 monomer and dimer were generated. A 94-bp (A1D dimer) and a 78-bp (A1M monomer) *Xho*I-*Xba*I fragment were gel purified and used as probes or competitors. Probes were end-labeled with [$\alpha$-$^{32}$P]dATP by filling in the 5′ overhangs with Klenow fragment of DNA polymerase I. The same probes were used as competitors without labeling. The other competitor, a tetramer of Box II of *RbcS*-3A from pea, was purified as a *Hin*dIII-*Xho*I fragment from plasmid KH4IIG90 (provided by Dr. Nam-Hai Chua). For nonspecific competitors, poly(dI.dC)·poly(dI.dC) (Phamacia LKB Biotechnology, Inc.) and pBS$^+$ (Stratagene) digested with *Pvu*II were used.

### Gel Mobility Shift Assay

Binding reactions (25 μL final volume) contained 1 ng (or about 10 fmol) of end-labeled probe, 2 to 4 μg of poly(dI.dC)·poly(dI.dC), 60 mM KCl, 20 mM Hepes (pH 7.6), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and specific competitor DNAs as indicated in the figure legends. Reactions were incubated at room temperature for 10 min with poly(dI.dC)·poly(dI.dC) and/or other competitors in the absence of labeled probe and then further incubated for 20 min after addition of probes. Reactions were loaded on a 4% nondenaturing polyacrylamide (bisacrylamide:acrylamide, 1:40) gel in 0.3 × buffer (1× = 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Following electrophoresis at 18 mA with constant buffer recirculation, gels were dried onto 3MM paper and exposed to XAR-5 x-ray films (Kodak) for varying periods with intensifying screens at −70°C.

## RESULTS

### Characterization of Transgenic Tobacco Plants

A series of binary vectors that contain variable lengths of *GapB* promoter fragments (Fig. 1) were constructed and used

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          BH2(-664)
          |
-664      AAGCTTTGGAGGTAAGTTATTGCTAAGCCTCTTGATTCATATTTATTACA
-614      CTTTTTGTTGAATTTACTTATGAACAATGTTGTCTACTGTTAATGCAGAA
                                        B8(-536)
                                        |
-564      AACACGTAAGGTACTAGCTGAGAAAATTGCTCAGCTTAACTCTGCTATCG
                                                  B11(-465)
                                                  |
-514      ACGATGTCTCCTCTCAGCTCAAATCAGAAGATACTCCGAATGGTGCAGCT
-464      CTAAGCACCGATGAAATCGAGGCTACAGCTGAAATCATCTGTTTTTAGGA
                                    B36(-389)
                                    |
-414      TTTGAAATTGAATCATGGGAGATTACTTACTATGATCCCAATAATTGTTT
                                              B145(-330)
                                              |
-364      TCCTTTCTGTGTAATGTTGTACAACTTTTCGTCTACTATCTTCAAATGAC
-314      TGCTTTCTTCCTTCTTCTTTTTTTCTCGAAATCGCGGTGTTGAAGGATAT
          B35(-263)
          |
-264      ATCACAGTTATATGCGAAACCAGACGTTATGAAGACTATATAATATCCTG
          Bb(-214)    B146(-204)                  B19(-175)
          |           |                           |
-214      ATGAAAACTATATGACATGAAAAGAAGGATTAAAAAAATGAGTCTCTTGA
                                                  B131(-116)
                                                  |
-164      GAAACTTGTGATCACTGGCTCACAGAATCTTATCCCCATATGTATCTTAC
-114      CTACATAATAGCCACATATTACTTTGCTTCACTTTGAACCATTGGTTGGT
                                    B2(-40)
                                    |
-64       TTTGATCAAAACCAAAGGATGTGTGTATGTATGTATATATTCTAACGCTC
-14       AAAACTGATCACTAGCATTCCATGATTTTTCCTTCTTGTGATCACTTGCA
+44       GCTATGGCCACACATGCAGCTCTCGCCGTCTCAAGAATCCCGGTCACACA
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**Figure 1.** Nucleotide sequence of the 5′ upstream region of the *GapB* gene from *A. thaliana*. The transcription start point (+1) as determined by S1 nuclease mapping, the putative TATA box, and translation initiation codon (ATG) are underlined. Numbering of the nucleotide is based on the distance from the transcription start point. The four direct repeats within the LRE are also underlined. The vertical bars show break points of *GapB* promoter deletion mutants. The numbers in parentheses are bases from the transcription start point.

to produce transgenic tobacco plants by leaf disc transformation (Bevan, 1984). Fifteen independent kanamycin-resistant transgenic plants from each construct were chosen for further analysis. Figure 2 shows that GUS activities are highly variable among independent transformants with the same construct. Between 15 and 50% of transgenic lines from each construct have no GUS activity, with the exception of the B19, B131, and B2 lines, in which none of the kanamycin-resistant transformants showed GUS activity. These results suggest that essential regulatory elements have been deleted from the B19, B131, and B2 constructs. To determine whether the variability in GUS activity among transgenic lines with the same construct affects regulation patterns, we chose three independent lines from among the BH2 plants (BH2-3, BH2-5, and BH2-13) that exhibited different levels of GUS activity for further analysis. Figure 3A shows that *Gus* mRNA levels, as determined by RT-PCR (for details, see below), from light-grown transgenic tobacco plants (lanes L) are 30-fold higher than those of dark-treated plants (lanes D). In addition, the relative differences in levels of GUS activity from leaves, stems, and roots for all three lines are very similar (Fig. 3B). These results indicate that independent transgenic lines with the same construct exhibit similar regulatory patterns. For subsequent light induction studies, we chose one transgenic line representative of each *GapB* promoter-*Gus* construct that exhibited near-average GUS activity and propagated them vegetatively to produce enough isogenic plants for further analysis.

### Quantitation of Light Induction by RT-PCR

To quantitate *Gus* mRNA levels under different light conditions, transgenic tobacco plants were dark treated for 5 d and then transferred to continuous white light for 24 h. Total RNAs from dark-treated plants or light-treated plants were isolated and analyzed by the competitive RT-PCR method (Becker-Andre and Hahlbrock, 1989; Gilliand et al., 1990; Siebert and Larrick, 1992). A plasmid clone, pΔGus/BS$^+$, that contains an internal deletion of the pUC19/Gus was used as the competitor template. The chosen primer pair will produce 585- and 422-bp PCR products from *Gus* messages and competitor templates, respectively (for details, see "Materials and Methods").

Figure 4 shows amplification of 2 μg of total RNA isolated from dark-treated and light-treated BH2 plants with variable amounts of competitor template in RT-PCR reactions. Levels of *Gus* mRNA in these plants can be determined by calculating the amounts of competitor template required to achieve equal intensity with the *Gus* cDNA band. The results indicate that there are 0.2 and 0.00625 pg *Gus* cDNA/μg total RNA from light-treated and dark-treated BH2 plants, respectively. This 32-fold induction by light is similar to that of the endogenous *GapB* gene in *A. thaliana* (Dewdney et al., 1993), suggesting that the BH2 construct contains all essential *GapB* promoter elements. To determine the effects of various *GapB* promoter deletions (Fig. 1) on light induction, RNA from dark-treated and light-grown transgenic plants with each of these constructs was amplified with equal amounts of competitor included in each PCR reaction (Fig. 5A). Magnitudes of light induction for each transgenic line can be calculated,

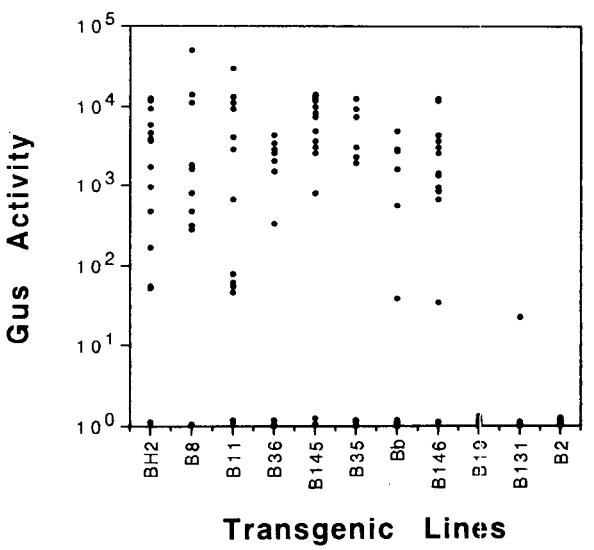


**Figure 2.** GUS activities among different transgenic lines. GUS activities from leaves of 15 independent transgenic tobacco plants for each construct shown in Figure 1 were determined according to the method described by Jefferson et al. (1987). GUS activities were measured by enzymic conversion of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone, which was quantified with a Hoefer TKO-100 spectrofluorimeter. The activity is expressed as pmol 4-methylumbelliferone min$^{-1}$ mg$^{-1}$ protein.

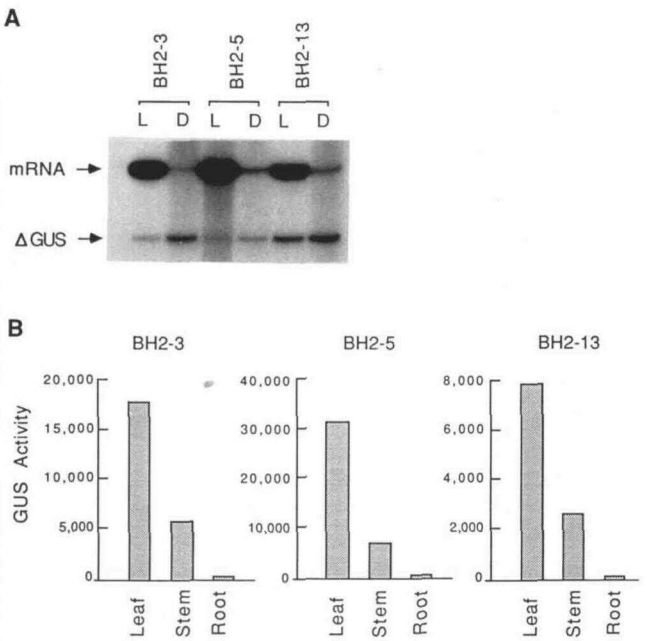


**Figure 3.** Expression of the *Gus* gene in transgenic tobacco plants. A, Total RNA (2 μg) from dark-treated (lanes D) or 24-h white-light-treated (lanes L) transgenic tobacco lines BH2-2, BH2-5, and BH2-13 was isolated and subjected to RT-PCR analysis. The arrows indicate positions of PCR products for *Gus* mRNA (mRNA) and competitor templates (ΔGUS). B, GUS activities from leaves, stems, and roots of BH2-2, BH2-5, and BH2-13 plants were determined according to the method described by Jefferson et al. (1987). The activity is expressed as pmol 4-methylumbelliferone $min^{-1}$ $mg^{-1}$ protein.

since equal amounts of competitor template were included in each reaction (Siebert and Larrick, 1992). In addition, the amount of *Gus* message for each reaction shown in Figure 5A can be estimated by comparing the relative intensity of the *Gus* band and the competitor band and calibrating against the titration curve from Figure 4. To confirm this estimation, a second set of RT-PCR (Fig. 5B), in which the reactions shown in Figure 5A were repeated except that competitor templates corresponding to the estimated amount of *Gus* mRNA for each reaction, was used. The results suggest that our calculations were fairly accurate, since the *Gus* band and the competitor band in each reaction should exhibit about equal intensity. Combining results from these experiments, relative levels of *Gus* mRNA from light-grown and dark-treated transgenic tobacco plants for each construct containing variable lengths of *GapB* promoter sequence can be quantified and are illustrated in Figure 6. The data show that deletions between BH2 and B11 (−664 to −465) resulted in decreases in light induction of the *Gus* gene from 32- to 19-fold. Deletions between B11 and B146 (−465 to −204) have no further effects on light induction. Deletions between B146 and B19 (−204 to −175) abolished light induction completely, as did deletions beyond B19 (B131 and B2). The results indicate that the region between −204 and −175 of the *GapB* gene contains sequence elements essential for light induction.

### Effects of BLRE from *GapB* on a Heterologous Promoter

We have previously identified three direct repeats with the consensus sequence 5′-CAAATGAA(G/A)A-3′ located within an upstream region of the *GapA* gene that is essential for light responsiveness (Conley et al., 1994). Examination of the *GapB* upstream sequence indicated that there are four similar repeats located between −236 and −179 (Fig. 1) with strong homology to the last seven nucleotides of the repeated elements of the *GapA* promoter. Deletion of all four repeats from the *GapB* promoter (B19, B131, and B2) abolished light induction completely (Fig. 6). To test the function of these four repeat elements, a *Kpn*I-*Sau*3AI *GapB* upstream fragment (designated BLRE) that contains the sequence between −263 and −152 was fused to a DNA fragment bearing the −92 to +6 sequence of the CaMV 35S promoter (Lam et al., 1990) in either orientation. The resulting chimeric promoter fragments were then linked to the *Gus*-coding sequence of the pBI101 binary vector (Fig. 7). These two constructs were designated BI and BII, respectively. As a control, two binary vectors that fused the −92 to +6 or −435 to +6 sequences of the 35S promoter to the *Gus*-coding sequence, designated −92(35S) and −435(35S), respectively, were constructed. Transgenic tobacco plants harboring these four constructs were made and assayed for their GUS activities in roots, since it has been shown that the −92(35S) promoter can confer expression of the *Gus* reporter gene only in roots (Lam et al., 1990). As in the case of the other constructs, between

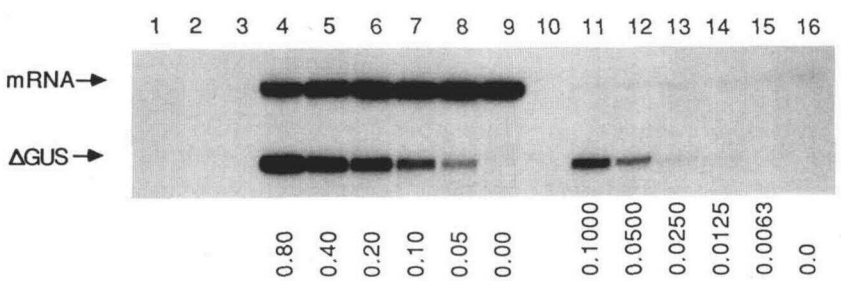


**Figure 4.** Titration of *Gus* mRNA levels in BH2 transgenic plants by RT-PCR. Total RNA (2 μg) from transgenic tobacco plants was subjected to RT-PCR analysis as described in "Materials and Methods." RNA from light-treated (lanes 3–9) and dark-treated (lanes 10–16) BH2 plants was used in RT-PCR reactions with variable amounts of competitor DNA included in each reaction. Lanes 3 and 10 are reactions without addition of RT. Lanes 1 and 2 are reactions using RNA from SR1 tobacco plants with (lane 2) or without (lane 1) addition of RT.

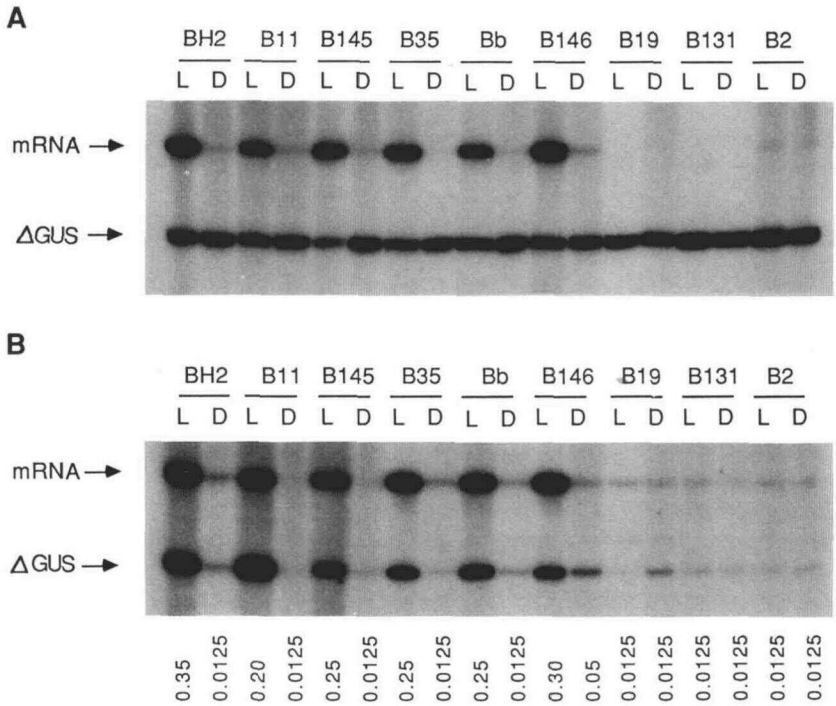


**Figure 5.** Measurement of light induction by RT-PCR. Transgenic tobacco plants containing different *GapB* promoter-*Gus* fusions (Fig. 2) were grown under different light conditions. Total RNA (2 μg) from light-treated or dark-treated plants was isolated and subjected to RT-PCR analysis. A, RNA from 24-h white-light-treated plants (L) and dark-treated (D) plants of each transgenic line with 0.015 pg of competitor DNA included in each reaction. The arrows indicate positions of PCR products for *Gus* mRNA (mRNA) and competitor templates (ΔGUS). B, The same as A except that a different amount of competitor DNA is used for each reaction. For reactions shown in B, amounts of competitor DNA used are shown at the bottom of each lane.

20 and 50% of transgenic lines from each construct lack GUS activity in roots (data not shown). We randomly chose three independent lines that had higher than background level GUS activity (compared to SR1 plants) from each construct for further characterization. Figure 8 shows that the −92(35S) promoter can confer only minimal transcriptional activity in leaves, an observation consistent with other published results (Lam et al., 1990). In plants bearing BI or BII, the average GUS activities in leaves are about 10-fold higher than those of −92(35S), indicating that the −263 to −152 upstream sequence of *GapB* placed in either orientation can enhance transcriptional activity of the −92(S35) basal promoter in leaves. The data also show that the average GUS activities in roots of the BI and BII transgenic lines are about 10-fold lower than those of −92(35S) lines, suggesting that the BLRE also functions to suppress transcription of the −92(35S) promoter in roots. To quantitate *Gus* mRNA levels under different light conditions, we chose one transgenic tobacco plant from each construct for RT-PCR analysis. Figure 9 shows that *Gus* mRNA levels from light-grown BI and BII plants (lanes L) are 10- and 8-fold higher, respectively, than those of the dark-treated control plants (lanes D). In contrast, there is no apparent difference in *Gus* mRNA levels from light-grown or dark-treated −92(35S) or −435(35S) plants. Compared to the −435(35S), the −92(35S) promoter confers only minimal transcriptional activity in leaves, an observation consistent with other published results (Lam et al., 1990). The patterns of light induction for the remaining BI and BII lines (data not shown) are similar to those shown in Figure 9. These results strongly suggest that the −261 to −149 sequence of *GapB* (BLRE) is a light-dependent and organ-specific enhancer-like element.

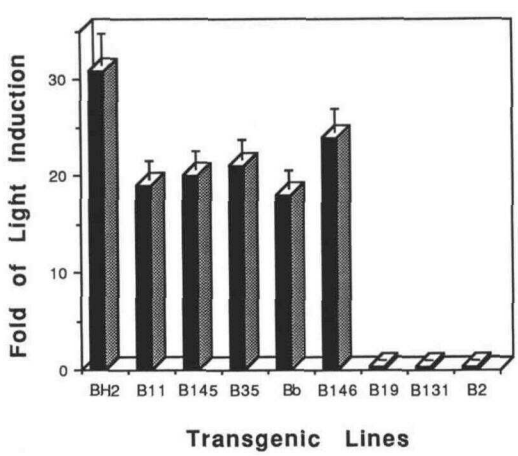


**Figure 6.** *Quantitation of light induction.* Relative *Gus* mRNA levels, as determined by RT-PCR reactions (Figs. 4 and 5) of the dark-treated and light-treated plants for each transgenic line were compared. The *Gus* mRNA levels from dark-treated plants of each line were used as the basal level to calculate magnitudes of light induction.

### Identification of GAPF in Tobacco Nuclear Extracts

To identify nuclear extract components that interact with the Gap boxes located within upstream regions of *GapA* and *GapB* genes, we chose two restriction fragments, ALRE, a 102-bp *Hin*dIII-*Eco*RI fragment that contains the −277 to −194 *GapA* sequence and 18 bp of linker sequences, and BLRE, a 156-bp *Hin*dIII-*Eco*RI fragment that contains the −261 to −154 *GapB* sequence and 48 bp of linker sequence, to use as probes in gel mobility shift assays. Figure 10 shows that each probe gave a prominent retarded band when to-

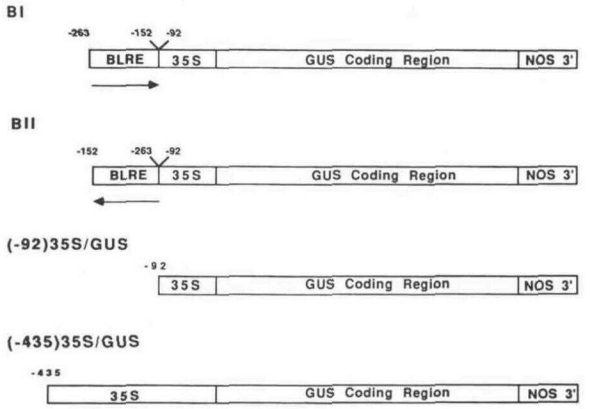


**Figure 7.** Construction of BLRE-35S chimeric promoters. The BLRE DNA that contains the sequence between −261 and −149 of the *GapB* promoter was fused to a DNA fragment bearing the −92 to +6 sequence of the CaMV 35S promoter (Lam et al., 1990) in either orientation. The resulting chimeric promoter fragments were then linked to the *Gus*-coding sequence of the pBI101 binary vector. These two constructs were designated BI and BII, respectively. (−92)/35S/GUS and (−435)/35S/GUS were constructed by fusing the −92 to +6 (−92/35S) or −435 to +6 (−435/35S) regions of the 35S promoter sequence to the *Gus* coding sequence. NOS, 3′ untranslated region of the nopaline synthase gene from Ti plasmid.

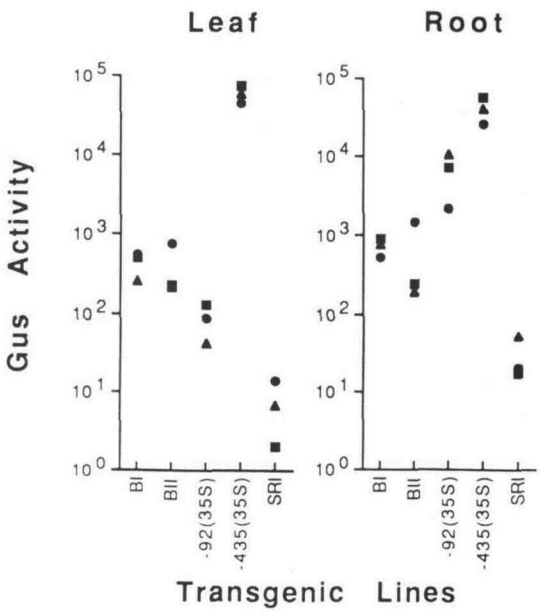


**Figure 8.** GUS activities of transgenic tobacco plants containing GapB-35S chimeric promoters. GUS activities from leaves and roots of three independent transgenic tobacco plants for each constructs shown in Figure 7 were determined according to the method described by Jefferson et al. (1987). The activity is expressed as pmol 4-methylumbelliferone $min^{-1}$ $mg^{-1}$ protein.

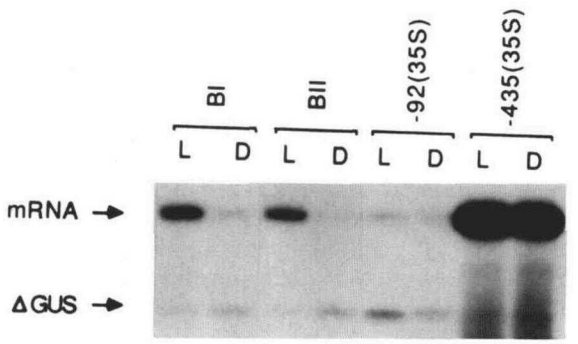


**Figure 9.** Light induction of the *Gus* reporter gene controlled by the BLRE-35S chimeric promoters. Total RNA (2 μg) from light-grown (L) or dark-treated (D) BI, BII, (−92)/35S, and (−435)/35S transgenic tobacco plants was isolated and analyzed by RT-PCR as described.

bacco nuclear extracts were used as the source of DNA-binding proteins (lanes 2 and 7). These binding activities disappeared when excesses of unlabeled respective DNA fragments (450-fold molar excess) were included in the binding reactions (lanes 3 and 8). In contrast, excessive amounts of linearized Bluescript plasmid DNA (4000-fold molar excess) had no apparent effects on these binding interactions (lanes 4 and 9). In addition, Figure 10 shows that BLRE can effectively compete with ALRE for protein binding (lane 5) and that ALRE also competes efficiently with BLRE binding (lane 10). These results suggest that the observed retarded bands represent specific DNA-protein interactions between ALRE and BLRE with protein factors present in the tobacco nuclear extracts and that ALRE and BLRE interact with the

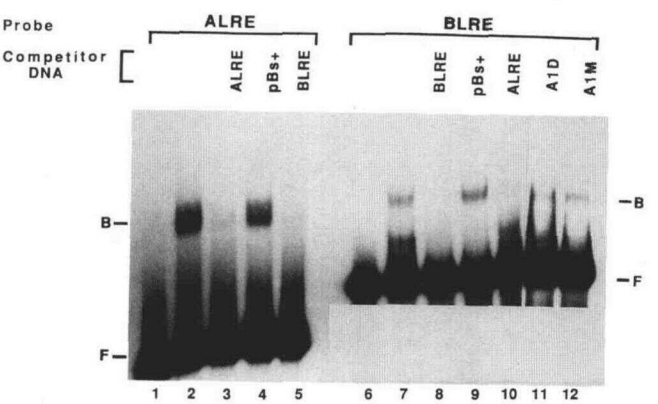


**Figure 10.** Binding of GAPF to ALRE and BLRE. Nuclear extracts (5 μg) prepared from leaves of greenhouse-grown tobacco plants were used in gel mobility shift assays, using $^{32}$P-labeled ALRE (lanes 1–5) or BLRE (lanes 6–10) fragments as probes. Lanes 1 and 5 are reactions without nuclear extracts added. Lanes 11 and 12 are reactions with BLRE as probes and the Gap box dimer (A1D) and monomer (A1M), respectively, as competitors. Competitor DNAs used in each reaction are illustrated at the top of each lane. The competitor/probe molar ratios for each reaction are as follows: lanes 3, 5, 8, and 10, 450×; lanes 4 and 9, 4000×; lanes 11 and 12, 360×. B, Bound; F, free.

same protein factor (designated GAPF). To test directly whether Gap boxes within the BLRE are binding sites for the GAPF, a 32-bp Gap box dimer (designated A1D) and a 16-bp Gap box monomer (designated A1M) were synthesized and subcloned into pBS+ (see "Materials and Methods"). Restriction fragments that contain A1D and A1M were gel purified and used as competitors or probes in gel mobility shift assays. When used as a competitor, a 360-fold molar excess of A1D efficiently competes for binding of BLRE to GAPF (Fig. 10, lane 11), and the same molar excess of A1M can also compete, although less efficiently, for binding of BLRE to GAPF (Fig. 10, lane 12). Similar results were observed for ALRE (data not shown). These results show that Gap boxes are binding sites for GAPF.

### The GAPF Is Distinct from the GT-1 Factor

The properties we have described for GAPF show a strong resemblance to those of the GT-1 factor, which binds to Box II and Box III within the LRE of the *RbcS-3A gene from* pea (Green et al., 1987, 1988; Lam and Chua, 1990). In addition to the Box II and Box III, GT-1 factor from tobacco binds to four other *cis*-acting elements (Boxes II*, III*, II**, and III**) present in the upstream region of *RbcS*-3A, all of which show little sequence homology to Box II and Box III (Green et al., 1987, 1988; Gilmartin et al., 1990). To test whether GAPF and GT-1 have the same binding specificity, an 84-bp DNA fragment that contains a Box II tetramer (Lam and Chua, 1990) was used as a competitor for binding of the ALRE and BLRE to the GAPF. Figure 11 shows that excessive amounts of the Box II tetramer did not compete out binding of ALRE or BLRE to the GAPF (lanes 5 and 8). Conversely, binding of GT-1 to the Box II tetramer is not competed out by an excess of unlabeled ALRE or BLRE (data not shown). These results indicate that GAPF is distinct from the GT-1-binding factor.

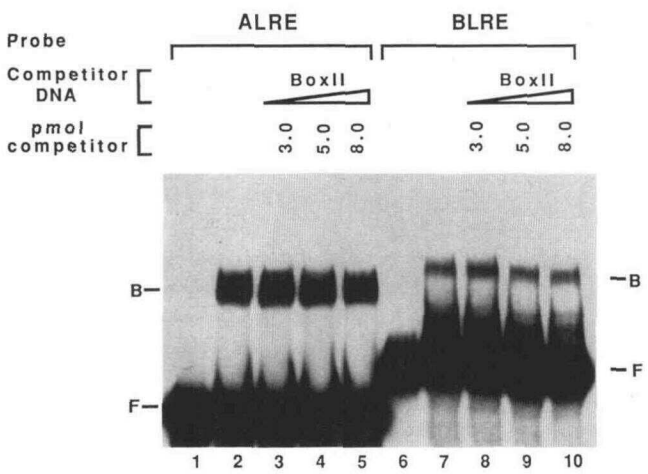


**Figure 11.** Competition of ALRE and BLRE with the Box II tetramer. Gel mobility assays using ALRE (lanes 1–5) or BLRE (lanes 6–10) as probes with variable amounts of Box II tetramer (Lam and Chua, 1990) as the competitors. The amounts of competitors are indicated on top of each lane. Lanes 1 and 6 are reactions without added nuclear extracts. Lanes 2 and 7 are reactions without Box II tetramer as the competitor. B, Bound; F, free.

### GAPF Is Present in Nuclear Extracts from Leaves and Roots of Light-Grown and Dark-Treated Tobacco Plants

To investigate whether GAPF is present in roots or in leaves of dark-treated tobacco plants, nuclear extracts from leaves and roots of light-grown and dark-treated tobacco plants were prepared and used in binding assays. Nuclear extracts from leaves of dark-treated tobacco plants contain DNA-binding proteins that interact with both ALRE and BLRE (Fig. 12, lanes 3 and 11). In addition, these binding activities can be competed out by excessive amounts of cold probes (lanes 4 and 12) but not by excessive amounts of linearized Bluescript plasmid DNA (lanes 5 and 13). Similarly, there is binding activity present in root nuclear extracts that interacts with both ALRE and BLRE (Fig. 12, lanes 6 and 14). These binding activities also can be competed out by unlabeled probes (lanes 7 and 15) but not by Bluescript plasmid DNA (lanes 8 and 16). However, the binding activities of nuclear extracts from leaves of dark-treated plants and from roots are weaker than those prepared from leaves of light-grown plants (cf. lanes 3 and 11 with lanes 2 and 10, lanes 6 and 14 with lanes 2 and 10). Under the same reaction conditions and using the identical nuclear extracts, Box II tetramer binds to nuclear extracts from roots and leaves with about equal efficiency (lanes 17–19), which is consistent with published results (Green et al., 1987, 1988). Based on these results we conclude that either GAPF or a factor with similar binding specificity is present in nuclear extracts from roots and leaves of dark-treated tobacco plants.

## DISCUSSION

We have presented evidence to show that a 664-bp *GapB* promoter fragment from *A. thaliana* is sufficient to confer both light induction and organ-specific expression of a *Gus* reporter gene in transgenic tobacco plants. Promoter deletions between BH2 and B11 (−664 to −465) resulted in a 1.5-fold decrease in light induction of the *Gus* mRNA (Fig. 5). An examination of these sequences shows that there are three sites with sequence similarity to the ACGT core (between −561 and −558 of the sense strand; between −471 and −468 and −571 and −568 of the antisense strand) of the G-box (Giuliano et al., 1988) within this region. However, since there is a 10% SE in our RT-PCR quantitation, the significance of the 1.5-fold difference in light induction between BH2 and B11 and the possibility that any of these G-box-like motifs are involved in *GapB* promoter function must be investigated further. Deletions between B11 and B146 (−465 to −204) have no further effect on light induction, whereas deletions between B146 and B19 (−204 to −175) and deletions beyond B19 (B131 and B2) (Fig. 5) resulted in a 32-fold reduction (compared to BH2) in light inducibility.

These results strongly suggest that the region between −204 and −175 of the *GapB* gene contains sequence elements essential for light induction. An examination of the DNA sequence in this region reveals the presence of four direct repeats with the consensus sequence 5′-ATGAA(G/A)A-3′ (Fig. 1). Deletion of one copy (Bb) or two copies (B146) of these repeats from the *GapB* promoter has no apparent effect on light induction (Fig. 6), whereas deletion of all four copies

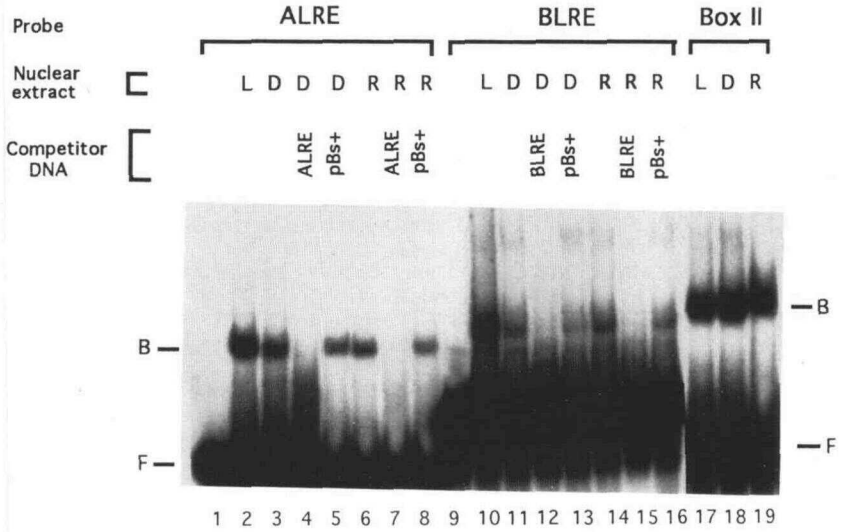


**Figure 12.** Organ and light specificity of GAPF. Binding of ALRE (lanes 2–8) and BLRE (lanes 10–16) to GAPF using nuclear extracts prepared from leaves from light-grown (L) or dark-treated (D) tobacco plants or from roots (R). Lanes 1 and 7 are control reactions without added nuclear extracts. Lanes 17 to 19 are the reactions with Box II tetramer as probe to normalize the abundance of GAPF. B, Bound; F, free.

of the direct repeats (B19) abolishes light induction completely. Analysis of the *GapA* gene, which is coordinately regulated with the *GapB* gene under several different growth conditions that we have examined (Shih and Goodman, 1988; Dewdney et al., 1993), reveals that three copies of similar repeats with the consensus sequence 5′-CAAATGAA(G/A)A-3′ exist in its promoter region (Conley et al., 1994). Deletion of one copy of these repeats from the *GapA* promoter reduces light induction by 3-fold, whereas deletion of all three repeats from the *GapA* upstream region abolishes light induction completely (Conley et al., 1994). Taken together these results suggest that the promoter regions containing these repeats (Gap boxes) are essential for light regulation of the *GapA* and *GapB* genes in *A. thaliana.* In addition, the presence of Gap boxes in similar regions of the promoters of both genes raises the question of whether the Gap boxes play some role in light regulation of *GapA* and *GapB* gene expression.

We have linked the *GapB* promoter sequence between −263 and −152 (which contains four copies of Gap boxes) in both orientations to the −92 to +6 sequence of the CaMV 35S promoter. Both resulting chimeric promoters confer light induction of the *Gus* reporter gene in transgenic tobacco plants (Figs. 8 and 9). In addition, our results show that the −263 to −152 region is able to enhance transcriptional activity of the −92(35S) promoter only in leaves and to suppress transcriptional activity in roots (Fig. 8). The −92(35S) promoter is not light regulated and can confer expression in roots but not in leaves (Lam and Chua, 1990). These results suggest that the −263 to −152 region of the *GapB* promoter contains sequence elements that are sufficient to confer light regulation and organ-specific expression of the *GapB* gene in *A. thaliana.* However, whether the four Gap boxes function in light/dark regulation or in general transcriptional regulation requires further investigation, since the data we have presented do not allow us to exclude the possibility that sequence elements other than Gap boxes within the −263 to −152 region function as LREs.

We have also shown that a nuclear factor (GAPF) from tobacco can bind specifically to *GapA* and *GapB* promoter fragments containing Gap boxes in gel mobility shift assays. The binding sites for GAPF are the Gap boxes within these two promoter fragments as shown using DNA fragments consisting only of a 32-bp Gap box dimer (A1D) or a 16-bp Gap box monomer (A1M) as the competitor (Fig. 10; Conley et al., 1994). In vitro binding of the *GapA* promoter fragment (Fig. 10, ALRE) to GAPF appears to be stronger or more efficient than that of the *GapB* promoter fragment (Fig. 10, BLRE), even though the *GapB* fragment contains one extra Gap box. Under in vivo conditions, expression of the *GapA* gene is higher than that of the *GapB* gene (H.-B. Kwon and M.-C. Shih, unpublished data). We believe, therefore, that further detailed analyses of these promoters by footprinting or mutational studies should be useful in determining the exact role(s) that Gap boxes and other promoter sequences play in regulation of these two genes.

Binding sites for the GT-1 factor, which binds to Box II and Box III within the LRE of the pea *RbcS*-3A gene (Green et al., 1987, 1988; Lam and Chua, 1990; Gilmartin et al., 1992; Perisic and Lam, 1992), are present in promoter regions of many light-regulated genes (for reviews, see Gilmartin et al., 1990, 1992), including the *RbcS* genes from *A. thaliana* and tomato (Schindler and Cashmore, 1990; Manzara et al., 1991). We have observed that expression of *GapA/B* and *RbcS* genes are coordinately regulated by light in both *A. thaliana* and tobacco (Shih and Goodman, 1988; T.R. Conley and M.-C. Shih, unpublished data). However, despite extensive computer searching, no GT-1-binding sequences can be identified in the promoter regions of the *A. thaliana GapA* or *GapB* genes. We have also shown that GAPF is distinct from the GT-1 factor. The results suggest that light induction of *GapA* and *GapB* genes in *A. thaliana* involves GAPF but not a GT-1-like factor. This raises the interesting possibility that different *cis*- and *trans*-acting elements are involved in regulation of *GapA/B* and *RbcS* genes, even though they are similarly regulated by light.

Based on computer analysis, Grob and Stüber (1987) identified a sequence motif, 5′-ATGATAAGG-3′ (designated the

GA-1 site by Gilmartin et al., 1990), that is present in almost all *RbcS* and *Lhc* genes (Dean et al., 1985; Castresana et al., 1987; Grob and Stüber, 1987; Giuliano et al., 1988; Gidoni et al., 1989; Gilmartin et al., 1990; Manzara et al., 1991). Although none of these elements have been characterized functionally, Grob and Stüber (1987) postulated that this element is the common LRE shared by all light-regulated genes in which the phytochrome-mediated pathway is involved. The Gap boxes we have identified show sequence similarity with the GA-1 site. However, the Gap boxes exist as multiple direct repeats in the upstream regions of *GapA* and *GapB* genes, whereas the GA-1 site appears as a monomer in the upstream regions of *RbcS* and *Lhc* genes. Therefore, it would be interesting to determine whether the Gap box and GA-1 site interact with the same factor and whether there is a common LRE among photosynthetic genes that are similarly regulated by light. However, future detailed analysis is required to prove that the GA-1 elements found among different *RbcS* and *Lhc* genes are involved in light responsiveness.

The GT-1-binding protein has been shown to be present in equal abundance in nuclear extracts prepared from light-grown or dark-treated tobacco plants (Green et al., 1987, 1988; Gilmartin et al., 1992). One study indicates that binding of GT-1 to the LRE of *RbcS*-3A is necessary but not sufficient for light responsiveness (Lam and Chua, 1990). Our results showed that GAPF-binding activities are present in nuclear extracts from leaves or roots of light-grown or dark-treated tobacco plants, although binding activities from leaves of dark-treated plants or from roots of light-grown plants appear diminished compared to nuclear extracts from leaves of light-grown plants. These distribution patterns are more similar to those of LRF-1, an activity binding to the upstream region of a *Lemna RbcS* gene (Buzby et al., 1990) and ABF-1, a nuclear factor binding to the promoter region of the pea *Lhc*AB80 gene (Argüello et al., 1992). Our results suggest the possibility that the interaction between the Gap boxes and GAPF is a determinant of the promoter activity of *GapA* and *GapB* genes in response to light. However, it must be pointed out that there is a significant amount of Gap box-binding activity present in nuclear extracts prepared from leaves of dark-treated plants and that the level of in vivo expression is very low for the *GapA* gene and undetectable for the *GapB* gene in leaves of dark-treated *A. thaliana* or tobacco plants (Shih and Goodman, 1988; Dewdney et al., 1993). In addition, Gap box-binding activity is present in nuclear extracts prepared from roots of tobacco plants; yet *GapA* and *GapB* are not expressed in roots of *A. thaliana*. These observations suggest that binding of GAPF to the Gap boxes within the −263 to −152 region of the *GapB* promoter is required but not sufficient for light regulation and organ-specific expression of the *GapB* gene from *A. thaliana*.

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