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Characterization of *cis*-Acting Elements in Light Regulation of the Nuclear Gene Encoding the A Subunit of Chloroplast Isozymes of Glyceraldehyde-3-Phosphate Dehydrogenase from *Arabidopsis thaliana*

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We have characterized *cis*-acting elements involved in light regulation of the nuclear gene (*GapA*) encoding the A subunit of chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in *Arabidopsis thaliana*. Our results show that a 1.1-kb promoter fragment of the *GapA* gene is sufficient to confer light inducibility and organ specificity in transgenic *Nicotiana tabacum* (tobacco) plants, using the β-glucuronidase gene of *Escherichia coli* as the reporter gene. Deletion analysis indicates that the −359 to −110 bp region of the *GapA* gene is necessary for light responsiveness. Within this region there are three copies of a decamer repeat (termed the Gap box) having the consensus sequence 5′-CAAATGAA(A/G)A-3′, which has not been characterized in the promoter regions of other light-regulated genes. A deletion (to −247) producing loss of one copy of these elements from the *GapA* promoter reduces light induction by two- to threefold compared with a promoter deletion (to −359) with all three Gap boxes present, while deletion of all three Gap boxes (to −110) abolishes light induction completely. Gel mobility shift experiments using tobacco nuclei as the source of nuclear proteins show that *GapA* promoter fragments that contain these repeats bind strongly to a factor in the nuclear extract and that binding can be abolished by synthetic competitors consisting only of a monomer or dimer of the Gap box. Furthermore, a trimer, dimer, and monomer of the Gap box show binding activity and, like the authentic *GapA* promoter-derived probes, show binding activities that are correlated with Gap box copy number. These results strongly suggest that these repeats play important roles in light regulation of the *GapA* gene of *A. thaliana*.

Light is one of the most important environmental factors affecting plant growth and development. One of the primary steps at which light exerts its control is regulation of gene expression at the transcriptional level. It has been shown that light is required for the efficient transcription of genes encoding enzymes of photosynthesis both in seedlings and in mature plants (for reviews, see references 13 and 30). Among these light-regulated genes, nuclear genes encoding the small subunit of ribulose 1,5-bisphosphate carboxylase (*RbcS*) and chlorophyll *a/b* binding proteins (*Cab*) have been well studied (11, 13, 14, 22, 23). Results from these studies showed that both phytochrome and blue light receptor-mediated pathways are involved in their regulation. Studies from different laboratories have also shown that *cis*-acting regulatory elements for light responsiveness are located within the 5′ upstream (promoter) regions of many light-regulated genes (13, 14). 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 that are similarly regulated by light, not all of the important *cis*-acting elements are conserved (22).

We are interested in studying light regulation of the two nuclear genes (*GapA* and *GapB*) encoding chloroplast isozymes of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Arabidopsis thaliana*. Higher plants possess two chloroplast GAPDH isozymes, with subunit structures of A_4 and A_2B_2, which function in photosynthetic carbon fixation (6). We have previously shown that the expression of *GapA* and *GapB* genes is coordinately regulated by light in *Nicotiana tabacum* (tobacco) and *A. thaliana* (8, 26). Continuous exposure of dark treated mature plants or etiolated seedlings to red, blue, or white light is required for sustained high-level expression of *GapA* and *GapB* genes in *A. thaliana*, with blue and white light much more efficient than red light. In contrast, a short light pulse can induce transient increases of *GapA* and *GapB* mRNA levels in etiolated seedlings but not in mature *A. thaliana* plants. In addition, induction by a short red light pulse cannot be reversed by subsequent far-red light treatment (8). These regulatory patterns are distinct from those of the pea *RbcS* genes (10, 19) and *A. thaliana Cab* genes (18), in which the effect of a short red light pulse can be reversed by a subsequent far-red light treatment. It would be interesting to determine whether similar regulatory elements are involved in light regulation of *RbcS* and *GapA* and *GapB* genes, since their gene products are key enzymes in the photosynthetic carbon fixation pathway.

We report here characterization of *cis*-acting elements involved in light regulation of the *GapA* gene of *A. thaliana*. Our results show that 1.1 kb of 5′ promoter sequence from the *GapA* gene is sufficient to confer light inducibility (measured as the difference in gene expression between dark-treated and light-treated plants) and organ specificity (measured as reporter enzyme activity) in transgenic tobacco plants in which the *Escherichia coli* β-glucuronidase gene (*Gus*) was used as the gene fusion marker. Deletion analysis allowed us to

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identify two regions of upstream sequence that are important for light regulation of gene expression. Deletion of the region from −1045 to −808 of the *GapA* promoter reduced light inducibility from 31-fold (with the full −1045 promoter) to about 15-fold (with the −808 promoter). When the sequence from −359 to −110 was deleted, only a low level of expression could be detected, and no difference in expression levels between dark-treated and light-treated plants was detectable.

Within the −359 to −110 region are three copies of decamer repeats with the consensus sequence 5′-CAAAT GAA(G/A)A-3′. Deletion of the most upstream of these repeats resulted in a decrease of light inducibility from 13-fold to 6-fold. Deletion of all three repeats resulted in complete loss of light inducibility. Gel mobility shift assays showed that there is a nuclear factor present in tobacco nuclear extracts that binds specifically (as shown by competition studies) to these repeated elements. These data suggest that the direct repeats, and possibly other sequence elements, within the *GapA* promoter comprise a previously uncharacterized promoter motif involved in light regulation of higher plant genes.

MATERIALS AND METHODS

Construction of *GapA* promoter-*Gus* fusions and transgenic tobacco plants. To eliminate potential effects from having variable polylinker sequences on the translational efficiency of transcripts (29) derived from the *GapA* promoter-*Gus* reporter gene constructs used in these experiments, site-directed mutagenesis was used to change nucleotides near the ATG codon of the coding region of the *Gus* gene in the plasmid pUC19/Gus from 5′-CCTTATGTTAC-3′ to 5′-CCCCATGGTAC-3′ to create an *Nco*I restriction site (plasmid pUC19/Gus*). This allowed us to take advantage of a naturally occurring unique *Nco*I site that includes the start codon of the *GapA* gene in subcloning various *GapA* promoter fragments into pUC19/Gus*. A 1.1-kb *Bam*HI-*Nco*I *GapA* promoter fragment that contains 1 kb of promoter sequence and the complete 5′ untranslated region of the *GapA* gene was ligated to *Bam*HI-*Nco*I-digested pUC19/Gus* to create pUC19/GapA-Gus*. The resulting 3-kb *Bam*HI-*Eco*RI fragment containing the *GapA* promoter-*Gus* fusion from pUC19/GapA-Gus* was then used to replace the *Bam*HI-*Eco*RI Gus fragment of pBI101 (17), resulting in the binary vector pmW. Promoter deletions were generated with either exonuclease III-mung bean nuclease or by using existing restriction sites. DNA sequencing of the complete promoter and to verify breakpoints of the promoter deletions used in these constructs was performed by the method of Sanger et al. (25), using the Sequenase enzyme and reagents from United States Biochemical (Cleveland, Ohio). Procedures similar to those used in the construction of pmW were used to link these DNA fragments to the *Gus* coding sequence of pBI101. The resulting pBI101 derivatives were mobilized into *Agrobacterium tumefaciens* LBA 4404 by triparental mating (2). These *Agrobacterium* strains were used in leaf disc transformation of wild-type *N. tabacum* SR1. Kanamycin-resistant calli derived from the leaf discs were selected on solid Murashige and Skoog medium containing 0.5 μg of benzyl adenine (BA) per ml, 3% sucrose, 100 μg of kanamycin per ml, and 200 μg of carbenicillin per ml, and regenerating plantlets were cultured on the same medium. Regenerated plants were transferred to soil mix and grown in environmental chambers under a 16-h light/8-h dark cycle at 25°C. Plants of similar age and development, with leaf lengths of 5 to 10 cm, were used for all experiments.

Dark and light treatment conditions. Environmental chamber-raised tobacco plants were dark treated by placing them in a 25°C dark incubator for 5 days. Plants were then transferred to other environmental chambers for light treatments. White light-treated plants received 150 microeinsteins of light m^{-2} s^{-1} from a combination of incandescent and cool white fluorescent lamps. The red light source was light from a 120-W GE flood light passed through a Ditric Optics 660-nm bandpass filter (bandwidth of 5.6 nm); the light intensity at leaf surface was 1 microeinstein m^{-2} s^{-1}. The blue light source was light from a 150-W Sylvania halogen floodlight passed through a Ditric Optics 430-nm bandpass filter (bandwidth of 8 nm); the blue light intensity at the leaf surface was 1 microeinstein m^{-2} s^{-1}. For red and blue light treatments, cooling was achieved by circulating chilled water through glass trays between the light source and the bandpath filters. Light levels were measured with a Lambda Instruments model LI-185 photometer.

RNA isolation and Northern (RNA) blot analysis. Total RNA from tobacco tissues was isolated by a LiCl precipitation protocol adapted from the procedures of Sambrook et al. (24) and described in detail by Yang et al. (32). Procedures used for RNA gel electrophoresis and Northern blot analysis have also been described (32). Prior to reverse transcription (RT)-PCRs, RNA samples were treated with DNase I twice to deplete contaminating genomic DNA (28). Standard precautions for handling RNA samples were observed (24). Quantitation of RNA blots was done by scanning autoradiograms with a Bio-Rad model 620 densitometer.

Construction of pΔGus/BS$^+$ internally deleted competitor. To construct a *Gus* plasmid clone with an internal deletion for use as a competitor template in RT-PCRs (27), a 560-bp *Hin*dIII-*Hin*cII fragment from pUC19/Gus (containing most of the N-terminal region of *Gus* coding sequence) was subcloned into the *Hin*cII site of pBS$^+$ (Stratagene) to create pGusH/Bs$^+$. A 173-bp *Sna*BI-*Eco*RV fragment was then deleted from the *Gus* sequences of pGusH/Bs$^+$ to create pΔGus/Bs$^+$.

Quantitative RT-PCR methods. Two primers, 5′-ACGTC CTGTAGAAACCCCAA-3′ (primer 1) and 5′-ACAGTCTT GCGCGACATGCG-3′ (primer 2), corresponding to nucleotide sequences of the *Gus* coding and antisense strands, respectively, were synthesized. First-strand cDNA synthesis was performed in a 20-μl reaction mixture containing 1 μg of RNA, 0.08 μg of primer 2, 40 μM concentrations of each of the four deoxynucleoside triphosphates (dNTPs), 2 μl of 10× buffer, 200 U of Moloney murine leukemia virus reverse transcriptase, and 20 U of RNase inhibitor. The reaction mixture was incubated at 42°C for 30 min, and the reaction was stopped by heating at 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, 10^6 cpm of ^{32}P-end labeled sense primer (specific activity was typically 1.5×10^8 to 2.5×10^8 cpm/μg), 5 μl of 10× DNA polymerase buffer (500 mM Tris [pH 9.0], 500 mM NaCl), 10 μl of 25 mM $MgCl_2$, dNTPs to a final concentration of 250 μM each, variable amounts of competitor DNA, and 2.5 U of *Taq* DNA polymerase. Final reaction volumes were brought to 100 μl by the addition of H_2O. PCR was performed in a Perkin-Elmer Cetus DNA Thermal Cycler programmed for 24 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C. Reaction samples were combined with DNA sequencing gel loading buffer (24) and denatured by heating at 94°C for 5 min. The products were separated by electrophoresis on a prerun 4% polyacrylamide–8 M urea denaturing gel. Following electrophoresis, the gels were exposed to XAR-5 X-ray film (Kodak) with intensifying screens at −70°C.

GUS enzyme assays. GUS enzyme activity assays were performed as described by Jefferson et al. (17). GUS activities

were determined by quantifying the enzymatic conversion of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone, using a Hoefer TK100 spectrofluorimeter.

Preparation of tobacco nuclei and nuclear extracts. Frozen tissues were powdered in liquid nitrogen with a mortar and pestle and resuspended in nuclear isolation buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES]-KOH [pH 7.6], 5 mM $MgCl_2$, 0.8 M sucrose, 10 mM 2-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, 2 mg of antipain per ml). The homogenate was filtered through 1-mm and 70-μm Nitex mesh (TETKO Inc.). The nuclei were pelleted from the homogenate at 3,000 × *g* for 10 min, then resuspended in nuclear isolation buffer containing 0.3% (vol/vol) Triton X-100, and purified on Percoll gradients. Purified nuclei were washed twice with nuclear isolation buffer to remove residual Percoll and Triton X-100. Nuclear extracts were prepared by a method modified from that of Green et al. (15). Nuclei were pelleted at 3,000 × *g* for 10 min. The pellet was resuspended in nuclear lysis buffer (110 mM KCl, 15 mM HEPES-KOH [pH 7.6], 5 mM $MgCl_2$, 2 mM dithiothreitol, phenylmethylsulfonyl fluoride, 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 of freshly ground ammonium sulfate per ml with shaking for 1 h on ice. Following centrifugation 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 dithiothreitol) 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. After protein concentrations were determined by the method of Bradford (2a), aliquots were frozen in liquid nitrogen and stored at −70°C.

DNA probes and competitors in gel mobility shift assays. Standard enzymatic and purification procedures involving DNA manipulation were performed essentially as described by Sambrook et al. (24). The ALRE probe (−276 to −195; three Gap boxes) was purified as a *Hin*dIII-*Eco*RI fragment from pBS$^+$/GapALRE-2. The AM9 probe (−247 to −195; two Gap boxes) was generated by PCR. A monomer, a dimer, and a trimer of a synthetic consensus Gap box (termed A1-M, A1-D, and A1-T, respectively) were generated by annealing two oligonucleotides (5'-AATTCCAAATGAAGAG-3' and 5'-CTCCCTTCATTTGGAATT-3') to generate a double-strand DNA fragment. To facilitate subcloning, 5'-AATTC-3' or its complement was added to the 5' end of each oligonucleotide to generate an *Eco*RI site. By subcloning one, two, and three copies of each synthetic box into the *Eco*RI site of the pBS$^+$ polylinker, plasmid clones containing the monomer, dimer, and trimer were generated. The orientation of the synthetic consensus Gap boxes in the dimer and trimer clones was verified by DNA sequencing; the Gap boxes in the dimer and trimer clones used as sources of probes and competitors for these experiments were in the same orientation. For use as probes or competitors, 78-bp (A1-M), 94-bp (A2-D), and 110-bp (A3-T) *Xho*I-*Xba*I fragments were gel purified. Probes were end labeled with [α-^{32}P]dATP by filling in the 5' overhangs with DNA polymerase I large fragment (Klenow). Identical unlabeled probes were used as competitors. The nonspecific binding competitors poly(dI-dC) · poly(dI-dC) (Pharmacia LKB Biotechnology Inc.) and *Pvu*II-digested pBS$^+$ (Stratagene) were used as described.

Gel mobility shift assay procedures. Binding reaction mixtures (25-μl volume) contained 1 ng (or about 10 fmol) of 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 dithiothreitol, 10% (vol/vol) glycerol, and competitor DNAs as specified in figure legends. Reaction mixtures 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 incubated for an additional 20 min after addition of labeled probes. Reaction mixtures were electrophoresed on 4% nondenaturing polyacrylamide gels (acrylamide/bisacrylamide ratio, 40:1) in 0.3× TBE buffer (1× TBE buffer is 89 mM Tris-HCl [pH 8.0], 89 mM boric acid, and 2 mM EDTA [pH 8.0]). Gels were run at 18 mA with buffer recirculation. Gels were dried onto Whatman 3MM paper or equivalent and exposed to Kodak XAR-5 X-ray film with DuPont Lightning Plus intensifying screens at −70°C.

Enzyme suppliers. Restriction and DNA modification enzymes were purchased from New England BioLabs (Beverly, Mass.) and Promega (Madison, Wis.). Sequenase, Moloney murine leukemia virus reverse transcriptase, and RNase inhibitor were obtained from United States Biochemical.

Nucleotide sequence accession number. The nucleotide sequence for the *GapA* gene from *A. thaliana* has been submitted to GenBank under accession number L14743.

RESULTS

The 1.1-kb *A. thaliana GapA* promoter sequence confers light responsiveness and organ-specific expression on a reporter gene in transgenic tobacco plants. To identify potential *cis*-acting sequence elements involved in light and organ-specific regulation of the *A. thaliana GapA* gene, a 1.1-kb *Bam*HI-*Nco*I *GapA* gene fragment containing all of the 5' untranslated sequence and 1,045 bp of additional 5' upstream sequence (Fig. 1) was linked to the *E. coli Gus* reporter gene and mobilized into the binary vector pBI101 (17). *Agrobacterium* strain LBA4404 bearing this plasmid (termed pmW) was used in tobacco leaf disc transformations to obtain transgenic plants. Fifteen independent transgenic plants obtained by this method were vegetatively propagated, and GUS activities were determined from leaf samples (Fig. 2, line mW). As shown in Fig. 2, GUS activities were highly variable among the 15 independent mW lines, with a small number of plants showing no expression above background. It is unlikely that the GUS-negative plants were escapees, since they had been cultured under continuous kanamycin selection. This kind of variation is commonly observed in transgenic systems and may be due to position effects of insert locations. To determine whether the 1.1-kb *GapA* promoter confers expression in an organ-specific manner, two mW lines (mW-3 and mW-12) were chosen, and GUS were assays performed on leaf, stem, and root samples. GUS activities were highest in leaves, lower in stems, and undetectable in roots (Fig. 3A). These relative levels of GUS activity correlate with expression patterns observed for the *GapA* gene in *A. thaliana* plants (5).

To determine whether the 1.1-kb *GapA* promoter is sufficient to confer light responsiveness on the reporter gene and whether the observed variation in GUS activity between different transgenic lines containing the same introduced DNA affects patterns of light-regulated gene expression, plants from the mW-3 and mW-12 lines were dark treated for 5 days and then given 24 h of continuous white light (see Materials and Methods). A 5-day dark treatment was used since our studies with *A. thaliana* show that levels of *GapA* mRNA require this extended period to decline to a basal level (5). Twenty-four hours of white light treatment at the intensity used in these experiments is sufficient to induce maximal levels of *GapA*

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        mW(-1045)
            |
-1045   GGATCCGATTCTAGCTTACACAGCCGGCGCTGAGGTTGAGCAGCTTCAGTGGTCATCTTC

-985    TCAGCCTGATTGGGTTGCCATTGCCTTCTCTAACAAGCTGCAGATTCTCCGGGTCTGATT

-925    ATGTTTTACAGATTTCTGTTGGATACTTGGATGTAAAAACTCGTTGCTATTGTTGATTAA
                                                        m1(-808)
                                                            |
-865    TGATCTTTTGATCTCTGACTCTAAGCCTCATTGAGTTTCTTTCATAGTATGGTCTGTGTA

-805    GGAACTATGTACCTTCAAAATTGTGAATAACTAGCTTTAGCATTGGATTTGATTAGACGT

-745    ATGGAAGTTAGTTTTAACAGAGAGCATGTGGCATAACGGGGAACTTGAAGTAGATTATGA

-685    CTAACACCAGCATTTATGTTTCTCTGCCCTCGAGCTGATGGAGTTGCAGGATTTTGAAGG

-625    AGTGTGTGTATAGAGGTAGTTCTTAACAGAATAATATTTCTCTGCCTCACCATCGTATGG
                                  mH(-535)
                                      |
-565    AATGTGTAGCTTTCTATGGTAGTTGTTATAAGCTTTGTCTCCATTGTCCATGTCATTTTT
                m7(-488)
                    |
-505    GCAATGATACTTAAAACACTTTATGGGTATGTAGATTTGTGAAATTAGGCATTAGTAACA
                                      m8(-410)
                                          |
-445    TCGTACAGATGATGTATTCTTCATTACTTCAATACAGGCATATAGATTCTCTGTCCAACT
                                  mD(-359)
                                      |
-385    TAAAAGGCATACAAGTTCCGTGGTTTAAAAAATCAGCTTTCCTAAATCCTTTGGCCAATA

-325    CTCTCTCAGCCAGTCCTAAGAACCTCTGCTCTAAAAAGTCTGAGCTTTGAAAGGCAGACA
                m9(-247)
                    |
-265    AATGGAGAGCAGCCTTATCCTCCTTTTATAACTGTGACCACACACACAAATGAAGAAGCC

-205    CAAATGAAAGGCCCAAAAATCTAAAAAAAATAGGTGAAGGAGGGACTTTGTGAATATGTGA
                                      m12(-110)
                                          |
-145    TCATAGAAACATGAGGAGACAAGAGAGTGCTAAAATCACAACCATAGAAACAAGTAAGAA
         mAlu(-83)
            |
 -85    AGCTATCCAGAATCTAATCTTATTTGCCTCCAAGTCATCAATTCTTTATCCTATATTACT

 -25    AGTCTCTGCTTCACCAATCCTTTTTGCTCTCCTGTCTGCTACAAACCTTAACTTGGATTC

  36    TTTCTGGTCACCATGGCTTCGGTTACTTTCTCTGTCCCCAAGGTACC
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FIG. 1. Nucleotide sequence of the 5′ upstream (promoter) region of the *GapA* gene from *A. thaliana*. Standard numbering is used and is based on the distance from the transcription start point (+1), as determined by S1 nuclease mapping. The transcription start point, putative TATA box, translation initiation codon (ATG), and Gap boxes are underlined. Breakpoints for *GapA* promoter-*Gus* gene fusions discussed in the text are shown in boldface.

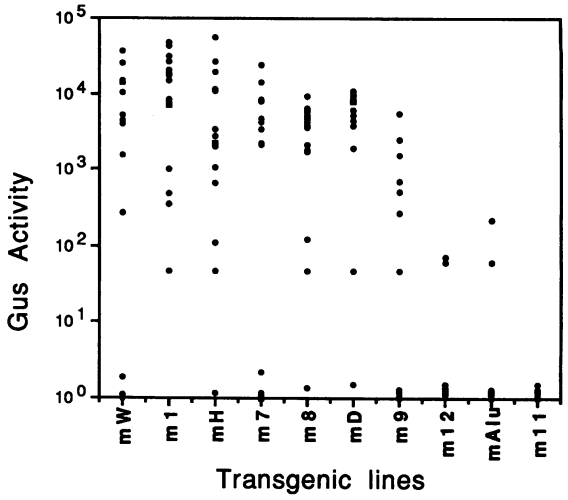

FIG. 2. GUS enzyme activity in transgenic tobacco plants. GUS activities from 15 independent transgenic lines for each construct discussed in the text and shown in Fig. 1 were determined as described by Jefferson et al. (17). GUS activities were measured by enzymatic conversion of 4-methylumbelliferyl glucuronide to 4-methylumbelliferone, which was quantified with a Hoefer TK100 spectrofluorimeter. Enzyme activity is expressed as picomoles of 4-methylumbelliferone per minute per milligram of protein. The GUS activities of 15 independent m11 lines of tobacco are also plotted and were used as a control for these experiments; the m11 construct (not shown in Fig. 1) contains a *GapA* promoter deleted to −22 (lacking the putative TATA box) and linked to the *Gus* gene as described in the text.

expression in both seedlings and mature *A. thaliana* plants (8). For each experiment, several isogenic plants for each line were used and pooled tissue samples were obtained; the dark- and light-treated samples came from the same set of plants. RNA isolated from dark- and light-treated leaf tissue was analyzed by the competitive RT-PCR method to determine levels of *Gus* gene expression. Results typical of those experiments are shown in Fig. 3B. Light induction (comparing levels of gene expression in the light-treated plants with those in the dark-treated plants) was approximately 31-fold in line mW-3 and 27-fold in line mW-12. The magnitude of light induction in the transgenic tobacco plants is similar to that observed for the *GapA* gene in *A. thaliana* plants (8). From the results of these experiments we concluded that the 1.1-kb *GapA* promoter is sufficient to confer organ specificity and light responsiveness on a reporter gene and that independent lines of transgenic tobacco exhibit similar regulatory patterns of the chimeric *GapA* promoter-*Gus* gene.

Characterization of transgenic tobacco plants containing *GapA* promoter deletions. A series of binary vectors containing different sizes of *GapA* promoter fragments was generated and used to construct transgenic tobacco plants. Breakpoints of all of the deletions were determined and are shown in Fig. 1. GUS activities from 15 independent transformants for each promoter construct were determined and are illustrated in Fig. 2. As in the case of the mW transgenic plants, GUS activities are highly variable among independent transgenic lines bearing the same construct; between 10 and 30% of the transgenic lines derived from each construct exhibit no GUS activity. In contrast, all 15 transgenic lines from m12 and mAlu, which contain *GapA* promoter sequences up to −110 and −83, respectively, have extremely low or no GUS activities. In addition, there is a significant drop in mean values of GUS activities between mD and m9 transgenic plants (six- to eightfold difference) compared with the differences among mW, m1, m7, m8, and mD lines (less than threefold difference). These results suggested that the regions between mD and m9 (−359 to −247) and between m9 and m12 (−247 to −110) contain elements important to *GapA* promoter function.

Quantitation of light induction by the competitive RT-PCR method. To identify sequence elements that are necessary for light responsiveness, we chose one transgenic plant that expressed near average GUS activity from eight of the nine *GapA* promoter-*Gus* constructs (all except mH) and propagated them vegetatively to produce enough isogenic plants for light induction studies. These transgenic tobacco plants were dark treated for 5 days and then transferred to continuous white light for 24 h. Total RNA was isolated from dark- and light-treated plants and analyzed by the competitive RT-PCR method (1, 12, 27). In this method, quantitation of mRNA is achieved by including competitive templates which can be amplified by the same primer pair as for the RNA of interest. The PCR products from these two templates can be distinguished by size or differential hybridization. We have con-

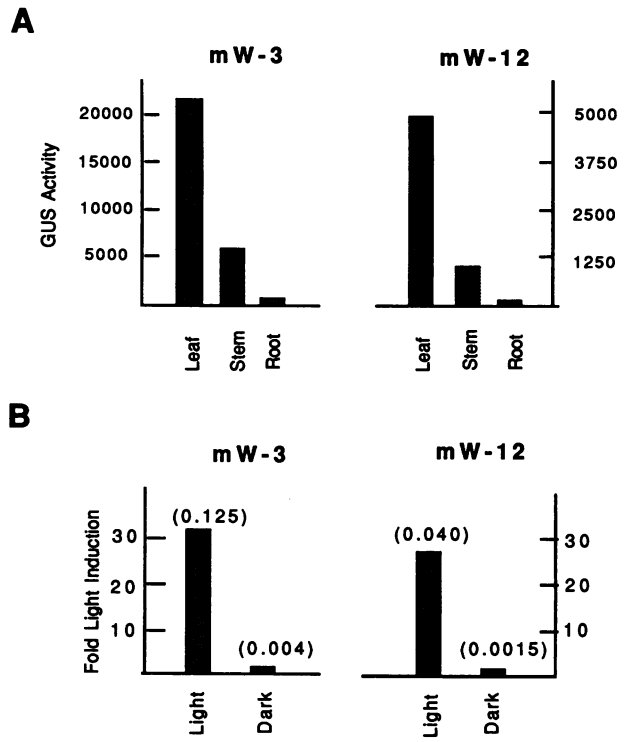

FIG. 3. Organ-specific expression and light induction of the 1.1-kb *GapA* promoter-*Gus* gene construct in mW-3 and mW-12 lines of tobacco. (A) GUS enzyme activities from leaves, stems, and roots of tobacco plants were determined by the method already described. The activity is expressed as picomoles of 4-methylumbelliferone per minute per milligram of protein. (B) Light induction of *Gus* reporter gene expression as measured by competitive RT-PCR and plotted as fold light induction. Numbers shown in parentheses are picograms of *Gus* mRNA per microgram of total RNA.

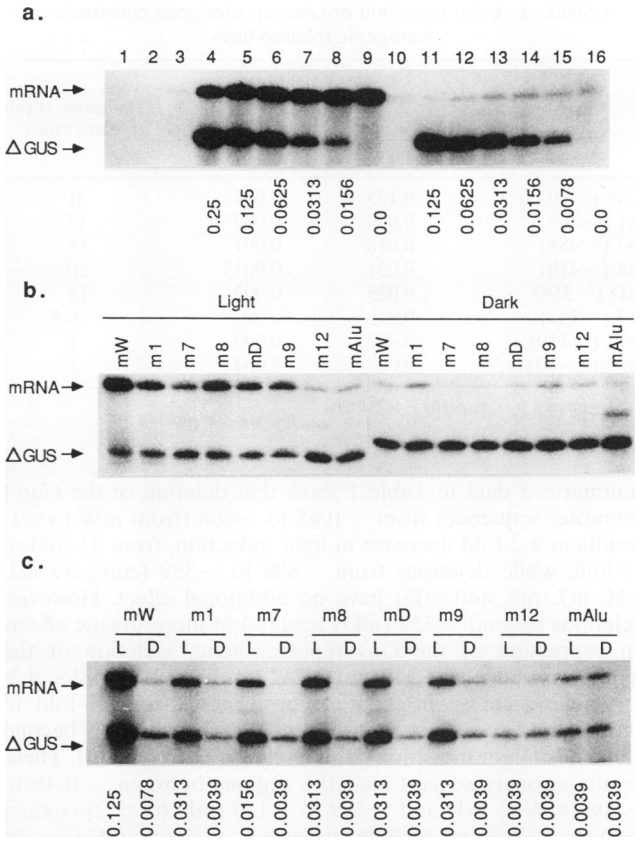

FIG. 4. Determination of *Gus* mRNA levels in transgenic tobacco plants by competitive RT-PCR. Total RNA (1 μg) from dark- and light-treated transgenic tobacco plants was subjected to competitive RT-PCR analysis as described in Materials and Methods. (a) RNA from light-treated (lanes 3 to 9) and dark-treated (lanes 10 to 16) mW plants was used in RT-PCRs with various amounts (picograms) of competitor DNA in each reaction as shown. Lanes 1 and 2 are reactions using RNA from wild-type SR1 tobacco plants without (lane 1) or with (lane 2) prior cDNA synthesis. Lanes 3 and 10 are reactions with RNA samples on which cDNA synthesis was not performed. (b) RNA (1 μg) from 24-h white light-treated plants (Light) and 5-day dark-treated plants (Dark) of each transgenic plant used in cDNA synthesis reactions and then amplified by PCR with 0.015 pg of competitor DNA included in each reaction. (c) Same as panel b except that the amount of competitor DNA used in each reaction was based on the estimated level of *Gus* gene expression. Arrows indicate positions of PCR products derived from *Gus* mRNA (mRNA) and competitor templates (ΔGus).

structed a plasmid clone, pΔGus/BS$^+$, that contains an internally deleted *Gus* structural gene fragment as the competitor template. The chosen primer pair (see Materials and Methods) will produce 585- and 422-bp PCR products from *Gus* messages and competitor templates, respectively. Three sets of experiments were performed to quantify *Gus* mRNA levels of transgenic tobacco plants. First, total RNA (1 μg) isolated from dark-treated and light-treated mW plants (which contain the 1.1-kb *GapA* promoter) was amplified together with variable amounts of competitor template in RT-PCRs (Fig. 4a). These titration experiments allowed us to determine the *Gus* mRNA level in light-treated and dark-treated mW plants by calculating the amount of competitor template required to achieve equal intensity with the *Gus* cDNA band. The results indicate that there are about 0.125 and 0.004 pg of *Gus* cDNA per μg of total RNA from light-treated and dark-treated mW plants, respectively (line mW-3). This 31-fold induction by light is similar to that of the endogenous *GapA* gene in *A. thaliana* (8), suggesting that the mW construct contains all essential *GapA* upstream elements necessary for light regulation. In a second set of experiments, RNAs from dark-treated and light-treated transgenic plants from each of the constructs were amplified with equal amounts of competitor included in each PCR tube (Fig. 4b). Magnitudes of light induction for each transgenic line can be calculated, since equal amounts of competitor template were included in all reactions (27). In addition, the amount of *Gus* message 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 the titration curve from Fig. 4a. To confirm this quantitation, a third set of RT-PCRs (Fig. 4c) in which the reactions shown in Fig. 4b were repeated except that competitor templates corresponding to the estimated amount of *Gus* mRNA for each reaction were used. In this set of reactions, the *Gus* cDNA band and the competitor band in each reaction should exhibit about equal intensity. The results obtained (Fig. 4c) suggest that our calculations based on Fig. 4b results were fairly accurate except that values for some of the dark samples were slightly underestimated (for example, lanes mW-D and m8-D). Combining results from these three experiments should allow us to quantify levels of light induction in transgenic tobacco plants efficiently and fairly accurately. The

TABLE 1. Light induction of *Gus* reporter gene constructs in transgenic tobacco lines

Line	Expression (pg of *Gus* mRNA/μg of total RNA)[a]		Induction (light/dark ratio)
	Light	Dark	
mW (−1045)	0.125	0.004	31
m1 (−808)	0.030	0.002	15
m7 (−488)	0.016	0.001	15
m8 (−410)	0.031	0.0015	20
mD (−359)	0.025	0.002	13
m9 (−247)	0.013	0.002	6.5
m12 (−110)	0.002	0.002	1
mAlu (−83)	0.004	0.004	1

[a] Estimated by competitive RT-PCR.

TABLE 2. Effects of red and blue light on reporter gene expression (*Gus*) in transgenic tobacco lines mW and mD

Treatment[a]	Induction (light/dark ratio)[b]	
	mW (−1045)	mD (−359)
W24	31	13
D		
R4	1.5	1.0
R12	2.7	2.0
R24	5.5	4.0
B4	2.7	2.0
B12	13	7.5
B24	20	10

[a] W24, continuous white light for 24 h; D, 5-day dark treatment; R, continuous red light for 4 (R4), 12 (R12), or 24 (R24) h; B, same as R, using blue light (light sources defined in Materials and Methods).
[b] As measured by competitive RT-PCR.

summarized data in Table 1 show that deletion of the *GapA* promoter sequences from −1045 to −808 (from mW to m1) results in a 2-fold decrease in light induction, from 31-fold to 15-fold, while deletions from −808 to −359 (compare m1, mH, m7, m8, and mD) have no additional effect. However, deletions beyond −359 (mD) resulted in more drastic effects on expression of the *Gus* reporter gene. Deletion of the sequences between −359 and −247 results in an additional 2- to 3-fold decrease in light responsiveness, from 13-fold to about 6-fold (compare mD and m9), while deletions beyond m9 abolish light induction completely (m12 and mAlu). These results strongly suggest that the regions between −1045 to −808 (mW to m1) and −359 to −110 (mD to m12) contain sequences essential for light induction of the *GapA* gene. In our hands, the competitive RT-PCR method produces highly reproducible results, with experimental variations between different experiments generally on the order of 10% or less.

Effects of red and blue light on expression of *GapA* promoter-*Gus* chimeric genes in transgenic tobacco plants. We have previously shown that continuous illumination with white, blue, or red light induces expression of the *GapA* gene in both 7-day-old etiolated seedlings and dark-treated mature *A. thaliana* plants, with blue and white light much more efficient than red light (8). We chose mW (the transgenic line with the 1.1-kb promoter fragment; line mW-3 was used in this analysis) and mD (containing the −359 promoter; the transgenic line with the minimum sequence required for a high level of responsiveness to white light) plants to investigate whether they can respond to blue and/or red light treatment. Transgenic tobacco plants were dark treated for 5 days and then transferred to continuous blue or red light (see Materials and Methods for light source specifications). Total RNA from samples taken after 4, 12, and 24 h of red and blue light treatments was isolated, and the levels of *Gus* mRNA were quantified by competitive RT-PCR. Results of those experiments are summarized in Table 2. Patterns of blue and red light induction of *Gus* mRNA in the mW and mD transgenic lines are very similar, even though the magnitudes of light inductions are different. Illumination with blue light for 24 h resulted in 20- and 10-fold increases in *Gus* mRNA levels (over dark-treated levels) in mW and mD lines, respectively. In contrast, after continuous illumination with red light for 24 h, levels of *Gus* mRNA increased only by 5.5- and 4-fold in mW and mD lines, respectively. As a comparison, after exposure to white light for 24 h, *Gus* mRNA levels increased by 31-fold in the mW line and 13-fold in the mD line. These kinetic patterns and the relative extent of induction by different light spectra are very similar to those of the *GapA* gene in *A. thaliana* (8), suggesting that the pmD construct (−359 promoter) contains *GapA* sequence elements required for responsiveness to blue and red light. In the control experiment, the m12 line, which contains sequences only up to −110 (Fig. 1) and was previously shown to not respond to white light (Fig. 4c), fails to respond to either blue or red light (data not shown).

A tobacco nuclear extract factor binds to *GapA* promoter fragments that have Gap boxes present. We next sought to determine by gel mobility shift assay whether the direct repeats (Gap boxes) or other sequences located within the *GapA* promoter could be sites at which *trans*-acting factors involved in regulation of expression of *GapA* interact. Two DNA probes derived from the *GapA* promoter and encompassing all or some of the Gap boxes (Fig. 1) were used in the binding experiments shown in Fig. 7. ALRE is a 82-bp *Hin*dIII-*Eco*RI fragment containing *GapA* sequences from −276 to −195. AM9 is 53 bp in size and encompasses the −247 to −195 region of the *GapA* promoter (probe preparation is described in Materials and Methods). The ALRE probe contains three copies of the Gap boxes, while the AM9 probe lacks the most distal copy of the Gap box. Each probe gave a prominent retarded band when tobacco nuclear extracts were the source of DNA-binding proteins (Fig. 5, lanes 2 and 10). These experiments also showed that the binding efficiency of the AM9 probe appears to be less than that of the ALRE probe (Fig. 5; compare lanes 2 and 10). Binding of the labeled ALRE probe is also abolished by unlabeled ALRE and AM9 probes, although with different degrees of efficiency. Larger amounts of unlabeled AM9 competitor than of unlabeled ALRE competitor are required (Fig. 5, lanes 6 to 8) to completely abolish ALRE probe binding (Fig. 5, lanes 3 to 5).

Gap boxes are binding sites for a tobacco nuclear extract factor. The decreased binding of the AM9 probe, which differs from the ALRE probe by having one fewer Gap box, suggested to us that the Gap boxes are functional sites for the in vitro binding seen in our gel mobility shift assays. To directly test this hypothesis, an oligonucleotide was used in the construction of a Gap box monomer and dimer to be used as competitors in ALRE binding assays. As described in Materials and Methods, restriction fragments containing the dimer (A1-D) and the monomer (A1-M) were gel purified and used as competitors in gel mobility shift assays. We found that a 360-fold molar excess of either A1-D or A1-M efficiently competed for binding of ALRE to the tobacco nuclear extract factor (Fig. 6, lanes 3 and 4). It should be pointed out that in preliminary experiments, we found that the presence of even very large amounts (up to a 4,000-fold molar excess) of

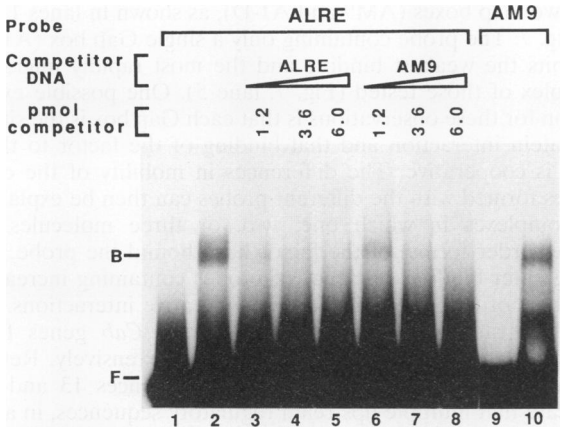

FIG. 5. ALRE and AM9 (*GapA* promoter fragments) bind to a tobacco nuclear factor. The ALRE fragment (lanes 1 to 8) and AM9 fragment (lanes 9 and 10) were used as probes in gel mobility shift assays with tobacco nuclear extract (5 μg) as the source of binding proteins. Lanes 1 and 9 are ALRE and AM9 probe control reactions without added nuclear extract. Lanes 2 and 10 are ALRE and AM9 probe control reactions with nuclear extract (5 μg) but without specific competitor added. Unlabeled ALRE (lanes 3 to 5) and AM9 (lanes 6 to 8) were used as competitors in the ALRE binding experiments illustrated; the amount of ALRE or AM9 competitor is shown above each lane. B and F indicate positions of bound and free probes.

nonspecific competitors such as poly(dI-dC) · poly(dI-dC) and linearized plasmid DNA (pBS^+) had no apparent effect on ALRE binding (data not shown); the binding of ALRE and AM9 in gel mobility shift assays is therefore most likely due to specific interactions with the Gap box sequences that are present and not to specific or nonspecific interactions with other *GapA* promoter or plasmid-derived polylinker sequences also found in ALRE and AM9.

In vitro binding efficiencies are correlated with Gap box copy number. In the experiment illustrated in Fig. 5, we found that the ALRE and AM9 probes differed in their binding efficiencies. The ALRE probe contains three Gap boxes, while the AM9 probe lacks the most distal of these boxes and has

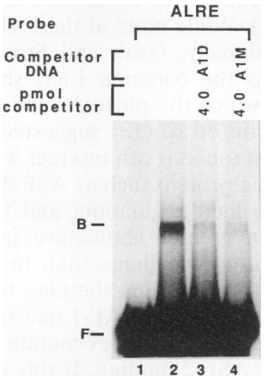

FIG. 6. Tobacco nuclear factor binding to the ALRE probe is competed for by a synthetic consensus Gap box sequence. Lanes: 1, free ALRE probe (no nuclear extract); 2, ALRE incubated with 5 μg of tobacco nuclear extract; 3 and 4, effects of preincubating tobacco nuclear extract (5 μg) with either the Gap box dimer (A1-D) or the Gap box monomer (A1-M). The amount of competitor is shown above lanes 3 and 4. B and F indicate positions of bound and free probes.

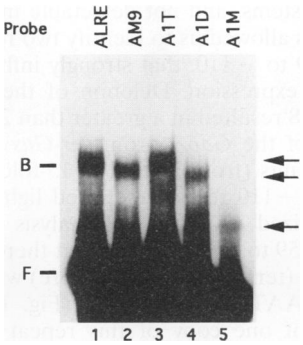

FIG. 7. In vitro binding efficiency of a tobacco nuclear factor decreases with decreasing Gap box copy number. The ALRE and AM9 probes are derived from the *GapA* promoter region and contain three and two copies of the Gap boxes; A1-T, A1-D, and A1-M probes are synthetic and contain three, two, and one copy of the consensus Gap box (see Materials and Methods for details on probes). B and F indicate positions of bound and free probes, and arrows mark the positions of complexes differing in electrophoretic mobility.

reduced binding efficiency. In our in vivo light induction studies (Fig. 4c and Table 1), we found that the mD tobacco line (−359 promoter; three Gap boxes present) exhibited a 13-fold light induction, while the m9 tobacco line (−247 promoter, two Gap boxes) had a 6-fold light induction. We decided to use the synthetic Gap boxes as probes in gel mobility shift assays to test whether Gap box copy number correlates with in vitro binding efficiency. Figure 7 illustrates binding of the *GapA* promoter-derived probes ALRE and AM9 and of the synthetic Gap box trimer (A1-T), dimer (A1-D), and monomer (A1-M). As in previous experiments, a more prominent retarded band is observed with the ALRE probe than when AM9 is used (compare Fig. 7, lanes 1 and 2, and Fig. 5, lanes 2 and 10, for independent experiments). Not apparent in the initial experiments but clearly visible in Fig. 7 (note arrows) are differences in the relative mobilities of the complexes formed between ALRE and AM9 probes and a tobacco nuclear factor (lanes 1 and 2). The ALRE probe (containing three Gap boxes) forms a more slowly migrating complex than does the AM9 probe (two Gap boxes). A similar pattern of complex formation is observed for the synthetic probes A1-T and A1-D; i.e., A1-D binds with reduced efficiency compared with A1-T or ALRE, and the electrophoretic mobility of the complex formed with A1-D is comparable to that formed with AM9. In the same experiment, weak binding can be observed between a nuclear factor and the A1-M probe (Fig. 7, lane 5), and the complex has an apparent mobility that is greater than that of the complexes formed when A1-T and A1-D probes are used in the binding reaction.

DISCUSSION

We have presented evidence that 1.1 kb of *GapA* promoter sequence from *A. thaliana* is sufficient to confer both light inducibility and organ specificity in transgenic tobacco plants. When transgenic tobacco plants containing this *GapA* upstream fragment driving a *Gus* reporter gene were transferred from dark to light conditions, the *Gus* mRNA levels in the light treated plants were 31-fold higher than those of dark-treated plants (Fig. 4c and Table 1). Regulation of *Gus* gene expression in these same transgenic tobacco plants also showed the patterns of organ specificity expected of *A. thaliana GapA*, with expression (measured by GUS enzyme assays) highest in

leaves, lower in stems, and not detectable in roots (Fig. 3A). Deletion analysis allowed us to identify two regions, −1045 to −808 and −359 to −110, that strongly influence light regulation of *GapA* expression. Deletion of the region between −1045 and −808 resulted in a greater than 2-fold decrease in light induction of the *GapA* promoter-*Gus* fusions in transgenic tobacco plants (from 31-fold to 15-fold), while deletion of the −359 to −110 region abolished light induction completely (summarized in Table 1). Analysis of the DNA sequence from −359 to −110 reveals that there are three direct decamer repeats (termed Gap boxes here) with the consensus sequence 5′-CAAATGAA(G/A)A-3′ (Fig. 1) within this region. Deletion of one copy of this repeat from the *GapA* promoter reduced light induction a further 2- to 3-fold (from 13-fold to 6-fold; compare mD and m9 in Fig. 4c and Table 1), while deletion of all three copies of the direct repeats abolished light induction completely (Fig. 4c, lanes m12 and mAlu). A separate analysis of the *A. thaliana GapB* gene, which is coordinately regulated by light with the *GapA* gene (8), reveals that four copies of similar direct repeats with the consensus sequence 5′-ATGAA(G/A)A-3′ exist in the *GapB* promoter (−261 to −173). Deletion experiments with the *GapB* promoter showed that loss of two of the four direct repeats significantly decreased light inducibility and deletion of all four direct repeats abolished light induction completely (20).

Gel mobility shift assays using *GapA* promoter sequences from −276 to −195 (ALRE probe) and from −247 to −195 (AM9 probe), which differ in part by one Gap box, showed that both of these fragments give prominent retarded bands when tobacco nuclear extracts are used as the source of nuclear proteins (Fig. 5, lanes 2 and 10). The efficiency of in vitro binding mirrored the extent of in vivo light induction in that the AM9 probe, containing one less Gap box than the ALRE probe, binds with reduced efficiency compared with ALRE, much as light induction is reduced when one Gap box is deleted from the *GapA-Gus* fusions in transgenic tobacco plants. As other sequences besides the direct repeats (Gap boxes) are present in the ALRE and AM9 probes, the binding between these probes and the tobacco nuclear factor could involve those nonrepetitive sequences, the direct repeats, or some combination of both. We then used oligonucleotides to construct a series of synthetic Gap boxes, comprising a monomer, a dimer, and a trimer of the consensus Gap box sequence. Binding of the ALRE probe with the tobacco nuclear extract factor was eliminated when either the Gap box dimer (A1-D) or the monomer (A1-M) was present as a competitor during the preincubation (Fig. 6, lanes 3 and 4). The synthetic Gap boxes by themselves bind to a component of the tobacco nuclear extract when they are used as probes, with their apparent relative binding efficiencies directly correlated with Gap box copy number (Fig. 7, lanes 3 to 5); i.e., binding efficiency increases with increasing Gap box copy number. The parallel between the results of separate in vivo *GapA* and *GapB* promoter analyses together with results from in vitro gel mobility shift assays are what lead us to suggest that the direct repeats that we have termed Gap boxes are important for light induction of the *GapA* gene of *A. thaliana*.

In addition to the differences in binding efficiencies illustrated in Fig. 7, there are differences in mobilities of the complexes formed between ALRE and AM9 and the tobacco nuclear factor and between the mobilities of the complexes that are observed when synthetic consensus Gap box probes are tested (Fig. 7; note positions of arrows). Probes containing three Gap boxes (ALRE and A1-T) form a more slowly migrating complex with nuclear factor than do probes containing two Gap boxes (AM9 and A1-D), as shown in lanes 1 to 4 of Fig. 7. The probe containing only a single Gap box (A1-M) exhibits the weakest binding and the most rapidly migrating complex of those tested (Fig. 7, lane 5). One possible explanation for these observations is that each Gap box is the site of a protein interaction and that binding of the factor to those sites is cooperative. The differences in mobility of the complexes formed with the different probes can then be explained as complexes in which one, two, or three molecules (or higher-order forms) of the factor have bound the probe, and the greater binding efficiency of probes containing increasing numbers of Gap boxes is due to cooperative interactions.

The upstream sequences of *RbcS* and *Cab* genes from several plant species have been analyzed extensively. Results from these studies (for reviews, see references 13 and 22) indicate that multiple upstream regulatory sequences, in addition to basal promoter elements such as the TATA box, are required for light regulation. Functions of these *cis*-acting regulatory elements can be divided into two groups (13): the light-responsive element (LRE), which is absolutely required for light induction; and accessory elements, which affect only the level of expression conferred by the LRE. The GT-1 factor binding site from the promoter region of the pea *RbcS3A* gene is the only LRE that has been identified (15, 21), although several potential candidates exist (3, 9, 31). The repeated elements that we have identified (Gap boxes) in the promoter regions of *GapA* and *GapB* genes from *A. thaliana* may comprise an LRE for the following reasons. First, we have shown that the extent of light induction of the *Gus* reporter gene correlates positively with the copy number of Gap boxes within the *GapA* upstream region. Second, using gel mobility shift assays, we have identified a nuclear factor from tobacco that specifically binds to the Gap boxes. The binding affinity of this nuclear factor to various *GapA* promoter fragments and to synthetic Gap boxes is also directly related to the copy number of the Gap boxes within these fragments. While these results provide strong evidence that the Gap boxes play a role in light regulation of the *GapA* gene, a more definitive proof is whether the Gap box multimer can confer light responsiveness on a minimal promoter that is normally not light regulated (21). Those experiments are currently under way.

The Gap box consensus sequence does show strong a similarity to a conserved sequence motif, 5′-ATGATAAGG-3′, that is present in almost all *RbcS* and *Cab* genes (4, 7, 11, 13, 14, 16, 22). This element was designated as the GA-1 site by Gilmartin et al. (13). While none of these elements have been characterized functionally, Grob and Stüber (16) proposed that this element is the common LRE shared by all light-regulated genes in which the phytochrome-mediated pathway is involved. Gilmartin et al. (13) suggested that two distinct nuclear factors from tobacco can interact with this element: a GATA motif-binding protein such as ASF-2 (21), which is not directly involved in light regulation; and the GAF-1 factor, which is present in greater abundance in nuclear extracts prepared from light-grown plants than in those from dark-treated plants. It is possible that the Gap boxes that we have identified interact only with a GAF-1 like factor and not with ASF-2, since none of the Gap boxes contain the crucial GATA motif necessary for ASF-2 binding. If this is indeed the case, the results from our transgenic tobacco studies should provide direct in vivo evidence to indicate that GA-1 like elements are involved in light regulation of gene expression. However, further detailed analysis is required to prove that all of these GA-1 elements and the GAF-1 factor are involved in light regulation and to determine whether the Gap box and GA-1 box interact with the same factor.

Our previous results (8) showed that continuous illumination with white, blue, or red light can induce expression of *GapA* and *GapB* genes in dark-treated mature and 7-day-old etiolated seedling stage *A. thaliana* plants, with blue and white light much more efficient than red light. These results suggest that both phytochrome- and blue light receptor-mediated pathways are involved in *GapA* regulation. These two pathways could act on separate *cis*-acting regulatory elements or they could converge to act on the same *cis*-acting regulatory elements in transcriptional-level regulation of *GapA* gene expression. Our transgenic tobacco studies show that the mD line, which contains the *GapA* promoter sequence up to −359, is sufficient to confer both red and blue light responses (Table 2). In contrast, the m12 line (deletion up to −110; Fig. 1), in which the three Gap boxes have been deleted, is not responsive to light, regardless of light spectra and intensity (data not shown). These results suggest that the *GapA* promoter region between −359 and −110 contains sequence information required for responding to red and blue light and that further analysis of this region might be revealing in terms of dissecting the mechanism by which red and blue light signals are transduced at the molecular level.

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T.R.C. and S.-C.P. contributed equally to this work.

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