Mutations Affecting Light Regulation of Nuclear Genes Encoding Chloroplast Glyceraldehyde-3-Phosphate Dehydrogenase in Arabidopsis[1]

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Expression of nuclear genes that encode the A and B subunits of chloroplast glyceraldehyde-3-phosphate dehydrogenase (*GAP*A and *GAP*B) of Arabidopsis is known to be regulated by light. We used a negative selection approach to isolate mutants that were defective in light-regulated expression of the *GAPA* gene. Two dominant mutants belonging to the same complementation group, *uga*1-1 and *uga*1-2, were then characterized. These two mutants showed a dramatic reduction in *GAPA* mRNA level in both mature plants and seedlings. Surprisingly, mutations in *uga*1-1 and *uga*1-2 had no effect on the expression of *GAP*B and several other light-regulated genes. In addition, we found that the chloroplast glyceraldehyde-3-phosphate dehydrogenase enzyme activity of the mutants was only slightly lower than that of the wild type. Western-blot analysis showed that the GAPA protein level was nearly indistinguishable between the wild-type and the *uga* mutants. These results suggested that posttranscriptional control was involved in the up-regulation of the GAPA protein in the mutants. The *uga*1-1 mutation was mapped to the bottom arm of chromosome V of the Arabidopsis genome.

Transcription is one of the primary steps at which light regulates gene expression in plants (Terzaghi and Cashmore, 1995). Two classes of photoreceptors, phytochrome and blue light/UV-A receptor (cryptochrome), are involved in the regulation of photosynthetic genes (Batschauer, 1998; Briggs and Huala, 1999; Deng and Quail, 1999; Fankhauser and Chory, 1999). It has been suggested that eukaryotic phytochromes are Ser/Thr kinases with a two-component His kinase ancestry (Yeh et al., 1997; Yeh and Lagarias, 1998; Fankhauser and Chory, 1999; Fankhauser et al., 1999). Five phytochrome genes have been identified in Arabidopsis (Clack et al., 1994; Quail et al., 1995; Quail, 1997). Current evidence indicates that the different phytochromes may have distinct functions (Quail et al., 1995; Quail, 1997). Genetic and molecular studies have led to the identification of four blue-light photoreceptors in Arabidopsis (Briggs et al., 2001). CRY1 (HY4) and CRY2/PHH1 have partial overlapping functions in promoting anthocyanin formation and inhibiting hypocotyl elongation (Ahmad and Cashmore, 1993; Ahmad et al., 1995; Lin, 2000), whereas PHOT1/NPH1 and PHOT2 regulate phototropism, stomatal opening, and chloroplast movement (Liscum and Briggs, 1995; Briggs and Huala, 1999; Kinoshita et al., 2001; Sakai et al., 2001). In addition, the mutations in *CRY*1 and *CRY*2 genes affect blue-light-mediated regulation of photosynthetic gene expression (Ahmad et al., 1995; Conley and Shih, 1995; Mazzella et al., 2001).

Several mutants affecting the light signal transduction pathway appear to be defective in genes that encode transcription factors. *PIF3* was found not only to interact directly with PhyB but also with the promoters of many light-regulated genes (Ni et al., 1999; Martinez-Garcia et al., 2000). The *hfr1/rsf1/rep1* mutants, on the other hand, appeared to be specific for PhyA pathway (Fairchild et al., 2000; Fankhauser and Chory, 2000; Soh et al., 2000). The *HFR1* gene product is a bHLH protein and, therefore, a putative DNA-binding protein (Fairchild et al., 2000; Soh et al., 2000). It was also found to interact with *PIF3* (Ni et al., 1999; Fairchild et al., 2000; Martinez-Garcia et al., 2000). Other phytochrome-specific intermediates have also been cloned via the isolation of mutants. The *HY5* gene product was shown to be a basic Leu Zipper transcription factor that interacts with light-responsive promoters (Chattopadhyay et al., 1998b).

A number of cis-acting elements, including GT elements, G boxes, I boxes, CGF element, and CCA element, have been characterized from several photosynthetic genes, including *RBC*S and *LHC*B, the nuclear genes encoding the small subunit of Rubisco and light harvest complex proteins, respectively (Donald and Cashmore, 1990; Gilmartin et al., 1990; Anderson et al., 1994; Kenigsbuch and Tobin, 1995; Terzaghi and Cashmore, 1995; Wang et al., 1997b). Based on in vitro-binding assays, genes that encode GBF, GT1, and CCA1 factors have been identified in Arabidopsis. A survey of the Arabidopsis genomic sequences indicated that each of these genes belongs to a small gene family, with a highly conserved sequence in the putative DNA-binding domains. To

[1] This work was supported by the U.S. Department of Agriculture National Research Initiative Competitive Grants Program (grant no. 9600717 to M.-C.S.).

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Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.007849.

show which member(s) in the gene family is involved in light regulation, it is essential to establish a direct link between the in vitro-binding activities and the in vivo function of transcription activation. This line of evidence is mostly lacking, with the exception of *CCA*1, in which it was shown that transgenic Arabidopsis plants expressing antisense RNA for CCA1 showed reduced phytochrome induction of the endogenous *LHCB1-3* gene (Wang et al., 1997b).

We have been studying light regulation of two nuclear genes (*GAP*A and *GAP*B) that encode chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Arabidopsis. In higher plants, there are two chloroplast GAPDH isozymes, with subunit structures of A_4 and A_2B_2, which are key enzymes in the photosynthetic carbon fixation cycle (Cerff, 1982). In previous studies, we showed that the expression of these two genes is coordinately regulated by light at the transcriptional level in tobacco (*Nicotiana tabacum*) and Arabidopsis (Shih and Goodman, 1988; Dewdney et al., 1993). Several cis-acting elements and their cognate binding factors of both *GAP*A and *GAP*B genes were identified (Conley et al., 1994; Kwon et al., 1994; Park et al., 1996; Chan et al., 2001). In etiolated seedlings, a short light pulse can induce transient increases of *GAP*A and *GAP*B mRNA levels. However, this induction cannot be reversed by subsequent far-red light treatment (Dewdney et al., 1993). These regulatory patterns are distinct from those of the pea (*Pisum sativum*) *RBC*S genes (Kaufman et al., 1984) and Arabidopsis LHCB genes (Karlin-Neumann et al., 1988), in which the effect of a short red light pulse can be reversed by a subsequent far-red light treatment. Continuous exposure of dark-treated mature plants or etiolated seedlings to red, blue, or white light is required for sustained high-level expression of *GAP*A and *GAP*B genes in Arabidopsis, with blue and white light much more efficient than red light (Dewdney et al., 1993; Conley and Shih, 1995). Our results indicated that this effect is mediated by a combination of phytochromes and the blue light photoreceptor encoded by the *CRY*1 (*HY*4) gene (Conley and Shih, 1995). Results from saturation linker scan mutagenesis of the *GAP*B promoter constructs in transgenic Arabidopsis suggest that a single cis-acting element may respond to more than one photoreceptor (Chan et al., 2001).

In addition to the identification of cis-acting elements, we are interested in obtaining mutations that affect light regulation of *GAP*A and *GAP*B genes. Although a variety of photomorphogenic mutants are available in Arabidopsis, most of these mutants are defective in early steps in light-signaling pathways or are not defective in *GAP* gene expression (Conley and Shih, 1995; M.-C. Shih, unpublished data). Therefore, we used a negative selection scheme to isolate regulatory mutants that are defective in light activation of the *GAP*A gene. Here, we report the characterization of two of these mutants. Our results indicated that these two mutations affect very downstream steps in light signal transduction pathways leading to the activation of the *GAP*A gene.

RESULTS

Selection of Mutants Affecting *GAP*A Gene Expression

In the presence of allyl alcohol, wild-type plants with functional ADH enzyme will die because of the conversion of allyl alcohol to toxic aldehyde by ADH. In contrast, plants without functional ADH can survive allyl alcohol treatment. Negative selection schemes using ADH as a selectable marker were used to isolate *aar* mutants, which are defective in hypoxic induction of *ADH* (Conley et al., 1999), and *cue* mutants, which are defective in controlling the expression of *LHCB*3 (Li et al., 1995; Lopez-Juez et al., 1998). We designed a similar selection scheme to isolate regulatory mutants that are defective in light activation of the *GAP*A gene.

In the current scheme, we first transformed an Arabidopsis *ADH* null mutant, *adh*1-2, with a construct that puts ADH and β-glucuronidase (GUS) coding sequences under the control of separate *GAP*A promoters (see "Materials and Methods" for details). Several independent transgenic lines that have GUS and ADH activity were obtained. In all of these lines, the expression of *ADH* and *GUS* transgenes was regulated by light similar to that of the *GAP*A gene. One of these lines, AG-5G, was chosen for mutagenesis. In 5-d-old etiolated AG-5G seedlings, the accumulation of ADH activity reached a steady-state level after 12 to 24 h of white light treatment, similar to that of the endogenous *GAP*A gene (Dewdney et al., 1993; Conley and Shih, 1995). Titration experiments indicated that 7.5 mM allyl alcohol is needed to cause 100% lethality of the 24-h light-treated seedlings.

To obtain mutants that underexpress ADH, a total of 50,000 M_2 seeds of AG-5G were germinated on filter papers in the dark for 5 d and then subjected to 24 h of white light treatment. The filters were then transferred onto medium containing 7.5 mM allyl alcohol. After 2 h, filters were moved onto a fresh agar medium. The surviving plants, which must have lacked ADH activity, were assayed for GUS activity in leaves. Among the 99 plants that survived allyl alcohol selection, 77 were GUS positive and 22 were GUS negative. Only seven of the latter mutants survived long enough to produce seeds, whereas the other 15 died or failed to set seeds after transfer to the soil. The lethality could be because of mutations in essential genes or the occurrence of multiple mutations in these plants. The surviving $adh^{-}gus^{-}$ plants, designated as *uga* (underexpressor of *GAPA*), are presumably defective in regulatory genes that control the expression of *GAP*A. We characterized two of these mutants, *uga*b3 and *uga*b9, as described below.

Table I showed that the F_1 progeny from crosses between line AG-5G and each of the two *uga* mutants had low GUS activity (GUS^-), indicating that all of them exhibited the mutant phenotype. These results suggested that both *uga*b3 and *uga*b9 mutations are dominant. However, the F_2 progeny from both crosses deviated significantly from the expected 1:3 ratio. GUS^+ and GUS^- plants in the F_2 progeny from the cross between AG-5G and *uga*b3 showed a 1:6 ratio, whereas the cross between AG-5G and *uga*b9 produced a 1:8 ratio. One possible explanation for this observation could be that the presence of the transgene resulted in the expression of ADH at abnormally high level in leaves. This may have caused a high rate of lethality to the individual plants that show the wild-type phenotype, hence yielding a lower than expected wild-type progeny. Table I also shows that the F_2 progeny of the *uga*b3 × *uga*b9 cross gave a GUS^+:GUS^- ratio of 0:132, i.e. all the F_2 progeny were mutant. This indicated that the mutations in b3 and b9 belonged to the same complementation group. The *uga*b3 and *uga*b9 mutants were hence renamed *uga1-1* and *uga1-2*, respectively.

Effects of *uga* Mutations on the Expression of *GAP*A

To quantify the effect of *uga* mutations on the expression of *GAP*A::*GUS* and *GAP*A::*ADH* transgenes, we compared the levels of ADH and GUS activities in wild-type AG-5G and *uga* mutants in 5-d-old etiolated seedlings subjected to 24 h of white light treatment. Both mutants showed a moderate reduction in ADH and GUS activity compared with the AG-5G line (Fig. 1A). When 4-week-old light-grown plants were assayed for reporter gene activities, the difference between the wild type and the mutants was far more dramatic. As shown in Figure 1B, the two mutants exhibited 30% and 23% of the wild-type level of steady-state ADH activity, whereas their GUS activity was reduced to 3% and 5% of that in the wild type. These results indicated that both mutants are impaired in the expression of both the *ADH* and *GUS* reporter genes, especially so in mature plants.

To determine the effects of *uga* mutations on the expression of the endogenous *GAPA* gene, mRNA levels from 5-d-old light-grown Arabidopsis seedlings were compared with those from 5-d-old etiolated seedlings. The results from one set of representative northern blots were illustrated in Figure 2A. These experiments were repeated three times and the resulting blots were quantified using the *GAP*A mRNA levels from light-grown AG5G as 100% (Fig. 2B). In greening seedlings, *GAP*A mRNA levels in *uga*1-1 and *uga*1-2 were 2- to 3-fold lower than the level in AG-5G (Fig. 2).

To determine the effects of *uga* mutations on the expression of *GAP*A in mature plants, mRNAs from light-grown 4-week-old plants were compared with those of plants that were light grown for 4 weeks

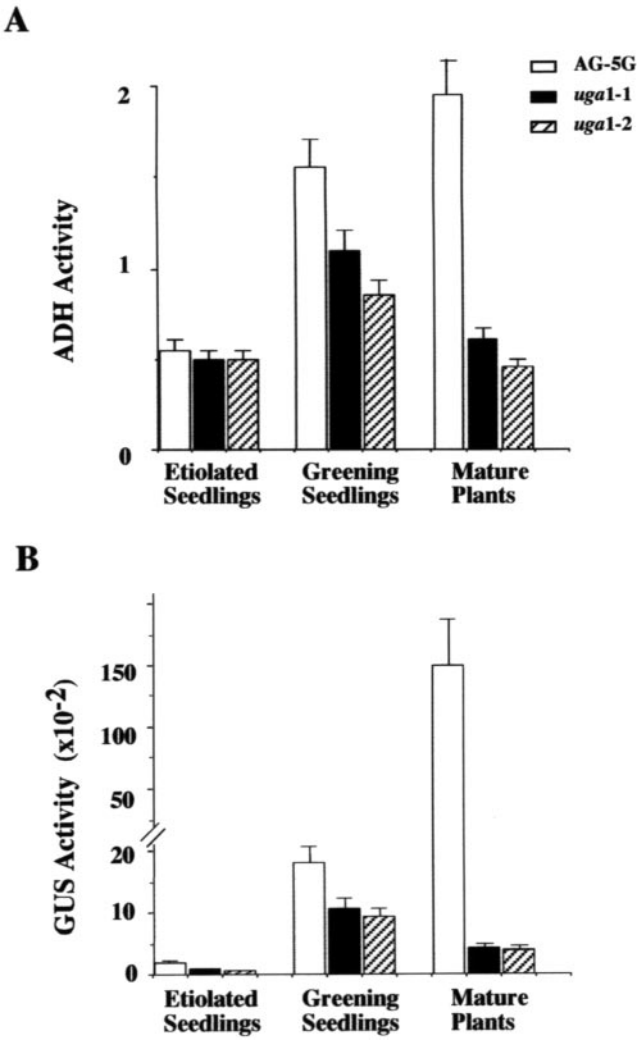

Figure 1. Effects of *uga* mutations on *GAP*A::*ADH* and *GAP*A::*GUS* transgenes. GUS and ADH activities of AG-5G and *uga* mutants in 5-d-old etiolated seedlings, greening seedlings, and 4-week-old plants were determined as described in "Materials and Methods." A, Unit of ADH enzyme is defined as an increase in A_{340} of 1 min mg protein^{-1}. B, GUS activity is expressed as pmol 4-methylumbelliferone min^{-1} mg^{-1} protein. The data presented are the average of three independent treatments. Plants grown at different times were used for replicated treatments. For each treatment, a total of about 500 plants was pooled and used for protein extracts preparation. Error bars = SDS.

Table I. *Genetic analysis of* uga *mutants*

GUS enzymatic assays were used to assess GUS^+ and GUS^- phenotype. F_1 and F_2 progeny with GUS activity comparable with the homozygous AG-5G line as shown in Fig. 1B were assigned as GUS^+, whereas those with GUS activity similar to homozygous mutants were assigned as GUS^-.

Cross	GUS^+:GUS^- in F_1	GUS^+:GUS^- in F_2
AG-5G × *uga*b3	0:13	6:37
AG-5G × *uga*b9	0:28	5:40
*uga*b3 × *uga*b9	0:15	0:132

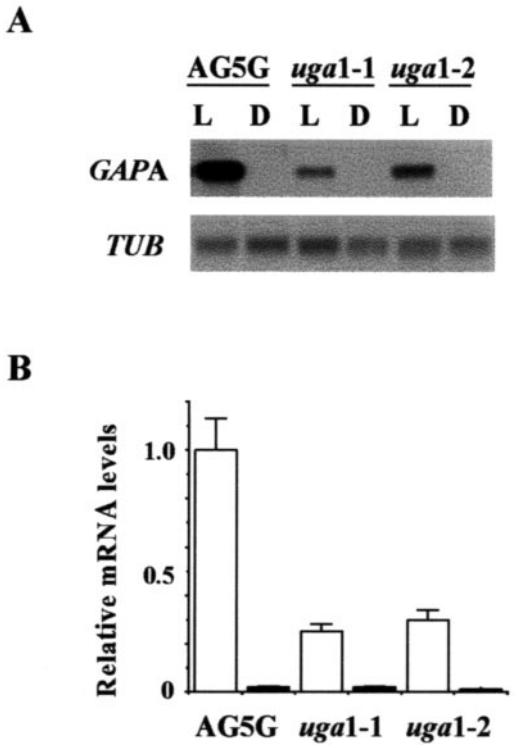

Figure 2. Effects of *uga* mutations on the expression of *GAP*A in seedlings. A, Total RNAs from seedlings grown in continuous light (L) or complete darkness (D) for 5 d were isolated and analyzed by northern-blot analysis. Representative data are from gels loaded with 5 μg RNA lane^{-1} and probed with radiolabeled *GAP*A or *TUB*. B, Each northern-blot analysis was repeated three times using RNA samples from plants grown at different times. Relative densitometric values were obtained by first taking the ratio of *GAP*A signal intensity over that of the corresponding TUB signal for each lane, and then dividing that by the ratio to obtain obtained for light-grown AG-5G. Therefore, relative densitometric value for light-grown AG5G is taken as 1. Error bars = SDs.

and then dark adapted for 5 d (Fig. 3A). The quantification data showed that levels of *GAPA* mRNA in *uga*1-1 and *uga*1-2 were more than 20-fold lower than that of AG-5G in 3-week-old plants (Fig. 3B). Consistent with our prior results (Dewdney et al., 1993; Conley and Shih, 1995), the data also showed that there was barely detectable *GAP*A mRNA in both etiolated seedlings (Fig. 2) and dark-adapted mature plants (Fig. 3). The combined results demonstrated that *uga*1-1 and *uga*1-2 mutations affect the expression of both the endogenous *GAP*A gene and the *GAP*A::*GUS* and *GAP*A::*ADH* transgenes. Therefore, it is likely that these mutations are defective in a regulatory gene that controls the expression of *GAP*A. However, the observation that the *uga* mutations had more severe effects on the mRNA levels of *GAP*A in mature plants than in seedlings suggested that the transcription complexes required for *GAP*A activation are not identical in these two stages.

Effects of *uga* Mutations on the Expression of Other Light-Regulated Genes

Because *GAP*A and *GAP*B gene products constitute subunits of the GAPDH holoenzyme (Cerff, 1982), it is reasonable to expect that these two genes are controlled by the same regulatory mechanism. Therefore, we compared the *GAP*B mRNA levels between wild-type and the two *uga* mutants in mature light-grown and dark-adapted plants by northern-blot analysis (Fig. 4A). Surprisingly, the levels of *GAP*B mRNA in *uga*1-1 and 1-2 were similar to that of wild type (Fig. 4, A and B). Next, we determined the effects of *uga* mutations on the expression of two other carbon fixation genes, *TIM* and *FBA*. We found that the kinetics of mRNA accumulation for these two genes during light induction were identical to those of *GAP*A and *GAP*B (M.-C. Shih, unpublished data). However, no significant difference in the transcription of these genes was observed between the *uga* mutants and AG-5G (Fig. 4).

Because combinatorial cis-acting elements are required to confer light responsiveness of light-regulated promoters in plants (Terzaghi and Cashmore, 1995; Puente et al., 1996; Chattopadhyay et al., 1998a), it is possible that *uga* mutations affect the expression of genes from different metabolic path-

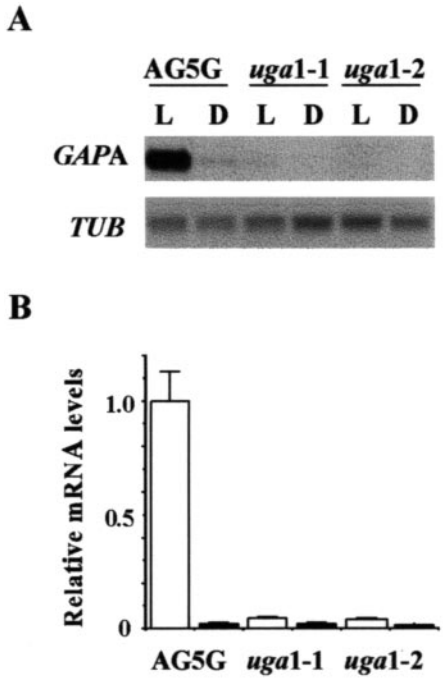

Figure 3. Effects of *uga* mutations on the expression of *GAP*A in mature plants. A, Total RNAs from light-grown 4-week-old plants (L) were compared with those of plants that were light grown for 4 weeks and then dark adapted for 5 d (D) were isolated and analyzed by northern-blot analysis. Representative data are from gels loaded with 5 μg RNA lane^{-1} and probed with radiolabeled *GAP*A or *TUB*. B, Each northern-blot analysis was repeated three times and quantified as described in Figure 2. The average densitometric value for light-grown AG5G is taken as 1. Error bars = SDs.

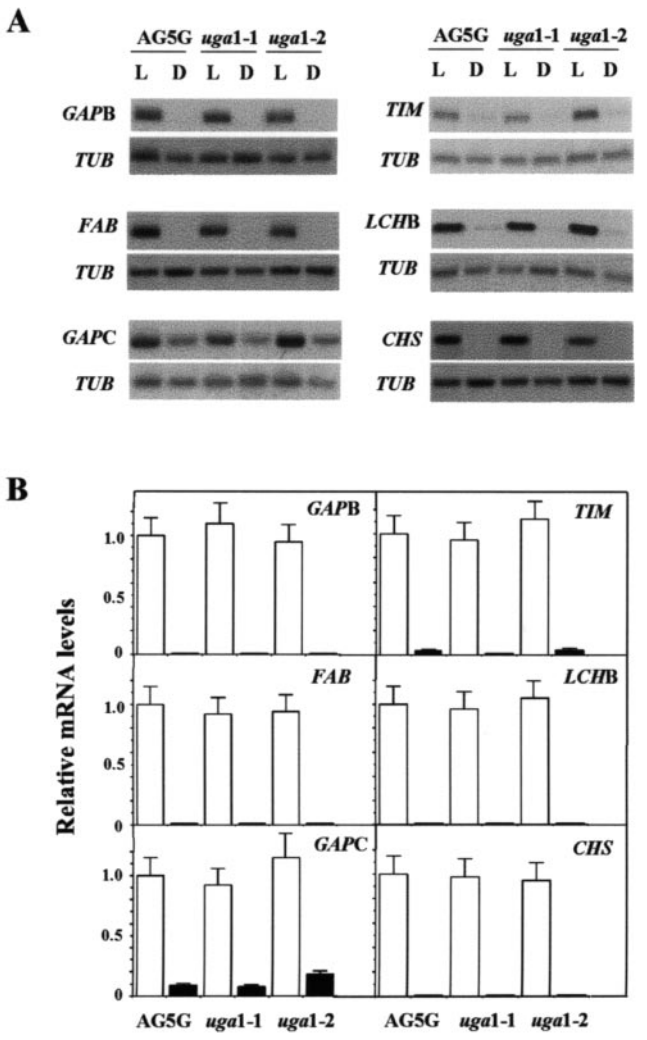

Figure 4. Effects of *uga* mutations on the expression of other light-regulated genes in mature plants. A, Northern-blot analyses of RNAs from 4-week-old light-grown (L) or dark-adapted (D) AG-5G, *uga*1-1, and *uga*1-2 were performed as described in Figure 3 with radiolabeled *GAP*B, *TIM*, *FAB*, *LCH*B, *GAP*C, *CHS*, and *TUB* probes. B, Each northern-blot analysis was repeated three times and quantified. The average densitometric value of each gene from light-grown AG5G is taken as 1. Error bars = SDs.

ways. Therefore, we performed northern-blot analyses for three other genes, including *GAP*C, *LHCB*3, and *CHS* (Feinbaum and Ausubel, 1988; Yang et al., 1993; Li et al., 1995), which are known to be regulated by light. In addition to light, the transcription of *GAP*C, which encodes the C subunit of GAPDH, could also be regulated by Suc (Shih and Goodman, 1988; Yang et al., 1993). As shown in Figure 4, A and B, there were no observable differences in the mRNA levels of these genes in either light-grown or dark-adapted mature plants between the two *uga* mutants and the AG-5G line. These data suggested that the *uga* mutations specifically affect the expression of *GAP*A.

Biochemical Characterization of *uga* Mutants

Because the *GAP*A mRNA levels decreased drastically in both *uga*1-1 and *uga*1-2 mutants, we decided to examine whether the chloroplast GAPDH activity in these mutants was similarly affected. As seen in Figure 5, the two mutants showed only slightly lower chloroplast GAPDH activities compared with AG-5G. This result suggested that posttranscriptional regulation of *GAPA* mRNA or posttranslational modification of the GAPDH enzyme could have occurred to compensate for the reduced *GAPA* mRNA level in the *uga* mutants. To distinguish between these possibilities, western-blot analysis was performed to quantify the protein levels of the A and B subunits (Fig. 6). The data showed that there were similar amounts of A and B polypeptides in leaf extracts from wild type, *uga*1-1, and *uga*1-2. These findings suggested that translational control of *GAP*A must have occurred in the *uga* mutants to compensate for their reduced levels of *GAP*A mRNA.

The *uga*1-1 Mutation Maps to the Bottom Arm of Chromosome V

We used the simple sequence length polymorphism (SSLP) mapping method (Bell and Ecker, 1994) to determine the chromosomal location of the *uga*1-1 mutation. The transgenic line AG-5G, the parental strain of *uga* mutants, is derived from Columbia ecotype (Col-O). We performed crosses between *uga*1-1 and Landsberg *erecta* (L*er*) to generate F_2 progeny as mapping populations. To score F_2 progeny, we needed a suitable marker. Unfortunately, *uga* mutants lack any visible phenotype and the cross to L*er* resulted in a loss of one or two copies of the trans-

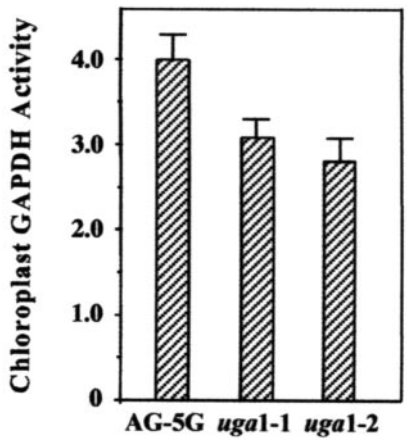

Figure 5. Chloroplast GAPDH activity of mature plants. Chloroplast GAPDH activity of 5-week-old plants from AG-5G and *uga* mutants was assayed as described by Cerff (1982). Each reading was obtained from the pooling of the aerial portions of 10 individual plants per line. The data shown are the average of two independent measurements from plants grown at different times. Specific activity is calculated as the rate of decrease of A_{366} per milligram protein extract. Error bars = SDs.

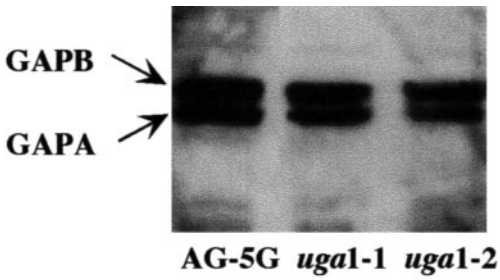

Figure 6. Western analysis of GAPA and GAPB in AG-5G and *uga* mutants. Total cellular proteins were isolated from leaves of mature AG-5G, *uga*1-1, and *uga*1-2 plants. Ten-microgram proteins from each sample were subjected to western-blot analysis using rabbit antibody raised against the GAPDH A_2B_2 tetramer. The arrows indicate the positions of A and B subunits of the chloroplast GAPDH.

gene in some of the F_2 progeny. As a result, GUS activity could not be used as a scoreable phenotype. However, knowing that the *GAP*A mRNA level differs by almost 20-fold between wild type and *uga*1-1 (Fig. 3), we used the *GAP*A mRNA levels to assess the genotype of the F_2 progeny.

Dot-blot analyses were used to compare *GAP*A mRNA levels of a population of F_2 progeny. We isolated total RNA from leaves of 94 F_2 progeny of *uga*1-1 × L*er*. Genomic DNA was isolated from each of these plants by the method of Edwards et al. (1991). RNA from F_2 progeny was subjected to slot-blot analyses using a P^{32}-labeled cDNA fragment of *GAP*A as the hybridizing probe. We found that 20 of 94 F_2 progeny had *GAP*A mRNA levels comparable with those of wild type and that the remaining 74 samples had very low levels of *GAP*A mRNA (Fig. 7A). Because of the dominant nature of *uga*1-1 mutation, the 74 plants with low mRNA levels should be either *UGA1*$^+$/*uga*1-1 or *uga*1-1/*uga*1-1 and the 20 plants with high *GAP*A mRNA should be homozygous *UGA1*$^+$. As opposed to the skewed ratios obtained using GUS expression as the phenotypic marker, the RNA dot blot gave a 1:3.3 ratio, which was close to the expected 1:3 ratio. This confirmed that the *uga1-1* mutation is a dominant, monogenic mutation.

Next, we performed PCR analysis of the 20 *UGA1*$^+$/*UGA1*$^+$ plants using 14 primer pairs corresponding to 14 SSLP markers that span the Arabidopsis genome over its five chromosomes, with at least one marker on each arm (see "Materials and Methods" for the list). Our results showed that the markers on chromosomes I through IV had no association with the L*er*/L*er* ecotype. However, in the case of the marker ciw9 that lies on the bottom arm of chromosome V, 18 of 20 F_2 progeny had a L*er*/L*er* ecotype at this locus, indicating that the *UGA1* gene was linked to this marker (Fig. 7B). Confirming the linkage of the *UGA1* gene to this marker, it was found that the ciw10 marker, which is also located on the bottom arm of chromosome V, was also linked to the *UGA1* gene, but not as tightly. Here, 11 of 20 samples showed the L*er*/L*er* ecotype (data not shown). The *uga1-1* mutation, therefore, is mapped to the bottom arm of chromosome V in the Arabidopsis genome in the vicinity of the ciw9 marker (at 88 cM).

DISCUSSION

We have identified two allelic mutations that affect the expression of the *GAP*A gene in Arabidopsis. Our results showed that the mRNA levels of both *GAP*A::*GUS* and *GAP*A::*ADH* transgenes and the endogenous *GAP*A gene in light-grown *uga*1-1 and *uga*1-2 mutants are greatly reduced. One possible explanation for this observation is that the effect of *uga* mutations on light induction of the *GAP*A gene is mediated at the transcriptional level. However, there were examples that light affects mRNA stability and this effect often involved the 5′- or 3′-untranslated region (UTR) sequences (Dickey et al., 1998; Anderson et al., 1999). The fact that the *GAP*A::*GUS* and *GAP*A::*ADH* transgenes in the AG5G line contain all or part of the 5′-UTR of *GAP*A (see "Materials and Methods") raised the possibility that the *uga* mutations might affect the mRNA stability of *GAP*A. We are in favor of the first interpretation, because results from our nuclear run-on experiments indicated that light effect on the steady-state *GAP*A mRNA level occurred mainly at the transcription level in both tobacco and Arabidopsis (Shih and Goodman, 1988; see also supplemental data). In addition, we have identified two cis-acting elements that are required for light induction of *GAP*A by deletional analyses of promoter constructs in transgenic plants (Conley et al., 1994; Park et al., 1996). A combination of these two elements could confer light responsiveness on a basal promoter that was not regulated by light (Park et al., 1996).

Results from our genetic analysis showed that both *uga*1-1 and *uga*1-2 mutations are dominant (Table I).

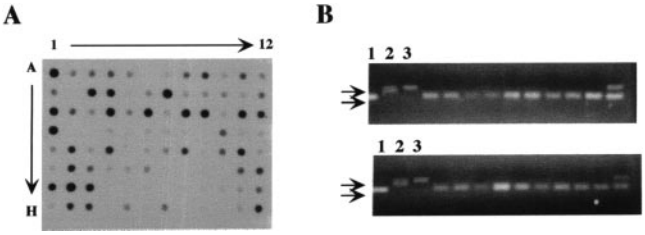

Figure 7. SSLP analyses of *uga*1-1 × L*er* F_2 progeny. A, Dot-blot analysis was performed as described in "Materials and Methods" to compare *GAP*A mRNA levels of 94 F_2 plants. RNA from AG-5G line was loaded on two corners of the filter (A1 and H12) to be used as a quantification standard. B, Gel electrophoresis of PCR products for the ciw9 SSLP marker. Genomic DNA from *UGA3*$^+$/*UGA3*$^+$ F_2 plants was used in PCR with ciw9 as the primer pair. PCR conditions were identical to those described by Bell and Ecker (1994). Lanes 1 through 3 were PCR products from reactions using genomic DNA from (1) Ler (2), Col-O, and (3) AG-5G, respectively, as templates.

With some exceptions, e.g. *shy*2 (Kim et al., 1996; 1998), most photomorphogenic mutants that have been isolated, such as *phyA*, *phyB*, *red1*, *fhy1*, *fhy3*, and *cue1*, are all recessive (Parks and Quail, 1993; Whitelam et al., 1993; Li et al., 1995; Wagner et al., 1997). There are a few ways to explain how this dominant phenotype could occur. First, the *UGA1* gene in its normal wild-type state could function as a positively acting intermediate in the signaling pathway leading to the light-activated transcription of *GAP*A. This would mean that the *uga* mutant gene product must act in a dominant negative manner. One possibility is that the resulting functional *UGA1* gene product is a multimeric protein comprising several subunits of the *UGA1* gene product. The binding of a mutated subunit could cause the entire protein structure to lose its function and, therefore, fail to effect the light-activated transcription of *GAPA*. Alternatively, we could propose that the *UGA1* gene product in its wild-type state normally represses *GAPA* transcription. Along with the action of other positively acting transcription factors, the *UGA1* gene product would maintain an acceptable level of *GAPA* mRNA under a given set of environmental conditions. Repression could be achieved either by interaction with other light-signaling molecules in the pathway or by direct interaction with the *GAPA* promoter. The mutation could have resulted in a much tighter interaction and, therefore, a more dramatic repression effect.

It should be pointed out that, although the RNA dot blot of the F_2 progeny generated from the mapping cross (*uga1-1* × L*er*) showed the expected 1:3 segregation ratio (Fig. 7A), the data obtained from F_2 progeny of the backcross (*uga1-1* × AG-5G) showed a 1:6 ratio as determined by the GUS assay (Table I). One possible explanation for this abnormal segregation ratio in the latter cross is that the AG-5G line contains the *GAP*A::*ADH* transgene. The regulation of endogenous ADH levels in a plant is tightly controlled in terms of tissue specificity and in its response to hypoxia and other environmental stresses (Dolferus et al., 1994; Chung and Ferl, 1999; Conley et al., 1999; Ellis et al., 1999). In AG-5G, however, where the *ADH* gene is driven by a *GAPA* promoter, the ADH activity is expressed about 70-fold higher than that in the Col wild-type plant (C.S. Chan and M.-C. Shih, unpublished data). Furthermore, its expression occurs throughout the entire plant instead of being tissue specific, which could have resulted in physiological abnormality in AG-5G. In fact, AG-5G plants were observed as slow-growing compared with the true wild type, Col. Assuming that the overexpression and misexpression of *ADH* in AG-5G had increased lethality, the introduction of *uga* mutations, which decrease *ADH* expression, might have actually increased the survival rate of AG-5G.

Specificity of the *uga* Mutations

Among a number of light-regulated genes examined here, *uga*1-1 and *uga*1-2 affect only the expression of *GAP*A. This was seen in the dramatic reduction in the steady-state *GAPA* mRNA level in mature plants (Fig. 3), whereas little or no effect was seen in the expression of *GAP*B and several other light-regulated genes, namely *LHC*B3, *FBA1*, and *TIM* (Fig. 4). Furthermore, we found that several photomorphogenetic phenotypes, including hypocotyl length, chlorophyll content, and chloroplast, appeared to be normal in the *uga* mutants (data not shown). In contrast, in most other light-signaling mutants, more than one gene or class of genes is affected. For example, the *cue1* mutant (now known to be a mutation in the *PPT* gene), which was isolated based on defective *LHC*B3-promoter driven reporter activity, was not only defective in endogenous *LHC*B3 transcription but also in the transcription of *RBCS* and *RBCL* (Li et al., 1995). The *psi*2 mutant, which was isolated based on elevated *LHC*B2-LUC (luciferase) activity, was found to be hypersensitive in *LHC*B1, *LHC*B2, *CHS*, and *RBCS* expression when compared with the wild-type equivalent (Genoud et al., 1998). However, we cannot eliminate the possibility that other genes that have not been examined in this study are unaffected by the *uga* mutations. The implications of this specificity are severalfold. First, the *UGA1* gene is likely to lie downstream in the light-signaling pathway leading to the transcriptional activation of *GAPA*. The second implication is that although *GAPA* and *GAPB* may be coordinately regulated at the transcriptional level (Dewdney et al., 1993), there probably exist distinct portions of their pathways that are independent of each other.

Importance of Translational Control of the GAPA Protein

Although the *uga* mutants showed drastic reduction in steady-state *GAPA* mRNA levels, the *uga* mutants appeared to survive very well, even though the A_4 isozyme accounts for 80% of the total chloroplast GAPDH activity in the plant. The assay of chloroplast GAPDH enzyme activities in 5-week-old plants revealed that chloroplast GAPDH activity was only modestly reduced in the mutants compared with AG-5G (Fig. 5). Furthermore, western-blot analysis also revealed that the *GAPA* protein levels in the *uga* mutants were indistinguishable from that in AG-5G (Fig. 6). These results suggested that step(s) between the end of transcription and the completion of translation is the critical step in determining the final GAPDH levels in the *uga* mutants.

There are two ways in which such posttranscriptional regulation could have been achieved. First, one can envision a system whereby differential rate of transcription does not play any significant role in the regulation of the final *GAPA* protein levels. This

could occur if the *GAPA* mRNA were always made in excess of what is required by the cell. Alternatively, because *GAPA* mRNA degradation is relatively fast (Dewdney et al., 1993; Conley and Shih, 1995), it is possible that the differences in transcription rates between AG-5G and the *uga* mutants may have little significance. Instead, translational control led to nearly equal levels of the *GAPA* protein between wild type and mutants. We could not argue in favor of either model based on our current results.

Many nuclear genes in plants are regulated at the posttranscriptional level (Gallie and Bailey-Serres, 1997). In Arabidopsis, *ACS5*, which encodes 1-aminocyclopropane-1-carboxylic acid synthase, is shown to be regulated posttranscriptionally (Woeste et al., 1999). The cytokinin-inducible soybean (*Glycine max*) *CIM1* gene is regulated by the stability of the *CIM1* mRNA rather than by the *CIM1* transcriptional level per se (Downes and Crowell, 1998). In a study of the thylakoid peptide plastocyanin and the Rieske polypeptides, mRNA transcript levels may have increased 10-fold upon illumination, but association of transcripts with polysomes only increased 2- to 3-fold, suggesting that mRNA uptake into polysomes is an important step of posttranscriptional control (Palomares et al., 1993). In the case of the proton-ATPase gene, regulation by translation rate in response to developmental and environmental cues is signified by the presence of a long 5′-UTR that contained an upstream open reading frame (Michelet et al., 1994). There is a 47-bp UTR in the *GAPA* transcript, suggesting translation as a possible mechanism of control. As reviewed by Bailey-Serres (1999), translation of mRNA is emerging as an important mode of gene regulation where initiation is frequently the step at which regulation is achieved. Some features that influence translation rate include the interactions between the 5′ and 3′ ends of the message, and variation in the cap-binding protein of which there are three types in Arabidopsis, as triggered by developmental and other environmental cues (Gallie and Bailey-Serres, 1997).

MATERIALS AND METHODS

Generation of Transgenic Arabidopsis Plants

An Arabidopsis *adh1-2* mutant in a Col background obtained from Dr. Dan Voytas (Department of Genetics, Iowa State University) was used as the starting strain. A binary construct carrying two consecutive reporters, ADH and GUS, each driven by the *GAPA* promoter, was constructed as follows. The *GAPA*::*GUS*/pBI101, which linked about a 1-kb promoter sequence and the complete 47-bp 5′-UTR of *GAPA* to the GUS coding sequence (Conley et al., 1994), was used as the starting plasmid. A DNA fragment that contains the −1,045 to +30 of *GAPA* was generated by PCR and linked to a DNA fragment containing the complete coding sequence of *ADH*. The *GAPA*::*ADH* DNA fragment was then cloned into the *Bam*HI site of the *GAPA*::*GUS*/pBI101. The resulting pBI101 derivative was mobilized into *Agrobacterium tumefaciens* by triparental mating (Bevan, 1984) and then transformed into the *adh1-2/adh1-2* starting strain using the floral dip method as described by Clough and Bent (1998). T_1 progeny containing at least one copy of the transgene were selected by kanamycin resistance. Each transgenic line was carried on to the T_2 generation, where a transgenic line with a single transgene insertion was selected based on a 3:1 segregation of the kanamycin resistance phenotype at the T_2 generation. Within the transgenic T_2 population, a homozygous line, designated as AG-5G, was selected based on a 4:0 segregation pattern at the F_3 and then again at the F_4 generation. The bulked seeds of this line constitute the parental strain, which has an *adh1-2*/*adh1-2*/Col background and carries two reporters, ADH and GUS, each driven by the *GAPA* promoter.

Mutagenesis and Generation of M_2 Progeny

Twenty thousand seeds of the AG-5G line were subjected to ethane methane sulfonate mutagenesis according to the method described by Somerville and Ogren (1982), with a few modifications. In brief, seeds were soaked in 0.1 mM ethane methane sulfonate for 16 h with rocking at room temperature, washed with 100 mM sodium thiosulphate, and rinsed thoroughly with water. Seeds were then treated at 4°C for 3 d before being sown onto soil at a density of 0.5 cm^{-2} and maintained in a growth chamber at 22°C. M_1 plants were carried on to the next generation by selfing, after which M_2 seeds were harvested into four separate pools.

Allyl Alcohol Selection

M_2 seeds were imbibed on Whatman No. 1 filter paper (Whatman, Clifton, NJ) soaked in 3.5 mL of Murashige and Skoog liquid medium containing 2% (w/v) Suc in glass petri dishes at a density of about 500 seeds per plate, carefully spread out using a sterile plastic pipette tip. Control plates containing AG-5G seeds as well as seeds of the *adh1-2* mutant were similarly prepared to be later used for comparison of lethality in allyl alcohol. Seeds were cold incubated at 4°C in the dark for 3 d and then transferred to a dark growth chamber at 22°C for 5 d. The etiolated seedlings were then subjected to 24 h of white light treatment as described in the following section. We found that the expression of the *GAPA* gene reached a maximal level after 24 h of white light treatment (Dewdney et al., 1993; Conley and Shih, 1995). The filters were then transferred onto medium containing 7.5 mM allyl alcohol in Murashige and Skoog + 2% (w/v) Suc. This concentration was the minimum concentration of allyl alcohol that would cause >99% lethality to AG-5G seedlings. After 2 h, the filters were transferred onto fresh agar Murashige and Skoog medium containing 2% (w/v) Suc. Allyl alcohol resistant mutants were isolated on d 4 to 5. These allyl alcohol resistant plants were then subjected to GUS histochemical staining over a 24-h staining period.

Light and Growth Conditions

Plants on soil were kept at 22°C under 16-/8-h light/dark cycle. White light was provided by three cool-white 35-W fluorescent lamps at 50 μmol $m^{-2}s^{-1}$. Blue light was used at 5.5 μmol $m^{-2}s^{-1}$ supplied by four fluorescent lamps with a blue plexiglas 3-mm filter (Rohm-Haas no. 2423, Ditric Optics, Hudson, MA) as previously described (Conley and Shih, 1995). In the case of etiolated seedlings, about 200 (for enzyme assays) or 400 (for RNA extraction) seeds were imbibed on 3.5 mL of Murashige and Skoog + 2% (w/v) Suc liquid medium soaked on two pieces of Whatman No. 1 filter paper in a glass petri dish, vernalized for 3 d, and then transferred to a light-proof incubator at 22°C for etiolation.

RNA Isolation and Northern-Blot Analyses

Total RNA was isolated by the Triazol LS method (Life Technologies/Gibco-BRL, Cleveland) using about 50 to 200 mg of plant tissue for each extraction. Northern analysis was performed as previously described (Conley and Shih, 1995). Five micrograms of RNA was loaded per lane, unless otherwise stated. Gels were blotted overnight onto nylon Hybond N^+ membranes (Amersham Pharmacia Biotech, Piscataway, NJ) with 10× SSC as the transfer buffer. The *GAPA*, *GAPB*, *GAPC*, *RBCS*, *LCHB*, and *TUB* cDNA probes were as described by Conley and Shih (1995). The *TIM* fragment was excised by *Sal*I and *Not*I resulting in a 1.4-kb fragment; *FBA1* was 1.1 kb in length after excision by *Hin*dIII. Normalization for loading was accomplished by stripping the original probe off the filter by dipping in deionized water at 80°C and checking for counts using a Geiger counter. The filter was then reprobed with *TUB*, the transcription of which is unaffected

by light (Conley and Shih, 1995). The bands on the autoradiograph of each northern were quantified using Scion Image version 1.62 software (National Institutes of Health, Bethesda, MD). Relative mRNA levels were then determined by taking the ratio of the band intensity specific for the gene probe of interest minus the background intensity to that for *TUB*.

Enzymatic Assays

GUS enzyme assay and histochemical staining were performed as described by Jefferson et al. (1987). ADH enzyme assays were performed as described by Xie and Wu (1989). This assay uses ethanol as the substrate and measures the production of NADH. Measurement of NADH formation was performed in a DU 64 spectrophotometer (Beckman Instruments, Fullerton, CA). A unit of ADH is defined as the production of 1 nmol of NADH min^{-1} mg^{-1} protein. Chloroplast GAPDH assays were performed as described by Cerff (1982). To determine chloroplast-specific GAPDH activity, NADPH was used as the starting cofactor instead of NADH. Specific activity is calculated as the rate of decrease of A_{366} per milligram protein extract.

Western-Blot Analysis

The aerial portions of 10 to 15 plants from each mutant line were harvested and homogenized in liquid nitrogen using a mortar and pestle after which cold homogenization buffer (15 mM HEPES [pH 7.6], 40 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) was added at 10 mL g^{-1} of fresh tissue. A 10-fold volume of 4 M ammonium sulfate was added drop-wise with stirring. The mixture was then centrifuged at 20,000 rpm in a SW41 swing bucket rotor (30,000*g*) at 4°C for 30 min. The supernatant was filtered through a Miracloth (Calbiochem, La Jolla, CA) after which freshly ground ammonium sulfate was added slowly at 0.33 g mL^{-1} to precipitate proteins. Proteins were then spun down at 19,000 rpm in an SW41 at 4°C for 30 min and resuspended in 1 mL of buffer (20 mM HEPES [pH 7.6], 40 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 10% [w/v] glycerol). Aliquot (400 μL) of the extract was desalted using ultra-free low-bind (10-kD cutoff) filter apparatus (no. UFC3LGC00, Millipore, Bedford, MA). Ten micrograms of protein per sample was used for western analysis as previously described (Wang et al., 1997a) using the semiwet transfer system. The membrane was incubated with a 1:3,000 (w/v) dilution of the rabbit antibody generated against the GAPDH A_2B_2 isozyme in blocking buffer at room temperature with swirling for 1 h. Under these conditions, this antibody reacts specifically with A and B subunits (Wang et al., 1997a). The bands were visualized with ECL western-blotting detection solution (Amersham-Pharmacia Biotech) and quantified with National Institutes of Health Scion Image software version 1.62.

Mapping Cross and F_2 Progeny

The *uga1-1* mutant was crossed to the L*er* wild type. The F_1 seeds resulting from this cross were grown and selfed to produce F_2 seeds. The homozygous recessive F_2 progeny resulting from the mapping cross were selected based on the results from the RNA dot-blot analysis.

For dot blot analysis, a 9- by 12-cm nylon Hybond N^+ membrane (Amersham) was prewet in 10× SSC, blotted dry on Whatman No. 1 filter paper, and assembled on the dot blot apparatus (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's manual. The 96 wells were then rehydrated by the addition of 500 μL of 10× SSC into each well and applying vacuum until dry. Three micrograms of RNA per sample derived from F_2 progeny of the mapping cross was subjected to alkaline denaturation by the addition of 500 μL of ice-cold 10 mM NaOH and 1 mM EDTA and kept on ice. A total of 94 samples of the F_2 progeny were dot blotted onto the nylon membrane together with the AG-5G RNA sample dotted at the top left and bottom right corners as positive controls. Hybridization with the *GAPA* probe was as described above for northern analyses. The F_2 individuals that were homozygous recessive for the *uga* phenotype (wild type for *GAPA* mRNA expression) were then matched to the corresponding numbered plant material reserved for DNA isolation. DNA was isolated using the method of Edwards et al. (1991).

PCR of SSLP Markers

About 1 to 10 ng of template DNA was used for PCR using standard reaction conditions provided by Promega (Madison, WI) at 2.5 mM of $MgCl_2$. Three control tubes using DNA isolated from wild-type L*er*, wild-type Col, and the AG-5G line were set up and run concurrently with the F_2 samples for band size comparison. The primers used for amplifying SSLP markers are as follows: chromosome I, ciw12 and nga111; chromosome II, ciw2, ciw3, and nag168; chromosome III, nag162 and nga6; chromosome IV, ciw5, ciw7, and nga1107; and chromosome V, CTR1, ciw8, ciw9, and ciw10 (Lukowitz et al., 2000). Typically, the annealing temperature was set at 2°C above the higher melting temperature (Tm) of the two primers if they were less than 2°C apart from each other. If the Tms of the two primers were more than 2°C apart, the average between the two Tms was used as the annealing temperature. The PCR cycles are 30 cycles of 95°C, 1 min; 55°C (or other annealing temperature), and 1 min; 72°C, 1 min; followed by 5-min extension at 72°C. About 10 μL of the PCR reaction was resolved on a NuSieve GTG 4% (w/v) agarose gel in 1× Tris-acetate EDTA buffer. Each tier on the DNA agarose gel was run with three control lanes, which carried the respective PCR products of L*er*, Col, and AG-5G for band size comparison. F_2 wild-type recessive progeny from the mapping cross could then be scored as L*er*, heterozygous, or Col ecotypes on the agarose gels for each SSLP marker.

Statistical Analysis

All comparisons between data of mutants versus AG-5G were done by ANOVA one-way analysis with Bonferroni's method, whereas phenotypic ratios of genetic crosses were tested by Chi square analysis. The SigmaStat software (SPSS Sciences, Chicago) was used in each case.

Distribution of Materials

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

ACKNOWLEDGMENTS

We thank Drs. Erin Irish and Jonathan Poulton for comments on the manuscript. We thank the Arabidopsis Biological Resources Center (Ohio State University, Columbus) for providing cDNA clones of *FBA* and *TIM*.

Received April 30, 2002; returned for revision May 20, 2002; accepted July 12, 2002.

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