shygrl1 Is a Mutant Affected in Multiple Aspects of Photomorphogenesis[1]

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We have used a counter-selection strategy based on aberrant phytochrome regulation of an *Lhcb* gene to isolate an Arabidopsis mutant designated *shygrl1* (*shg1*). *shg1* seedlings have reduced phytochrome-mediated induction of the *Lhcb* gene family, but normal phytochrome-mediated induction of several other genes, including the *rbcS1a* gene. Additional phenotypes observed in *shg1* plants include reduced chlorophyll in leaves and additional photomorphogenic abnormalities when the seedlings are grown on medium containing sucrose. Mutations in the TATA-proximal region of the *Lhcb1*3* promoter that are known to be important for phytochrome regulation affected reporter gene expression in a manner similar to the *shg1* mutation. Our results are consistent with the possibility that the mutation either leads to defective chloroplast development or to aberrant phytochrome regulation. They also add to the evidence of complex interactions between light- and sucrose-regulated pathways.

Plants require light not only as an energy source, but also as a cue for growth and development. In the absence of light, plants follow a distinct program of development that is characterized by elongated growth, closed cotyledons, lack of chloroplast development, and low transcriptional activity. Upon exposure to light, seedlings undergo photomorphogenesis, a developmental program that is visually characterized by short hypocotyls and green, open cotyledons. Transcription of nuclear- and chloroplast-encoded genes, many of which have functions related to photosynthesis, is induced as greening commences. Light is perceived by different photoreceptors. Three types that have been characterized are the phytochromes, cryptochromes, and phototropins (Briggs and Huala, 1999; Deng and Quail, 1999).

Components of the phytochrome-signaling pathways have been identified in Arabidopsis using genetic, biochemical, or molecular biological approaches (Neff et al., 2000). PKS1 (Fankhauser et al., 1999) and NDPK2 (Choi et al., 1999) are kinases that interact with phytochromes. CCA1 (Wang et al., 1997), PIF3 (Ni et al., 1998), and HFR1/REP1 (Fairchild et al., 2000; Soh et al., 2000) are transcription factors for which some target genes have been identified (Wang et al., 1997; Martinez-Garcia et al., 2000). FAR1 (Hudson et al., 1999), FIN219 (Hsieh et al., 2000), PAT1 (Bolle et al., 2000), and SPA1 (Hoecker et al., 1998; 1999) have been characterized at the genetic and molecular levels, but their biochemical functions remain a puzzle. Like phytochrome mutants, a number of phytochrome-signaling mutants have long hypocotyls when grown under white, red, or far-red light. Some, such as *cr88* (Lin and Cheng, 1997) and *ndpk2* (Choi et al., 1999), exhibit a pale-green phenotype in addition to long hypocotyls. In many cases, the mutants display only a subset of the phenotypes associated with loss of a specific phytochrome, indicating the existence of branched pathways. The emerging picture indicates a complex network of pathways, some overlapping and some independent, leading from phytochrome perception to a physiological response (Casal, 2000).

Phytochrome regulation of transcription of the *Lhcb* gene family may be used as a system to identify additional loci that function in photomorphogenesis (Karlin-Neumann et al., 1991). This nuclear gene family encodes light-harvesting chlorophyll (Chl) *a/b*-proteins associated with photosystem II, and it is induced within one-half of an hour of phytochrome activation in dark-grown seedlings (Silverthorne and Tobin, 1984). In addition to phytochrome, *Lhcb* genes are regulated, at both transcriptional and posttranscriptional levels, by such factors as developmental stage, blue light, abscisic acid, cytokinins, plastid development, and circadian rhythms (Flores and Tobin, 1988; Taylor, 1989; Brusslan and Tobin, 1992; Gao and Kaufman, 1994; Weatherwax et al., 1996; Kubasek et al., 1998; Millar, 1999). The *Lhcb* promoter has been used previously to identify mutants in light and circadian clock regulation (Li et al., 1995; Millar et al., 1995; Lopez-Juez et al., 1998). Various efforts have identified the TATA-proximal region of *Lhcb* genes to be important for phytochrome regulation of transcription, and two motifs, CCAAT and GATA,

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have been demonstrated to be crucial to the phytochrome-mediated activity of promoter fragments (Anderson et al., 1994; Kehoe et al., 1994; Anderson and Kay, 1995; Degenhardt and Tobin, 1996). In vitro-binding assays showed that many proteins in plant extracts associate with these promoter fragments (Terzaghi and Cashmore, 1995).

To identify components of the phytochrome signal transduction pathways, we have designed a counterselection strategy that takes advantage of the phytochrome responsiveness of the *Lhcb1*3* gene. We used transgenic plants transformed with a 1.3-kb promoter fragment of the *Lhcb1*3* gene, which is strongly induced in etiolated seedlings by brief, saturating red illumination (Karlin-Neumann et al., 1988), fused to the coding region of the *Nia1*2* nitrate reductase gene. Nitrate reductase (NR), an enzyme that reduces nitrate to nitrite, can also convert the nontoxic compound chlorate to chlorite, which is toxic to plants. We had previously found that the growth of the transgenic line was severely inhibited when grown on chlorate under conditions in which the phytochrome system was activated by intermittent red (IR) light (Heimer et al., 1995). This strategy has led to the identification of a locus that we have designated *shygrl1* (*shg1*) because when mutated, it causes short stature, yellow-green leaves, and reduced accumulation of *Lhcb* genes.

RESULTS

Isolation of Mutants with Reduced *Lhcb1*3::Nia1*2* Expression

Seeds of the transgenic line A4 (Heimer et al., 1995), which were homozygous for the *Lhcb1*3::Nia1*2* chimeric gene construct depicted in Figure 1 were mutagenized. Plants grown from these seeds were selfed, and the progeny of these plants (the M2 generation) were used to select potential mutants of interest. Seedlings were germinated and grown under IR on medium with Gln as the nitrogen source and chlorate as the selection substrate. Because endogenous *NR* genes are not expressed when plants are grown on Gln, this growth condition ensures *NR* expression predominantly from the introduced construct. Therefore, seedlings that had normal phytochrome-induced expression of *NR* from the *Lhcb* promoter fragment were severely growth inhibited. Seedlings that grew to a height greater than 6 mm under these conditions were expected to have impaired transcription of the *Lhcb1*3::Nia1*2* construct and low levels of NR activity. These seedlings were transferred to soil and allowed to produce seed. These M3 progeny were then assayed for NR activity. To eliminate mutations that affected endogenous NR directly, lines that had little or no NR activity when grown on nitrate under white light were not studied further.

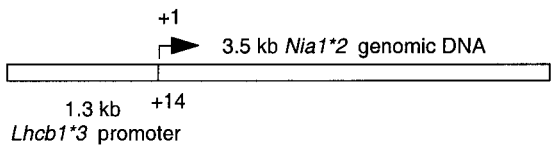

Figure 1. The *Lhcb1*3::Nia1*2* chimeric gene construct. Part of the *Lhcb1*3* (1.3 kb) promoter, which extends through +14 of transcription start, was fused to 3.5 kb of *Nia1*2* genomic DNA containing its own transcription termination sequences. Construction of the transformation vector and selection of the stably transformed A4 line was described in detail in Heimer et al. (1995).

Two mutant lines had characteristics expected of a mutant defective in phytochrome signaling. In Table I, their NR activities are compared with the untransformed wild-type (WT) parent Wassilevskija (WS) and the transgenic parental line A4 when grown with Gln or nitrate. One mutant line, 60, had levels of NR activity that were approximately one-third of the parental A4 line when grown on Gln under IR light, which suggests that the phytochrome induction of the introduced transgene was greatly reduced. When grown on nitrate under continuous white light, the NR activity of line 60 was 1.5-fold higher than WS, the parental line of the A4 transgenic plants; thus, the mutant is not likely to be defective in NR proteins or cofactors and most probably is able to produce additional NR from the introduced transgene under these growth conditions. The NR activity of line 60 was 75% of the parental A4 line under continuous white light, suggesting that the mutation led to limited expression of the introduced construct even under continuous light. Line 58, the second mutant line, behaved similarly. Lines 60 and 58 also had a similar appearance, exhibiting short stature and yellow-green stems and leaves. Crosses of each mutant line to the untransformed parent ecotype WS showed that the mutants were recessive, and crosses of lines 60 and 58 to each other showed that they fell into one complementation group that we have designated *shg1* (data not shown). Therefore, we renamed lines 60 and 58 *shg1-1* and *shg1-2*, respectively.

Phytochrome Regulation of Endogenous *Lhcb1*3* Is Reduced in *shg1-1 Mutants*

To demonstrate that the *shg1* mutation affected the transcription of the *Lhcb1*3* gene in response to phytochrome, we tested the phytochrome induction of the endogenous *Lhcb1*3* gene. Dark-grown seedlings express *Lhcb1*3* at a very low level; a 1-min exposure to red light (R) causes an increase in this mRNA level that has previously been shown to be due to increased transcription (Karlin-Neumann et al., 1991). Figure 2 shows that the phytochrome-induced increase of *Lhcb1*3* mRNA in *shg1-1* was considerably less than in the transgenic WT A4 plants. The initial dark level of this transcript was also lower than that found in A4. These results are consistent with the idea that the mutation affects endogenous *Lhcb1*3* promoter activity, and not just the promoter activity

Table I. *Nitrate reductase activity of seedlings*

Seedlings were grown for 5 d on medium containing glutamine under intermittent red light (IR), or on medium containing nitrate under continuous white light (CWL). We used the transgenic wild-type A4, the untransformed parent of A4 (WS), and the mutant lines 58 and 60. Nitrate reductase activity was measured according to Heimer et al. (1995) and is expressed as the percentage of the activity found in A4 seedlings for each growth condition. The averages of duplicate assays are given. The ranges for each average value are denoted in parentheses.

Line	Growth Conditions	
	Glutamine and IR	Nitrate and CWL
A4	100	100
WS	9.8 (9.3–10.2)	49.7 (48.5–50.8)
58	25.6 (25.0–26.3)	64.0 (63.4–64.6)
60	34.7 (32.7–36.7)	74.8 (71.2–74.5)

of the introduced construct. It also suggests that *shg1-1* may be mutated in a component of the phytochrome signal transduction pathway leading to increased *Lhcb* transcription.

The yellow-green phenotype of *shg1-1* cosegregated with the reduced phytochrome induction of *Lhcb1*3* mRNA. The phenotypes of WT and *shg1-1* are shown in Figure 3, A and B. We grew F_2 plants from the selfed F_1 generation of a backcross between WT and *shg1-1*. We selected 10 green F_2 plants that yielded 100% green offspring and 10 yellow-green F_2 plants that yielded 100% yellow-green offspring for further analysis. Etiolated F_3 seedlings from these plants were assayed for R-induced *Lhcb1*3* mRNA accumulation. In all cases, yellow-green F_2 lines yielded seedlings that had a reduction in R-induced accumulation of *Lhcb1*3* mRNA compared with that found in seedlings from the green F_2 plants (data not shown). The appearance of the two traits, yellow-green and reduced phytochrome-mediated *Lhcb1*3* mRNA accumulation, in the same lines indicates that they cosegregate, and, unless due to mutation in a closely linked gene, are caused by the same mutation. The yellow-green phenotype of *shg1-1* can be observed from the seedling stage. As shown in Figure 3, C through E, the cotyledons and first leaves of *shg1-1* are noticeably paler than WT. In addition, all leaves of *shg1* are serrated (data not shown). This is in contrast to WT, which develops serrated leaves at a later stage. The flowering time of *shg1-1* did not differ noticeably from WT, and it had no marked fertility defect.

SHG1 Maps to Chromosome 2

We used PCR-based markers and a mapping population of 75 yellow-green F_2 siblings to map *shg1* to the middle of chromosome 2, approximately 4 cM from the *er* marker. This map position places *shg1* in the vicinity of *hy1*, a mutant defective in heme oxygenase 1 (Davis et al., 1999; Muramoto et al., 1999), which is pale green and has reduced *Lhcb* RNA accumulation (Chory et al., 1989a; Parks and Quail, 1991). Complementation analysis demonstrated that *shg1* is not allelic to *hy1* (data not shown). None of the *cue* mutants, which are pale-green mutants that exhibit reduced *Lhcb* gene expression (Li et al., 1995; Lopez-Juez et al., 1998), map to chromosome 2. Other photomorphogenic mutants such as *cr88* and *fin219*, which have some phenotypes similar to *shg1*, map to positions distinct from the region to which *shg1* map. *serrate (se)*, which is mutated in a putative single 2Cys-2His zinc finger transcription factor (M.J. Prigge and D.R. Wagner, personal communication), map to the vicinity of *SHG1*. Because *shg1* has serrated leaves like *se*, we crossed the *se* and *shg1-1* mutants to each other. Complementation tests showed that they are not allelic to each other (data not shown). Thus, *SHG1* is a new photomorphogenic locus.

shg1-1 Has Reduced Chl Levels

To characterize the pale-green phenotype of *shg1*, we measured levels of Chl and Chl intermediates in *shg1-1*. Figure 4A shows that cotyledons of *shg1-1* had eight to 10 times lower Chl levels than those found in WT cotyledons. However, leaves from 2-month-old *shg1-1* plants grown in the greenhouse showed only a small difference. At the seedling stage, the Chl *a/b* ratio of *shg1-1* was only slightly

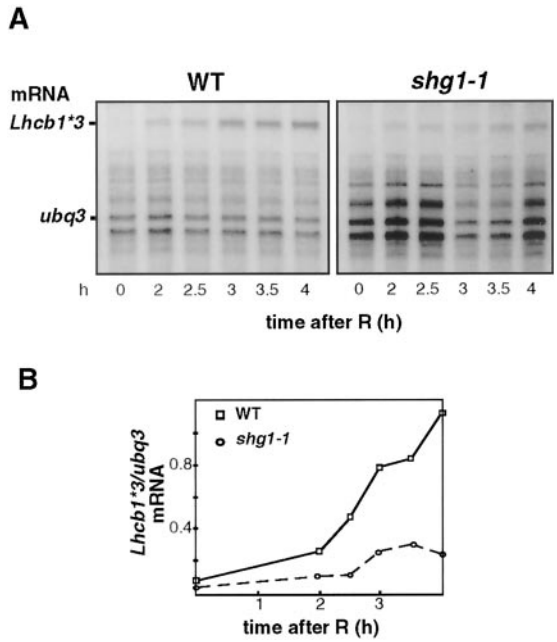

Figure 2. Time course of *Lhcb1*3* mRNA accumulation in the transgenic WT line A4 (WT) and mutant line 60 (*shg1-1*) seedlings after a 1-min R treatment (R). A, RNase protection assay (RPA) of total RNA from 5-d-old dark-grown seedlings given R and harvested at the indicated time points. The fragments protected by the *Lhcb1*3* and ubiquitin (*ubq3*) riboprobes are indicated. Quantification of *Lhcb1*3* mRNA relative to *ubq3* mRNA levels is shown in B.

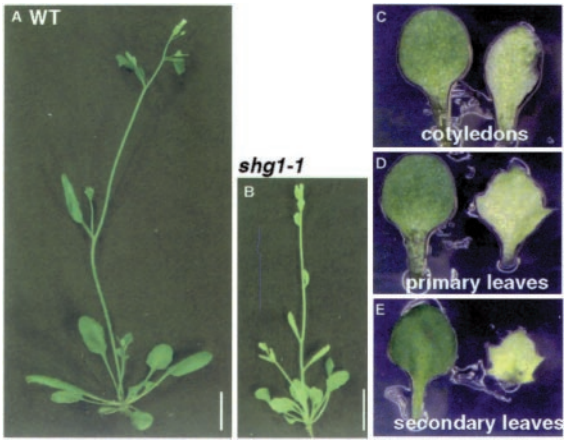

Figure 3. WT (A) and *shg1-1* (B) grown for 6 weeks under continuous white light, bar = 1 cm. Cotyledons (C), primary (D), and secondary (E) leaves of WT (left) and *shg1-1* (right) seedlings grown for 2 weeks on MS2S plates under continuous white light.

higher than WT, whereas at the adult stage, the Chl *a/b* ratios of *shg1-1* and WT were similar (Fig. 4B).

The pale phenotype of *shg1* suggested that it might be a Chl biosynthesis mutant. ALA feeding of etiolated seedlings showed a 4-fold reduction in protoporphyrin IX, Mg-protoporphyrin IX, and protochlorophyllide, compared with WT (Fig. 4C). We tested for accumulation of ALA and porphobilinogen (PBG) in *shg1-1*, and found that both of these intermediates were undetectable (data not shown). Previous work had shown that treatment of *Chlorella vulgaris* with levulinic acid, an inhibitor of ALA dehydratase, resulted in a large increase in ALA, equivalent to the molar loss of Chl (Beale, 1970). An increase of this size would have been easily detected in our assay; thus, we can conclude that neither ALA nor PBG are accumulating in *shg1*. Together, these results suggest that the pale phenotype of *shg1* is a result of reduced flux through the Chl biosynthetic pathway, and not likely to be a result of a leaky mutation in one of the early enzymes of Chl biosynthesis.

Phytochrome Regulation of Other *Lhcb* Genes, But Not of *rbcS1a*, *CCA1*, or *ATHB-2*, Is Affected in *shg1-1*

The low levels of *Lhcb1*3* mRNA and Chls in the seedling stage prompted us to check whether other members of the *Lhcb* gene family are similarly affected. Figure 5, A and B, shows the phytochrome-induced increase of *Lhcb1*1* and *Lhcb1*2* mRNAs in dark-grown WT and *shg1-1* seedlings. Because the sequences of these two *Lhcb* genes are so similar, they give a single band on the RNA gel blot. As seen for *Lhcb1*3* mRNA, the phytochrome induction of *Lhcb1*1* and *Lhcb1*2* mRNAs was reduced greatly. Therefore, different *Lhcb* genes are regulated similarly in *shg1-1*.

One possibility for the reduction in R-induced mRNA accumulation of *Lhcb* genes is the loss or reduction of a positive-acting factor for *Lhcb* transcription. CCA1 is such a transcription factor. It binds to a conserved motif in the *Lhcb1*3* promoter in vitro (Wang et al., 1997), and loss of CCA1 in a null mutant caused reduced phytochrome induction of *Lhcb1*3* (Green and Tobin, 1999). In dark-grown seedlings, *CCA1* itself is regulated by phytochrome (Wang and Tobin, 1998), and the kinetics of its induction are consistent with a role in activating *Lhcb* genes. However, phytochrome induction of *CCA1* was not affected in *shg1-1* (Fig. 5, A and C).

To determine whether *shg1-1* is also affected in the signaling pathway for other phytochrome-regulated genes, we tested the phytochrome regulation of *rbcS1a* and *ATHB-2*. Like *Lhcb* genes, *rbcS1a* is a nuclear-encoded gene for a chloroplast protein. Of the small family of genes encoding the small subunit of Rubisco, *rbcS1a* is the most strongly induced by phytochrome (Dedonder et al., 1993). Figure 5, D through E, shows that the increase in *rbcS1a* mRNA

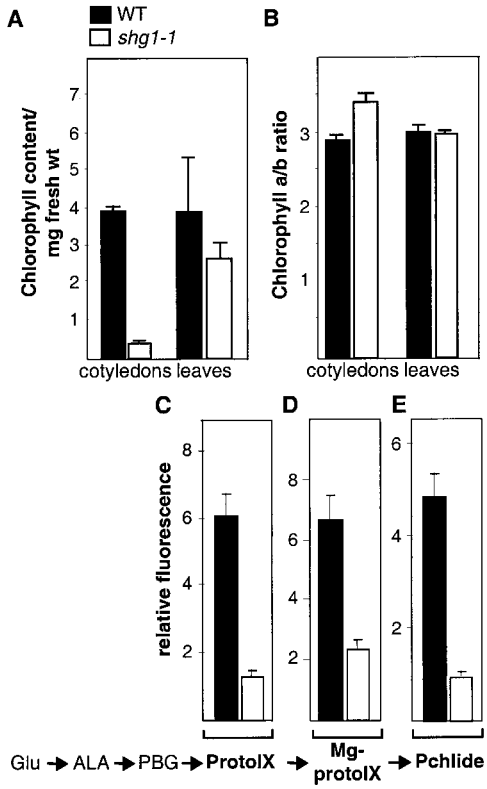

Figure 4. Levels of Chl and Chl intermediates in WT and *shg1-1*. A, Total Chl levels from cotyledons of seedlings grown for 7 d on MS plates under long-day conditions, and from rosette leaves of plants grown for 2 months on soil in the greenhouse. B, Chl *a/b* ratios of samples from A. Accumulation of Protoporphyrin IX (ProtoIX), Mg-protoporphyrin IX (Mg-protoIX), and Protochlorophyllide (Pchlide) in WT and *shg1-1* seedlings after ∂-aminolevulinic acid (ALA) feeding. A schematic diagram of the Chl synthetic pathway from Glu (Glu) to Pchlide is shown and each graph above ProtoIX (C), Mg-protoIX (D), and Pchlide (E) represents levels of that intermediate. Error bars represent SE and representative data from two independent experiments are presented.

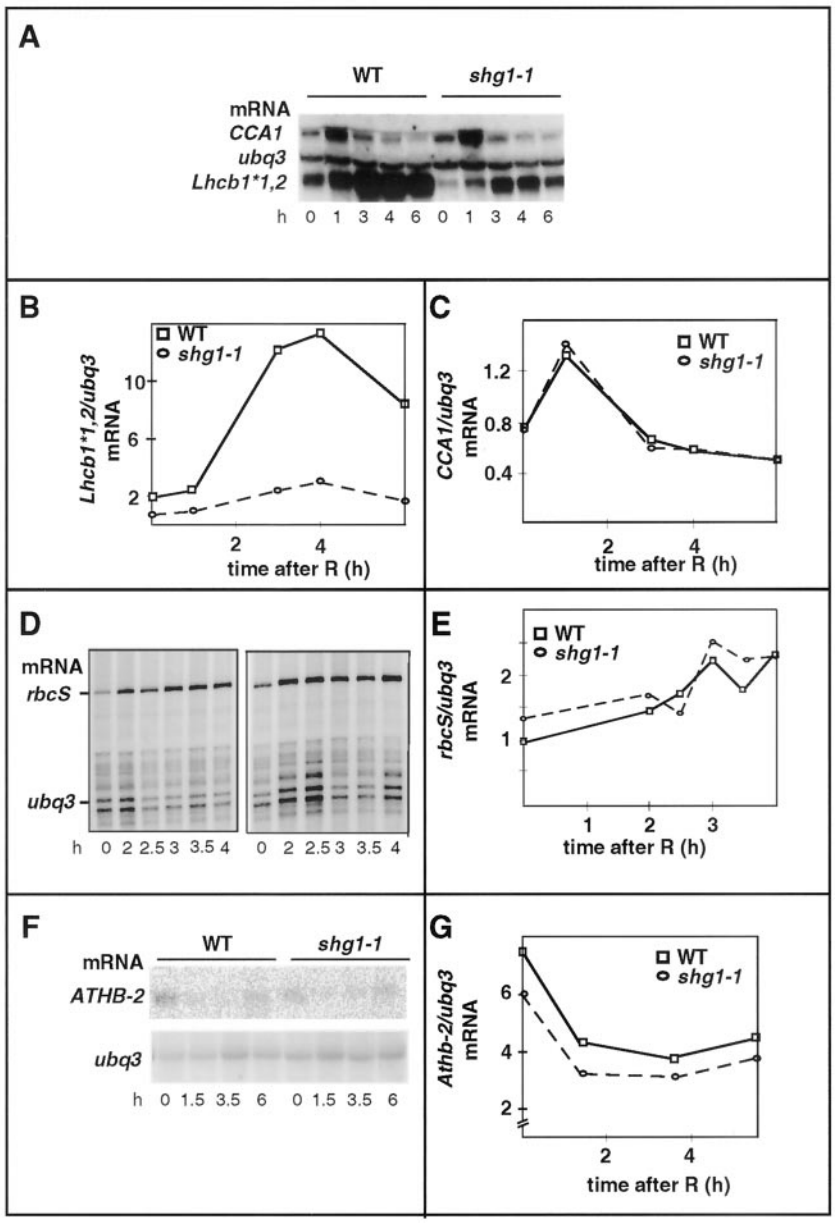

Figure 5. The *shg1-1* mutation affects phytochrome-regulated mRNA accumulation of other *Lhcb* genes but not of *rbcS1a, CCA1*, or *ATHB-2* genes. A, Total RNA was prepared from 6-d-old, dark-grown WT and s*hg1-1* seedlings harvested after R. RNA gel blots were successively hybridized with riboprobes specific for the *CCA1*, *ubq3*, and both *Lhcb1*1* and *Lhcb1*2* genes. Quantification of *Lhcb1*1,2* (B) and *CCA1* (C) mRNA levels in WT and *shg1*. D, RPA using an *rbcS1a* ^{33}P-labeled RNA probe which protects 160 bases. E, Quantification of *rbcS1a* mRNA normalized to *ubq3* mRNA levels. F and G, *ATHB-2* RNA gel-blot analysis and quantification. Representative data from two independent experiments are shown.

after R treatment of *shg1* seedlings approximated that found in WT. Thus, the phytochrome regulation of *rbcS1a* is not significantly affected in the mutant line. We also tested phytochrome regulation of *ATHB-2*. The *ATHB-2* gene, which encodes a homeodomain protein (Schena and Davis, 1992) not thought to be related to chloroplast function (Schena et al., 1993; Steindler et al., 1999), is down-regulated by phytochrome (Carabelli et al., 1993; 1996). As shown in Figure 5, F and G, *ATHB-2* was similarly regulated in WT and *shg1-1*. Thus, *SHG1* affects only a subset of phytochrome-regulated genes. Furthermore, the fact that the expression of the *CCA1* gene is not affected, although the *Lhcb* genes to which it can bind are, suggests that either *SHG1* acts downstream of CCA1 or it acts in a different pathway.

Mutation of Conserved Motifs in the *Lhcb1*3* Promoter Affects β-Glucuronidase (*GUS*) Reporter Gene Expression in a Way Similar to *shg1-1*

The reduced induction of the endogenous *Lhcb1*3* gene in *shg1* mutants was the molecular phenotype predicted to be found in a mutant involved in phytochrome signaling. To identify promoter elements that might be targets of the signal transduction pathway affected in *shg1* mutants, we introduced nucleotide changes in the context of a full-length (1.3-kb) *Lhcb1*3* promoter. These mutations were targeted to three different motifs implicated previously as important for phytochrome responsiveness. These motifs and the mutant constructs are shown in Figure 6A. The Mu1 construct has a 1-bp change in each of

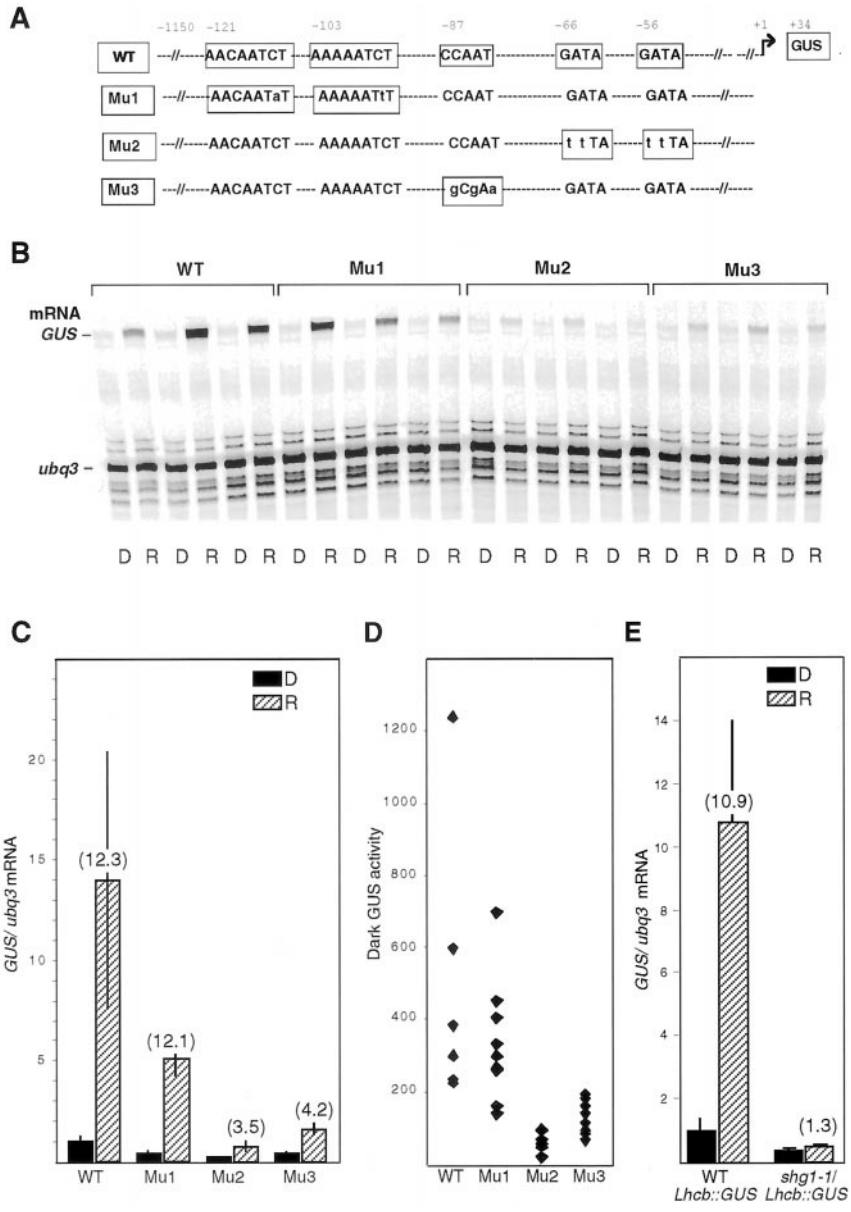

Figure 6. Mutating either CCAAT or both GATA promoter motifs in *Lhcb1*3::GUS* sharply reduces phytochrome-responsive and constitutive dark GUS expression. A, Diagram of the TATA-proximal regions of WT and mutant *Lhcb1*3::GUS* constructs. The *Lhcb1*3* promoter (−1,150 to +34) was fused upstream of the *GUS* gene in pBI101 (CLONTECH, Palo Alto, CA). The sequences of WT nucleotide motifs are shown in uppercase letters. The motif that is mutated in each mutant reporter is enclosed in a box, with the changed base denoted in lowercase. B, RPA of total RNA from 5-d-old seedlings given no light treatment (D) or 1 min R 3.5 h before harvest. Three independent lines transformed with either WT or mutant constructs Mu1, Mu2, and Mu3 were used; the fragments protected by *GUS* and *ubq3* riboprobes are indicated. C, Comparison of the activities of WT and mutant promoters from the RPA above. For each set of lines, fold-induction of *GUS* mRNA levels, shown in parentheses above each R column, were obtained by dividing relative GUS mRNA from R samples by relative *GUS* mRNA from D samples, which gives an indication of the phytochrome inducibility of the transgenic construct. For each line, the value for relative *GUS* mRNA was obtained by dividing the absolute *GUS* mRNA level with the absolute *ubq3* mRNA level and the average relative value for three lines per construct is shown. Error bars represent SE. D, GUS activity found in 7-d-old dark-grown transgenic lines containing either WT or mutant constructs. Six to eight independent lines were tested per construct. E, Phytochrome inducibility of *Lhcb::GUS* is abolished when expressed in the *shg1-1* mutant background. The WT *Lhcb::GUS* construct was crossed into the *shg1-1* mutant background. Five-day-old dark-grown WT *Lhcb::GUS* and *shg1-1/Lhcb::GUS* seedlings were given no (D) or R treatment, 4 h before harvesting for RNA extractions. *GUS* and *ubq3* mRNA levels were analyzed by RPA. Calculation of fold-induction levels, shown in parentheses, was done as explained in C. Error bars represent SE from three independent lines.

the two imperfect repeats of the motif $AA^{A}/_{C}AATCT$. This motif is conserved among *Lhcb* promoters and is part of a site to which the transcription factor CCA1 binds. A DNA fragment containing the Mu1 mutation competed less effectively for CCA1 binding in vitro than the WT equivalent, though more effectively than a fragment containing a 9-bp mutation (Wang et al., 1997). The Mu2 construct has two bases changed in the CCAAT sequence and the Mu3 construct has two bases changed in each of two GATA boxes. The WT and mutant promoters were fused to the *GUS* reporter gene, and the constructs were stably transformed into Arabidopsis. F_3 seedlings from multiple transgenic lines were analyzed for phytochrome regulation of the introduced gene for each construct.

We found that Mu2- and Mu3-transformed lines exhibited reduced dark expression and phytochrome induction of *GUS*. The results from one representative experiment, using three independent lines for each of the WT, Mu1, Mu2, and Mu3 constructs, are shown in Figure 6, B and C. In different WT *Lhcb::GUS* lines, the increase in levels of *GUS* mRNA after R treatment ranged from 9- to 18-fold, and in Mu1 lines the average increase was approximately 12-fold. Thus, it does not appear that the nucleotide changes in the Mu1 construct affected phytochrome responsiveness of that promoter. In contrast, both the Mu2 and Mu3 lines had consistently low increases in response to the R treatment, ranging from no induction to 5-fold induction. We have extended previous studies by showing that mutating these motifs in the

context of the full-length promoter has a strong effect on expression. Our results also demonstrate that the loss of signaling through these motifs results in a reduction in phytochrome inducibility of the *Lhcb1*3* promoter such that it should be detectable in our promoter-mediated mutant screen.

We also tested whether the mutations affected dark *Lhcb1*3* expression. Accumulation of *GUS* mRNA in the dark appeared to be reduced in the Mu 1-3 lines, but it was difficult to ascertain the accuracy of our measurements because the mRNA levels were so low. Therefore, we assayed for GUS activity rather than *GUS* mRNA levels in the dark. We used at least six independent lines per construct for these measurements. The results from one such experiment are shown in Figure 6D. The WT and Mu1 lines showed a similar range of GUS activity levels, whereas Mu2 and Mu3 lines had considerably lower levels. Some Mu2 lines had GUS activities that were lower than the WT average by 10-fold. *GUS* mRNA levels from light-grown Mu2 and Mu3 lines were also consistently lower than those from WT lines (data not shown). Thus, the CCAAT and GATA motifs in the *Lhcb1*3* promoter are not only important for phytochrome-induced expression, but also for maintenance of expression. Mutations that affect any of the proteins that mediate signaling through these motifs thus are likely to affect *Lhcb1*3* expression in dark and in light.

The results presented in Figure 6E suggest that SHG1 may act in such a signaling pathway. We crossed one transgenic line containing the WT *Lhcb::GUS* construct with *shg1-1*. By introducing a WT *Lhcb::GUS* into the *shg1-1* mutant background and analyzing different siblings from the cross, we found that phytochrome responsiveness of the construct was abolished. In addition, the level of *GUS* mRNA in the dark was reduced. This result confirms other experiments in which we observed reduced dark accumulation of the endogenous *Lhcb* mRNA in *shg1-1* and *shg1-2* (Figs. 2 and 5; data not shown). The *shg1* mutation causes changes in *Lhcb1*3* expression that are similar to those caused by mutating the CCAAT or GATA motifs. Therefore, it is possible that the *SHG1* locus encodes a protein that acts as a component of a phytochrome-signaling pathway that terminates at one of the DNA-binding sites on the *Lhcb1*3* promoter, possibly at the CCAAT or GATA site.

Suc Causes Additional Photomorphogenic Abnormalities in *shg1*

shg1 seedlings exhibit additional mutant phenotypes when grown in light and on medium containing Suc. As shown in Figure 7, A and B, one effect of a *shg1* mutation that is unmasked by growth on Suc is a long hypocotyl phenotype. The characteristic increase of hypocotyl elongation on Suc-containing medium (Kurata and Yamamoto, 1998) was exhibited by WT seedlings, which were 50% taller on MS medium supplemented with 2% (w/v) Suc (MS2S) than on Murashige and Skoog (MS) medium while main-

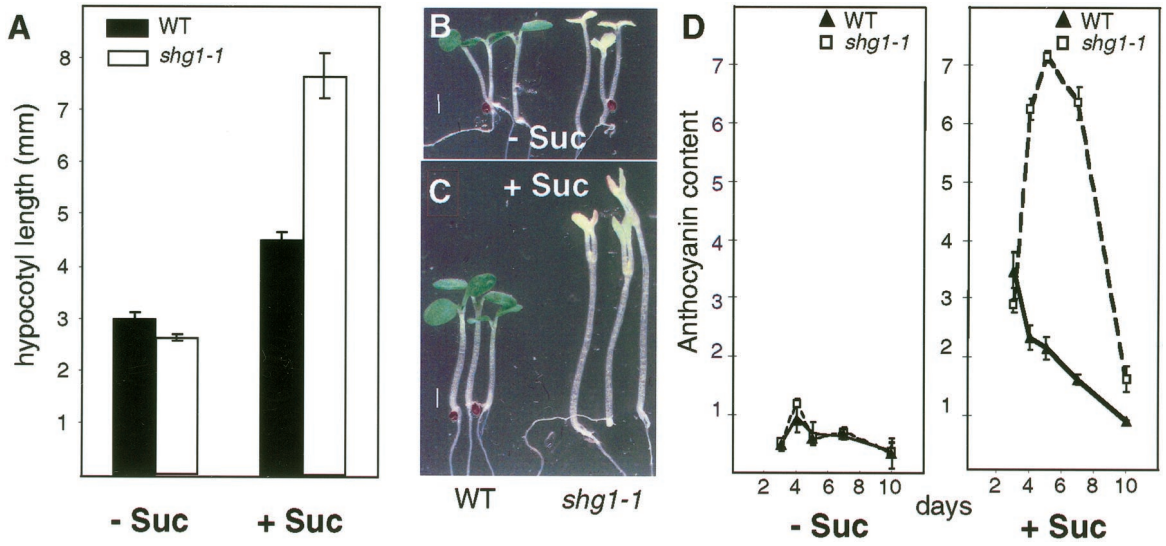

Figure 7. *shg1-1* seedlings exhibit additional photomorphogenic abnormalities when grown on medium containing Suc. A, Photomorphogenic growth of *shg1-1* seedlings is altered by the presence of Suc in the growth medium. WT and *shg1-1* seedlings were grown on MS plates containing no (− Suc) or 2% Suc (+ Suc) for 7 d under a short-day photoperiod. Mean hypocotyl lengths of 15 to 20 seedlings are shown with error bars denoting SE. Photographs of representative WT (left) and *shg1-1* (right) seedlings grown on medium without (B) or with (C) Suc (bar = 1 mm). D, Anthocyanin content of *shg1-1* seedlings is higher than WT when grown on medium containing Suc. WT and *shg1-1* seedlings were grown on MS plates containing no (− Suc) or 2% Suc (+ suc) for the indicated number of days under a short-day photoperiod. Anthocyanins from 20 seedlings were extracted and the difference between A_{535} and A_{650} represents anthocyanin content. Three different sets of 20 seedlings were measured for each treatment. Error bars denote SE.

taining the same morphology. However, *shg1-1* seedlings were 200% taller on MS2S than on MS alone. In addition, their petioles remained tightly closed in an upright position, and their cotyledons were only half open. This is in sharp contrast to *shg1-1* seedling morphology on MS, which is essentially a paler and smaller version of the WT morphology. Thus, growth on Suc-containing medium causes *shg1* seedlings to retain aspects of etiolation when grown in light.

Figure 7D shows that *shg1* seedlings accumulated high levels of anthocyanin only when the growth medium was supplemented with Suc. Light-grown WT and *shg1-1* seedlings accumulated similar levels of anthocyanin when grown on plates with MS medium only or on soil (data not shown). On MS2S, there was a substantial increase in anthocyanin levels. This response to Suc has been described previously for WT petunia (*Petunia hybrida*; Weiss, 2000) and Arabidopsis (Tsukaya et al., 1991). In *shg1-1* seedlings, however, the anthocyanin increase was similar to WT seedlings during the first 3 d of growth, but it continued to rise for a longer period, reaching a peak that reflected a 15-fold increase. This burst of anthocyanin accumulation peaked 4 to 5 d after germination, and then levels dropped to slightly above WT levels. By the 10th d, *shg1-1* levels were only twice those of WT plants. Dark-grown *shg1* seedlings were indistinguishable from WT when grown on medium with or without Suc (data not shown).

DISCUSSION

We have used phytochrome regulation of the *Lhcb1*3* gene as a system to identify potential phytochrome signaling intermediates. By generating a stably transformed line containing an *Lhcb1*3* promoter fused to a selectable marker and carrying out the genetic screen using IR, a light regime that supports high phytochrome-specific *Lhcb1*3* promoter activity while preventing full de-etiolation (Tobin, 1981; Karlin-Neumann et al., 1991), we aimed to select mutants that affect phytochrome regulation of gene expression. We identified *shg1*, a yellow green mutant which exhibited reduced phytochrome induction of *Lhcb1*3*.

We showed that *shg1* exhibited reduced phytochrome regulation of *Lhcb* genes, but normal phytochrome regulation of the *rbcS1a* gene. Our finding that *rbcS1a* is regulated normally provides genetic evidence for the separate regulatory pathways between *Lhcb* and *rbcS* that have been inferred from physiological experiments (Batschauer et al., 1986) and from the divergent light-regulatory elements found in each promoter (for review, see Arguello-Astorga and Herrera-Estrella, 1998). Coordinate regulation of *Lhcb* and *rbcS* genes has been typically found for phytochrome and photomorphogenic mutants (for *hy* mutants, Chory et al., 1989a, Sun and Tobin, 1990; *det*, Chory et al., 1989b; *gun*, Susek et al., 1993; *cop*, McNellis and Deng, 1995; and *sun*, Djikwel et al., 1997). Thus, SHG1 may affect a step in phytochrome signaling that is either after a branch point or separate from the pathway regulating *rbcS* expression.

The two other nuclear genes we tested, *CCA1* and *ATHB-2,* do not encode chloroplast proteins and were also normally regulated by phytochrome in *shg1-1*. CCA1 is a transcription factor that binds to a conserved motif in the *Lhcb1*3* promoter. We found that *CCA1* mRNA accumulation after R is identical in WT and *shg1-1*. The reduced *Lhcb* gene expression observed in *shg1* thus is unlikely to be due to defective phytochrome signaling to *CCA1*. The phytochrome regulation of *ATHB-2*, a homeodomain protein, was examined as an example of a gene that is involved in neither chloroplast function nor *Lhcb* expression (Steindler et al., 1999). We found that the expression of *ATHB-2* mRNA in *shg1* was also very similar to WT. Thus, phytochrome signaling to the other genes outside the *Lhcb* gene family is intact, suggesting that the lesion in *shg1* specifically affects *Lhcb* gene expression.

We considered that the reduction in Chl levels seen in *shg1* might stem from an impairment in chloroplast function. Lopez-Juez et al. (1998) showed a correlation between reduced Chl levels and abnormal plastid structure. In addition, mutations in chloroplast-localized proteins can cause Chl deficiency (recent examples include Sundberg et al., 1997; Jarvis et al., 1998). To address whether *shg1* might be mutated in any of the enzymes in the Chl biosynthetic pathway, we measured the levels of some of the Chl intermediates. Because the rate-limiting step in Chl synthesis is the synthesis of ALA, accumulation in an intermediate pool after feeding dark-grown seedlings with ALA would indicate blockage at the subsequent enzymatic step. We found no such accumulation (Fig. 4). ALA and PBG contents were also reduced in light-grown *shg1* (J.A. Brusslan, data not shown). Because *shg1* does green, we can conclude that the pathway for the stepwise synthesis of Chl is likely to be intact. The reduced flow of intermediates through the pathway may be the result of aberrant regulation of the Chl synthesis pathway, similar to that found for the *aurea* (*au*) and *yellow-green-2* (*yg-2*) mutants of tomato (*Lycopersicon esculentum*) (Terry and Kendrick, 1999).

Different aspects of greening such as Chl synthesis and transcription of nuclear-encoded photosynthetic genes are processes controlled by phytochrome (Mohr, 1984; Silverthorne and Tobin, 1984). Loss or reduction of phytochrome leads to reduced Chl accumulation as well as reduced phytochrome induction of gene expression (Chory et al., 1989a; Parks and Quail, 1991; Somers et al., 1991; Reed et al., 1993; Terry and Kendrick, 1996). Proteins that have been implicated in phytochrome signal transduction can

cause a pale-green phenotype when mutated. For instance, mutation of diphosphate kinase 2, a protein that interacts with phytochrome, caused a pale-green phenotype (Choi et al., 1999). Further evidence that a pale-green phenotype arises concomitant with a deficiency in functional phytochrome was provided by Lagarias et al. (1997) using plants overexpressing biliverdin reductase, an enzyme that inactivates phytochrome chromophores. They suggested that the reduction in Chl observed in these plants was a consequence of the loss of phytochrome regulation of its biosynthesis. These results indicate that disrupting phytochrome regulation in the plants may lead to improper regulation of such chloroplast functions as Chl biosynthesis.

Aberrant phytochrome regulation also affects growth in the dark. There is physiological evidence for phytochrome control of the "capacity" for Chl formation (for review, see Mohr, 1984). The *au* mutant of tomato has reduced protochlorophyll and abnormal proplastids (Terry and Kendrick, 1996, 1999; Montgomery et al., 1999). *Lhcb* mRNA in dark-grown *hy1* (Lopez-Juez et al., 1998) and *au* (Sharrock et al., 1988) seedlings is reduced. Our findings concerning the dark expression levels in *shg1* and the mutant *Lhcb1*3* promoter lines provide additional insights into regulation of gene expression. Dark expression of *Lhcb* mRNA is specifically affected in *shg1*. Moreover, disrupting either the CCAAT or both GATA boxes (Mu2 and Mu3, respectively) greatly reduced not only phytochrome responsiveness but also dark expression of *Lhcb1*3* expression. Thus, the CCAAT and GATA motifs in the *Lhcb1*3* promoter are not only important for phytochrome-mediated regulation, but also for maintenance of basal or dark expression. Mutations that affect any of the proteins that mediate signaling through these motifs are thus likely to affect *Lhcb1*3* expression. It has been postulated that a signal from the plastids regulates the *Lhcb* gene family (Taylor, 1989) and a Chl intermediate can serve as one such signal for light-induced nuclear genes in *Chlamydomonas reinhardtii* (Kropat et al., 1997). It is tempting to speculate that *shg1* and some of the *cue* mutants (*cue3*, *cue6*, and *cue8*; Lopez-Juez et al., 1998), which exhibit reduced Chl levels and reduced dark *Lhcb1*3* expression, are mutated in a phytochrome-signaling pathway that includes the plastid and terminates at one of the DNA-binding sites on the *Lhcb1*3* promoter.

The most remarkable light-grown phenotype of *shg1* is its response to Suc at the seedling stage. *shg1* seedlings have elongated hypocotyls and partially closed cotyledons when grown on medium containing Suc. These alterations in *shg1* seedling morphology, coupled with its pale-green color, make *shg1* seedlings appear more like etiolated than light-grown seedlings, and suggests that Suc is acting to block full de-etiolation normally caused by light signals. The repressive effects of Suc on de-etiolation have been shown to be a phytochrome-dependent response in WT seedlings (Dijkwel et al., 1997). When grown in increasing light fluences and on medium containing Suc, phytochrome-deficient biliverdin reductase-overexpressing seedlings can be taller than dark-grown seedlings (Montgomery et al., 1999). We also found that the burst in anthocyanin accumulation seen in *shg1* grown on Suc was more extensive than that of WT. Expression in transgenic Arabidopsis of a petunia chalcone synthase gene, encoding the enzyme for the first committed step in anthocyanin biosynthesis, was shown to be sugar-, particularly Suc-, dependent (Tsukaya et al., 1991). Furthermore, anthocyanin induction and the expression of chalcone synthase were demonstrated to be predominantly controlled by phy A (Kunkel et al., 1996). The increased hypocotyl length and anthocyanin accumulation in *shg1* can be interpreted as an exaggeration of the effects normally induced by Suc in light-grown WT seedlings. Thus, Suc and SHG1 may act in a subset of the pathways normally regulated by phytochromes, similar to *Suc-uncoupled* (*sun*) mutants (Dijkwel et al., 1997). On the other hand, the heightened pigmentation that *shg1* seedlings exhibit on Suc may constitute a stress response. In addition to light and Suc (for review, see Weiss, 2000), environmental stimuli such as stress can induce flavonoid biosynthesis (Shirley, 1996). Interpretation of the phenotypes induced by Suc in light-grown *shg1* seedlings is complicated further by the antagonistic relationship of some phyA and phyB pathways (Casal, 2000). Furthermore, it has been reported recently that the dominant negative interference of phy A-mediated hypocotyl growth inhibition in far-red by overexpression of phyB (Wagner et al., 1996) is dependent on the availability of metabolizable sugars such as Suc (Short, 1999). Further experiments are necessary to determine whether *shg1* is involved in the phyA photobiological pathway that is susceptible to the antagonistic action of phyB and Suc (Casal et al., 2000).

In summary, we have identified and characterized *shg1*, a pale-green mutant that shows reduced phytochrome induction of *Lhcb* genes as well as reduced *Lhcb* mRNA accumulation during growth in darkness. In these respects, *shg1* is similar to other pale-green mutants. Phytochrome regulation of other genes that we tested, however, was normal. This differential regulation of *Lhcb* genes distinguishes *shg1* from the other mutants. Because phytochrome is known to regulate the synthesis of Chl, expression of nuclear-encoded chloroplast proteins, and the development of plastids, it is difficult to ascertain whether the *shg1* mutant phenotype is caused by defective plastid function or by impaired phytochrome signaling. We provide evidence that reduced *Lhcb* gene expression, which may be indicative of defective plastids, may also be caused by disruption of phytochrome signaling to *Lhcb* promoters. In addition, the light-grown phenotype of the *shg1* mutant adds ge-

netic proof of the complex interplay of signals between phytochrome, Suc, and photomorphogenic development.

MATERIALS AND METHODS

Plant Material and Growth Conditions

The mutant screen was conducted with Arabidopsis ecotype Wassilevskija (WS) seeds. Seedlings were grown at 22°C to 24°C on MS, MS2S, or on soil. Seedlings for hypocotyl length and anthocyanin content measurements were grown under short-day (8-h light/16-h dark) conditions with a fluence of approximately 90 μmol m^{-2} s^{-1} from a combination of fluorescent and incandescent bulbs. Seedlings for Chl measurements were grown on MS under long-day (16-h light/8-h dark) conditions with a fluence of approximately 45 μmol m^{-2} s^{-1}. Etiolated seedlings analyzed for RNA were sown onto MS or MS2S plates, cold treated for 2 d, induced to germinate by a 30-min exposure to white light, then grown for another 5 d in the dark. Seedlings were given either no or 1 min R, then harvested for RNA extraction 3 to 4 h later. R treatments and sources were as described in Tobin (1981).

Mutant Screen

Seeds (600 mg, approximately 25,000) from the A4 transgenic line containing a chimeric Arabidopsis *Lhcb1*3::Nia1*2* gene (Heimer et al., 1995) were mutagenized by incubation in 20 mM ethyl methanesulfonate for 8 h. Seeds from 100 M_1 plants were pooled to constitute one family, and approximately 1,000 seeds per family were screened under conditions similar to those described by Heimer et al. (1995). We screened 125,000 M_2 seeds. Approximately 330 seeds were sown onto a prerinsed filter on a plate containing nitrate-free MS medium, 10 mM L-Gln, 0.05% (w/v) MES (2-[N-morpholino]ethane-sulfonic acid, Sigma, St. Louis), 0.7% (w/v) phytagar (Gibco-BRL, Rockville, MD), and 2% (w/v) Suc (pH5.8), cold treated for 2 d, exposed to white light for 30 to 60 min, then transferred to IR light (1 min R every 2 h) for 5 d. Seedlings that were taller than 6 mm were transplanted to soil. One hundred twenty-seven individuals survived and set seed. M_3 seeds were used to grow tissue for NR assays, which were performed as described by Heimer et al. (1995).

Mapping

We generated a mapping population by crossing mutant line 60 to the Columbia ecotype and identifying 75 yellow-green F_2 offspring. We isolated DNA from these lines using either leaf and inflorescence tissue or pooled F_3 seedlings, according to the protocol described by Ausubel et al. (1994). We used markers for simple sequence length polymorphisms and cleaved amplified polymorphic sequences to obtain the approximate map location of *shg1* (Konieczny and Ausubel, 1993; Bell and Ecker, 1994; *Arabidopsis thaliana* Database, Stanford University, Palo Alto, CA; http://www.Arabidopsis.org).

GUS Constructs

To construct the WT *Lhcb1*3::GUS* reporter construct, we introduced an *Xba*I site at +34 of *Lhcb1*3* using the primer pair Atcab28 (−698 to −678 of the promoter) and JX77 (5′ cgctctAGATTTGTTGTTGTAAGCCAAG 3′, spanning +11 to +34 of the coding region). The amplified product was cut with *Bgl*II and *Xba*I, and used to replace the *Bgl*II/*Xba*I fragment from a pBlueScriptKS(-) vector containing an *Eco*RI/*Bam*HI fragment spanning −1,150 to −1 bp of the *Lhcb1*3* promoter used by Kenigsbuch and Tobin (1995); this plasmid was named pWTcabXba. The *Lhcb1*3* promoter was excised from pWTcabXba as a *Hin*dIII/*Xba*I fragment and fused to the *GUS* gene by insertion into the polylinker region of pBI101 (CLONTECH).

The base substitutions in the Mu1 construct were created by amplifying two *Lhcb1*3* fragments using the primer pairs Atcab28 and Atcab32 (5′-AGCAAACAAACAATCT-AAACCCCAAAAAAAATTTATGACT-3′, spanning −129 to −90 of the upper strand of the *Lhcb1*3* promoter), and Atcab33 (sequence complementary to Atcab32) and JX77. These fragments were combined to serve as template for another round of PCR using Atcab28 and JX77. One product around 732 bp, presumed to contain the mutated nucleotide, was cut with *Bgl*II and *Xba*I, yielding a 283-bp fragment that was used to replace the analogous fragment in pWTcabXba. Using the same strategy, Mu2 and Mu3 base substitutions were introduced using the primers: MO1 (5′ TGACTAGGCGAAAGCAACCTCAGAGATTGATATT-TC 3′, spanning −94 to −55 of the upper strand); MO2 (sequence complementary to MO1) for Mu2 containing a mutated CCAAT box; MO3 (5′ CTCAGAGATTTTTATT-TCAATTTAAGACAGTATTTAGATTTC 3′, spanning −72 to −35); and MO4 (sequence complementary to MO3) for Mu3 containing two mutated GATA boxes. Mutant *Lhcb1*3* promoters were inserted into the *Hin*dIII/*Xba*I polylinker region of pBI101. All constructs were transformed into Arabidopsis ecotype Columbia by vacuum infiltration. For all the independent lines used in this study, the correct promoter::GUS junctions and TATA-proximal regions of the T2 lines were verified by sequencing.

Pigment Analysis

Anthocyanins were measured as described by Schmidt and Mohr (1981). Anthocyanins were extracted by immersing 20 seedlings in propanol:HCl:H_20 (18:1:81 percent volume) and boiling for 3 min. Extracts were left in the dark overnight at room temperature, clarified by centrifugation, and analyzed spectrophotometrically. The difference between A_{535} and A_{650} represents anthocyanin content. Three different sets of 20 seedlings were measured for each treatment.

Chls were extracted from cotyledons or leaf tissue by overnight immersion in dimethylformamide. Chl content was calculated from absorbance readings using the Porra equation (Porra et al., 1989).

ALA Feeding

Seeds (approximately 250) were sown onto MS2S plates, cold treated for 2 d, exposed to white light for 20 min, and then grown in the dark for 4 d at 24°C. Three milliliters of 10 mM ALA, 5 mM $MgCl_2$, and 10 mM $NaPO_4$ (pH 7.0) were added to each plate under a green safelight. Seedlings were grown in the presence of ALA for 12 h, then homogenized in 5 mL 9:1 acetone:0.1 M NH_4OH, and centrifuged at 3,000 rpm for 5 min. Fluorescence measurements were performed on the recovered supernatants with an SPEX fluorometer (Metuchen, NJ) using Ex400:Em632 for Protoporphyrin IX, Ex440:Em633 for protochlorophyllide, and Ex420:Em595 for Mg protoporphyrin.

Hypocotyl Length Measurement

Seedlings were grown on plates and pressed down to the surface of the plate to display the hypocotyls on the plane of the plate. Photographs were taken using a digital camera and hypocotyl lengths were determined using National Institutes of Health image software (public domain; Bethesda, MD).

Analysis of RNA

Isolation of total RNA from etiolated seedlings and RPAs were performed according to Brusslan and Tobin (1992) with some modifications. Riboprobes were synthesized using ^{33}P-UTP (ICN, Irvine, CA) and purified using G-50 spin columns. RNA gel-blot analyses were done as described by Wang and Tobin (1998). Intensities of bands were quantitated using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). The *ATHB-2* probe was synthesized by random priming the ATHB-2/360 plasmid, which contains the 360-bp *Hin*dIII insert of *ATHB-2* (corresponding to nucleotides 93–453, I. Ruberti, personal communication). The *Lhcb1*3* riboprobe was identical to the one designated *cab1* by Karlin-Neumann et al. (1991); the riboprobe that recognized both *Lhcb1*1* and *Lhcb1*2* was identical to that designated *cab 2,3* by Brusslan and Tobin (1992). The *CCA1* and *ubq3* riboprobes were identical to those from Wang and Tobin (1998). To produce a *GUS* riboprobe that protects a 176-bp product in the RPAs, we created a plasmid template containing a short fragment of *GUS*. An *Xba*I site was introduced at +12 of the *GUS* gene using the primer 5′ CGTCCTCTAGAAACCCCAACCCG 3′ and a *Bgl*II site at +174 using the primer 5′ CGAAGATCTGCATCGGCGAACTGATCG 3′ (+182 to +156). The PCR product was digested with *Xba*I and *Bgl*II, then ligated to pGEM112f(-) that had previously been digested with *Bam*HI and *Xba*I. This plasmid was linearized with *Xba*I to serve as template for a T7 polymerase transcription reaction. All experiments were repeated with similar results.

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