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Arabidopsis bZIP Protein HY5 Directly Interacts with Light-Responsive Promoters in Mediating Light Control of Gene Expression

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The Arabidopsis *HY5* gene has been defined genetically as a positive regulator of photomorphogenesis and recently has been shown to encode a basic leucine zipper type of transcription factor. Here, we report that HY5 is constitutively nuclear localized and is involved in light regulation of transcriptional activity of the promoters containing the G-box, a well-characterized light-responsive element (LRE). In vitro DNA binding studies suggested that HY5 can bind specifically to the G-box DNA sequences but not to any of the other LREs present in the light-responsive promoters examined. High-irradiance light activation of two synthetic promoters containing either the consensus G-box alone or the G-box combined with the GATA motif (another LRE) and the native Arabidopsis ribulose bisphosphate carboxylase small subunit gene *RBCS-1A* promoter, which has an essential copy of the G-box, was significantly compromised in the *hy5* mutant. The *hy5* mutation's effect on the high-irradiance light activation of gene expression was observed in both photosynthetic and nonphotosynthetic tissues. Furthermore, the characteristic phytochrome-mediated red light– and far-red light–reversible low-fluence induction of the G-box–containing promoters was diminished specifically in *hy5* plants. These results suggest that HY5 may interact directly with the G-box in the promoters of light-inducible genes to mediate light-controlled transcriptional activity.

INTRODUCTION

Plant photomorphogenic seedling development requires coordinated expression of large arrays of structural, metabolic, and regulatory genes (von Arnim and Deng, 1996). A number of light-responsive elements (LREs), such as the G, GT1, and GATA (or I) motifs (Figure 1A), are commonly found in the promoters of light-regulated genes and have been shown to be necessary for light-controlled transcriptional activity (Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995; Millar and Kay, 1996). Indeed, when these elements were placed in specifically paired combinations upstream of non-light-regulated basal promoters, they were sufficient to confer phytochrome-mediated light responsiveness and tissue specificity (Lam and Chua, 1990; Puente et al., 1996). Numerous DNA binding factors with high affinities for specific LREs have been identified, and genes encoding a number of these proteins have been cloned (Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995). For example, a Myb-related DNA binding factor named CCA1 has recently been demonstrated to interact with an essential LRE in the Arabidopsis light-harvesting chlorophyll *a*/*b* binding protein gene *Lhcb1*3* (or *CAB140*) promoter, and its role in phytochrome-controlled *Lhcb1*3* expression in vivo has been confirmed by using an antisense approach (Wang et al., 1997). Nevertheless, most of the DNA binding proteins still await genetic evidence for functional involvement in light-regulated gene expression and photomorphogenesis (Quail, 1994; Tobin and Kehoe, 1994; Terzaghi and Cashmore, 1995; Millar and Kay, 1996).

Arabidopsis *HY5* has been genetically defined as a positive regulator of photomorphogenesis based on the light insensitivity of *hy5* mutants (Koornneef et al., 1980; Ang and Deng, 1994). The phenotype of *hy5* seedlings includes defects in light inhibition of hypocotyl elongation, light-induced chlorophyll accumulation, and extensive root abnormalities (Koornneef et al., 1980; Ang and Deng, 1994; Oyama et al., 1997). Genetic analyses have suggested that HY5 acts downstream of multiple photoreceptor-mediated pathways and that it functionally interacts with pleiotropic CONSTITUTIVE PHOTOMORPHOGENIC/DEETIOLATED/FUSCA (*COP*/*DET*/*FUS*) genes, which are the negative regulators of photomorphogenesis (Koornneef et al., 1980; Chory, 1992; Ang and Deng, 1994; Wei et al., 1994; Pepper and Chory,

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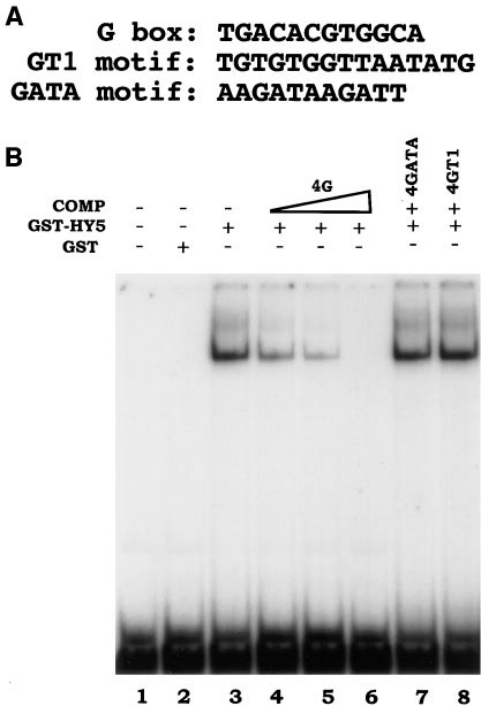

Figure 1. The GST–HY5 Protein Specifically Binds to the Consensus G-Box Tetramer in Vitro.

(A) Sequences of the three consensus LREs examined in this study. **(B)** Gel shift analysis with a 53-bp DNA fragment of the consensus G-box tetramer (4G) that was used as a probe. The amount of protein added in each reaction was none (lane 1), 4 μg of glutathione S-transferase (GST; lane 2), and 0.8 μg of GST–HY5 (lanes 3 to 8). The amounts of unlabeled competitors (COMP) were 100, 200, and 300 ng of 4G in lanes 4, 5, and 6, respectively, and 300 ng of GATA and GT1 tetramers (4GATA and 4GT1) in lanes 7 and 8, respectively. Increasing concentrations of the competitor are indicated by the triangle. Plus and minus signs indicate presence and absence of competitors, respectively.

1997). However, the exact role of *HY5* in light-regulated gene expression remains obscure.

When expression of multiple members of the *CAB* and ribulose bisphosphate carboxylase small subunit (*RBCS*) gene families was examined by RNA gel blot analysis, no significant reduction in mRNA levels of these two gene families was found between the *hy5* mutants and the wild-type siblings (Chory, 1992; Ang and Deng, 1994; Pepper and Chory, 1997). Because the *RBCS* and *CAB* gene families include large numbers of individual genes, the specific transcriptional regulation of a given member in particular tissue types could be overlooked easily during undiscriminating steady state mRNA analyses. By using gene-specific S1 nuclease protection analysis, Sun and Tobin (1990) showed that *hy5* mutants retain normal levels of phytochrome-mediated induction of *CAB* gene expression within the first 2 hr after light pulses. However, in a recent study using the *CAB2* promoter fused to the luciferase reporter (*CAB2*::*LUC*), Anderson et al. (1997) demonstrated that in the *hy5* mutant, although the acute response to light (2 to 4 hr after light pulse) was normal, extended expression of *CAB2*::*LUC* was attenuated to 50% of the wild type. This strongly indicates that HY5 plays a role in phytochrome-mediated *CAB2* gene expression. However, the effect of the *hy5* mutation on other light-regulated promoters and the way HY5 regulates the target promoters are not clear.

The *HY5* gene has been cloned and shown to encode a 168–amino acid protein representing a member of a new class of basic leucine zipper (bZIP) DNA binding proteins (Oyama et al., 1997). A number of bZIP proteins have been isolated from plants by their biochemical affinity to DNA sequences that have an ACGT core motif (Foster et al., 1994; Menkens et al., 1995). Although the specific functions of these genes in vivo have yet to be defined, the light-stimulated modification and the nuclear translocation of cytosolic G-box binding factors (GBFs), such as GBF2, have been demonstrated in cultured cells and protoplasts (Harter et al., 1994; Terzaghi et al., 1997). It has been proposed that this GBF2 factor is involved in light-induced transcriptional activity and that the limitation of nuclear access may be an important control of its activity by light.

The bZIP protein HY5 as a positive regulator of photomorphogenic development raised the possibility that it may bind directly to light-responsive promoters and mediate light-induced gene expression. In this report, we focus on three types of light-responsive promoters to study their interactions with HY5. We investigated the biochemical interaction of HY5 with the DNA fragments containing individual LRE sequences in vitro. Further, we analyzed their functional interactions in vivo by examining the effects of the *hy5* mutation on the activity of these promoters in stable transgenic plants. Our data strongly support that there is a direct interaction of HY5 with the well-defined LRE motif, G-box, and that this interaction is critical for the optimal light activation of the G-box–containing promoters.

RESULTS

HY5 Specifically Binds to the G-Box Commonly Found in Light-Regulated Promoters

To test whether the HY5 protein interacts directly with any of the LREs, we used a purified glutathione *S*-transferase–HY5 (GST–HY5) fusion protein and DNA fragments containing three different types of LREs (G-box, GATA, and GT1 motifs) in gel shift assays to study their interactions. As shown in Figure 1B, GST–HY5 was able to bind to the G-box tetramer (4G) with high affinity but not to the tetramers of GATA or GT1 motifs (data not shown). GST alone did not show any binding activity (Figure 1B, lane 2). Moreover, excess unla-

beled G-box tetramers, but not GATA or GT1 tetramers, were able to compete for the binding activity of GST–HY5 (Figure 1B, lanes 4 to 8). These results indicate that the interaction between HY5 and the G-box is specific.

We then tested whether HY5 was able to interact with the G-box in a native light-regulated promoter. A 196-bp minimal light-responsive promoter fragment of the Arabidopsis *RBCS-1A* gene has been shown to be necessary and sufficient to mediate the light response (Donald and Cashmore, 1990). This fragment contains a single essential G-box along with three GT1 and two GATA (or I) motifs. As evident in Figure 2, gel shift assays using the minimal light-responsive 196-bp promoter fragment as a probe resulted in a high-affinity protein–DNA complex with GST–HY5. Similarly, the interaction was efficiently competed by a 26-bp double-stranded oligonucleotide containing the G-box of the *RBCS-1A* promoter (Figure 2, lanes 4 to 6) and by the 4G fragment (Figure 2, lanes 7 to 9). The two other LREs, GT1 and GATA, whose variant forms are also present in the promoter fragment, failed to compete for HY5 binding activity (Figure 2, lanes 10 and 11). Finally, the footprinting analysis of this 196-bp promoter fragment confirmed that GST–HY5 only protected a single 20-bp region centered around the G-box from the DNase I cleavage (Figure 3). Together, these results demonstrate that HY5 specifically binds to the G-box in vitro. The data also indicate that the only HY5 binding site in the minimal light-responsive promoter region of *RBCS-1A* corresponds exactly to the single G-box, which has been shown to be critical for high-level expression in the light (Donald and Cashmore, 1990).

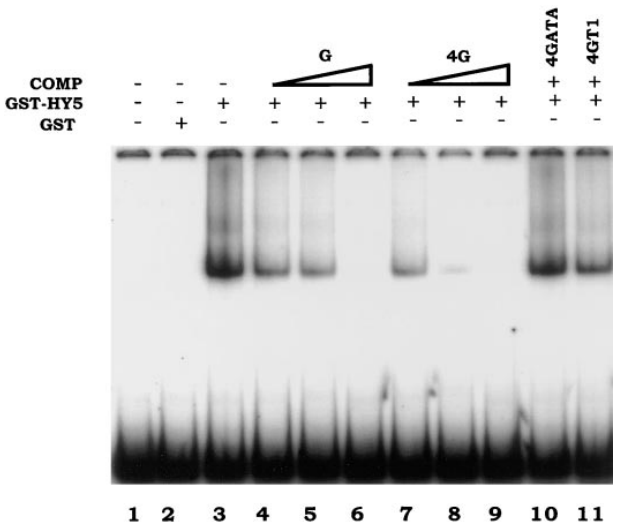

Figure 2. GST–HY5 Binds to the *RBCS-1A* Minimal Light-Responsive Promoter.

A 196-bp DNA fragment of the *RBCS-1A* minimal promoter was used as a probe. The amount of protein in each reaction was none (lane 1), 4 μg of GST (lane 2), and 0.8 μg of GST–HY5 (lanes 3 to 11). The amounts of unlabeled competitors (COMP) were G, 26-bp double-stranded DNA (5′-AATTATCTTCCACGTGGCATTATTCC-3′; underlining indicates the hexameric G-box core motif) oligonucleotide containing the G-box from the *RBCS-1A* promoter (80, 160, and 320 ng in lanes 4, 5, and 6, respectively), and 4G, consensus G-box tetramer (80, 160, and 320 ng in lanes 7, 8, and 9, respectively), and 320 ng of 4GATA and 4GT1 in lanes 10 and 11, respectively. Increasing concentrations of the competitor are indicated by the triangles.

HY5 Is Required for Optimal Light Expression of a Synthetic G-Box–Containing Promoter

To address whether the observed HY5 and G-box interaction in vitro has functional relevance in vivo, we asked whether the activities of the G-box–containing promoters were affected in *hy5* mutants. Stable transgenic Arabidopsis plants containing the tetrameric G, GT1, and GATA consensus motifs (Figure 1A) upstream of the nopaline synthase *NOS101* basal promoter and β-glucuronidase (*GUS*) reporter fusion constructs (Puente et al., 1996) were utilized. These transgenes were introduced into a *hy5* null mutant (*hy5-1*) background by genetic crosses, and mutant lines homozygous for the transgenes were generated and used for all of the experiments in this study.

Based on the GUS reporter enzymatic activity measurements, the promoter activities of G/*NOS101* in dark-grown seedlings of the wild type and *hy5* mutant were similar (Figure 4A). In the light, however, eightfold higher GUS activity was observed in the wild-type seedlings, whereas less than a twofold increase was detected in *hy5* mutants. Thus, the *hy5* mutation resulted in more than a threefold reduction in GUS activity in light-grown seedlings and had no effect on dark-grown seedlings. This result implies a critical role for HY5 in mediating high-irradiance light activation of the G/*NOS101* promoter. In contrast, the synthetic promoters containing other consensus LREs in place of the G-box, such as GATA/*NOS101* (Figure 4B) and GT1/*NOS101* (data not shown), exhibited the same activities in the light-grown wild-type and *hy5* mutant backgrounds. Therefore, the *hy5* mutant is not defective in general high-irradiance light induction of gene expression, but it specifically affects the optimal light expression of a G-box–containing synthetic promoter.

HY5 Plays a Crucial Role in Light Activation of Complex Light-Regulated Promoters Containing the Essential G-Box

Previous studies have shown that the synthetic promoters containing a single LRE element, such as G/*NOS101*, could respond only to continuous high-irradiance light and were unable to respond to low-fluence or pulsed light (Puente et al., 1996). In most native light-responsive promoters in plants, light responsiveness is usually defined by regions containing multiple LREs (Tobin and Kehoe, 1994; Terzaghi

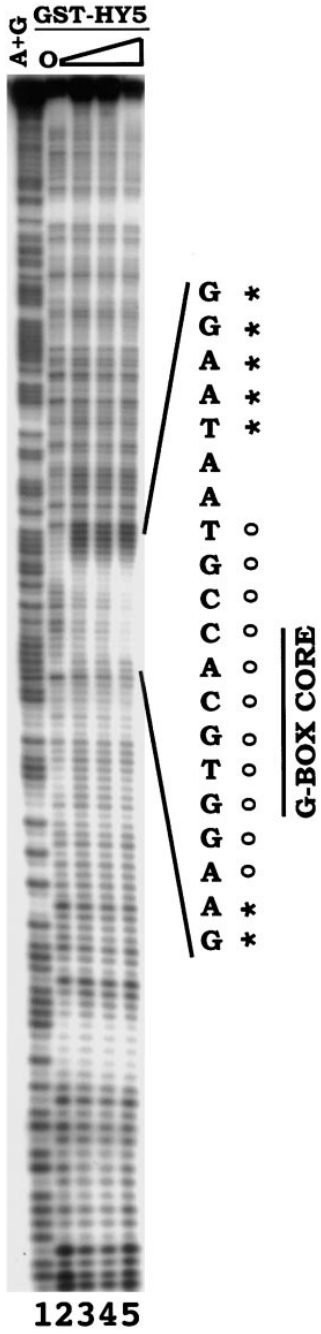

Figure 3. DNase I Footprinting Analysis of the 196-bp Minimal Light-Responsive Promoter Region of the *RBCS-1A* Gene with GST–HY5 Protein.

Lane 1 shows the A+G Maxam and Gilbert sequencing ladder of the same labeled fragment (bottom strand). Lanes 2 to 5 show the DNase I cleavage pattern with 0, 5, 10, and 15 μg of the GST–HY5 protein (increasing concentrations of the protein are indicated by the triangle). The sequence of the G-box–containing region in the promoter fragment is shown at right. The hypersensitive nucleotides are indicated with stars, and the protected nucleotides are indicated with open circles.

and Cashmore, 1995). Several recent studies suggest that a minimal autonomous promoter, which mimics the native promoters in response to light and developmental signals, must consist of at least one pair of distinct LREs in specific combinations (Degenhardt and Tobin, 1996; Puente et al., 1996; Feldbrugge et al., 1997). Thus, it would be important to determine whether HY5 plays a role in the light regulation of those promoters.

To examine the role of HY5 in light activation of promoters containing the G-box in the context of another LRE(s), we analyzed the effect of the *hy5* mutation on the expression of two representative light-responsive promoter–*GUS* transgenes. One is a synthetic promoter (G-GATA/*NOS101*), which has a combination G-box and GATA motif (Puente et al., 1996), and the other is the native *RBCS-1A* promoter (Donald and Cashmore, 1990). As shown in Figures 4C and 4D, the *hy5* mutation resulted in approximately half of the wild-type activity level for both promoters in 6-day-old seedlings grown in constant white light. In addition, the high-irradiance light inducibility of both promoters was significantly compromised in the *hy5* mutant background. When 4-day-old dark-grown seedlings were transferred to continuous white light for 48 hr, the G-GATA/*NOS101*–*GUS* transgene elevated the GUS activity level to approximately fourfold in wild-type plants, whereas the GUS activity level was elevated to only about twofold in the *hy5* mutants (Figure 4C). In the case of the *RBCS-1A* promoter–*GUS* transgene, 48-hr exposure to light increased GUS activity levels by approximately twofold, whereas very little increase in GUS activity, if any, was detected in the *hy5* mutants (Figure 4D). As a control, a synthetic promoter–*GUS* fusion (GT1-GATA/*NOS101*), which does not contain a G-box sequence (Puente et al., 1996), was introduced into the *hy5* mutant and examined. The *hy5* mutation caused no reduction in the light-activated expression of this transgene (data not shown). These results suggest that HY5 plays an important role in light activation of the promoters containing the G-box in the context of other LREs.

HY5 Is Essential in Mediating the Low-Fluence Phytochrome Response of Complex Light-Regulated Promoters Containing the G-Box

The phytochrome-mediated low-fluence light induction of gene expression is characterized by the ability of far-red light cancellation to affect red light pulse induction. To examine whether HY5 directly participates in this response and to rule out the possibility that the deficiency of light response in *hy5* mutants is due to a secondary effect of the light-triggered developmental or physiological process, we analyzed the effect of the *hy5* mutation in this phytochrome-mediated low-fluence response. Because single LRE-containing promoters do not respond to light pulse (Puente et al., 1996), only the paired LRE-containing promoters and the *RBCS-1A* promoter were examined. As shown in Figures 5A

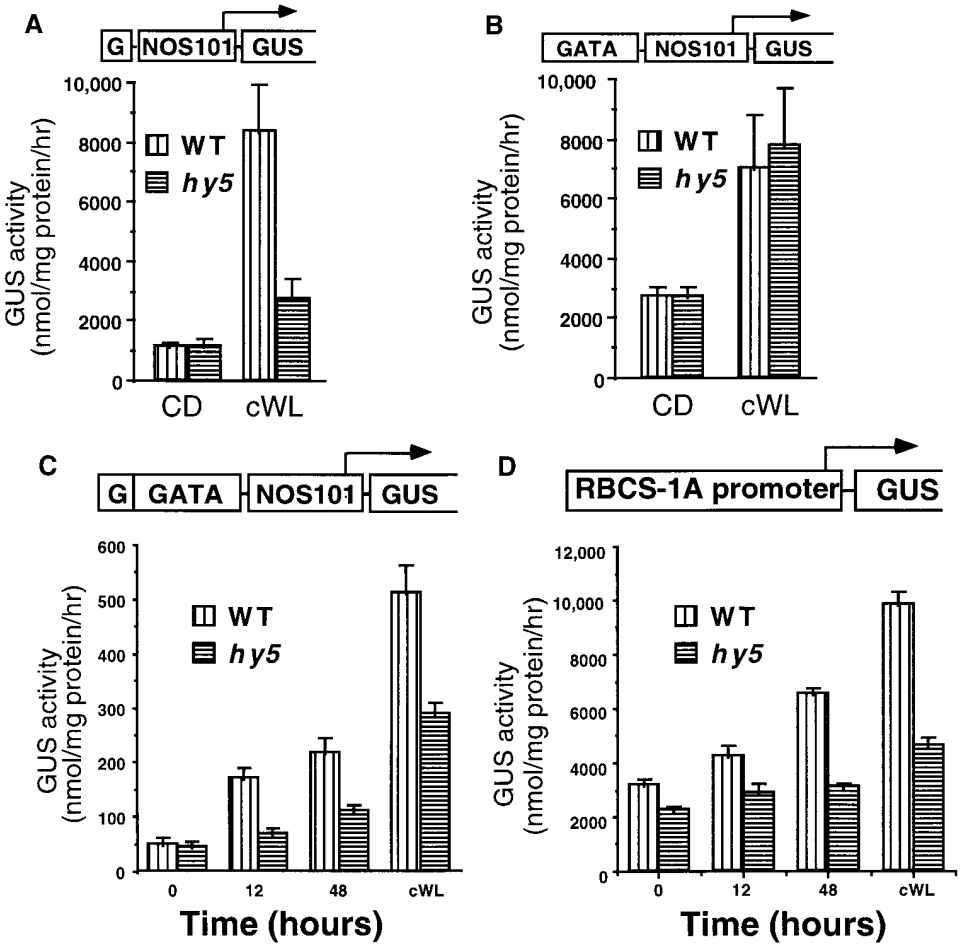

Figure 4. Effect of the *hy5* Mutation on Light-Activated Expression of the G-Box–Containing Promoters.

Promoter activity in the wild type (WT) and *hy5* mutant (*hy5*) backgrounds was estimated by quantitative GUS activity assays. The chimeric promoter–*GUS* reporter transgenes are diagrammed above each graph. The activities are the average of four independent repeats in one representative experiment (out of four), and the error bars indicate standard deviations.

(A) GUS activity of the G/*NOS101–GUS* transgene in 6-day-old seedlings grown under complete darkness (CD) or constant white light (cWL).

(B) GUS activity of the GATA/*NOS101–GUS* transgene in 6-day-old light- and dark-grown seedlings.

(C) and **(D)** Light induction kinetics of dark-grown seedlings containing G-GATA/*NOS101–GUS* and *RBCS-1A* promoter–*GUS* transgenes, respectively. Four-day-old dark-grown seedlings exposed to white light for 0, 12, and 48 hr and 6-day-old light-grown seedlings (cWL) were used for the GUS activity assay.

and 5B, a single red light pulse effectively induced G-GATA/*NOS101–GUS* and *RBCS-1A–GUS* expression approximately twofold, and a far-red light pulse immediately following the red pulse cancelled red light induction. The *hy5* mutation basically eliminated the red light pulse induction of both promoters. As a comparison, there was no significant effect of the *hy5* mutation on the red pulse induction of the GT1-GATA/*NOS101* promoter (data not shown), indicating that the effect of the *hy5* mutation was dependent on the presence of the G-box in the promoter.

Because our experimental conditions could not distinguish the acute response from the later extended expression (Anderson et al., 1997) following the red light pulse for the promoters we studied, we were unable to determine whether HY5 is specifically involved in only one particular response or in both responses. Nonetheless, because the single red light pulse treatment caused no detectable morphological changes in seedlings, the above data definitively confirm the essential role of HY5 in phytochrome-mediated low-fluence induction of gene expression, which is

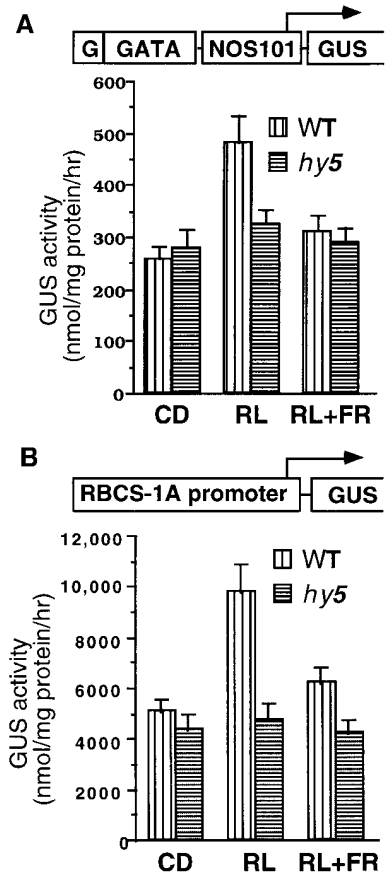

Figure 5. Red Light Pulse Induction and Its Far-Red Light Pulse Reversibility of Dark-Grown Seedlings Containing G-GATA/*NOS101*–*GUS* or the *RBCS-1A* Promoter–*GUS* Transgenes.

Four-day-old dark-grown seedlings were exposed to 2 min of red light (RL) alone or immediately followed by exposure to 10 min of far-red light (RL+FR). After the light treatment, the seedlings were transferred to complete darkness (CD) for an optimal time period for each line (18 hr for *RBCS-1A*–*GUS* and 48 hr for G-GATA/*NOS101*–*GUS*) before being harvested for the GUS assay. The activities are the average of four independent repeats in one representative experiment (out of four), and the error bars indicate standard deviations.
(A) GUS activity of the G-GATA/*NOS101*–*GUS* transgene.
(B) GUS activity of the *RBCS-1A* promoter–*GUS* transgene.

independent of morphological change and the state of chloroplast development.

HY5 Is Required for Light-Regulated Expression of the G-Box–Containing Promoters in Both Photosynthetic and Nonphotosynthetic Tissue Types

Because the morphological defect of the *hy5* mutants is most dramatic in the hypocotyls, stems, and roots (Ang and Deng, 1994; Oyama et al., 1997; Ang et al., 1998) and less evident in the cotyledons/leaves, we thought it would be of interest to examine whether the effect of the *hy5* mutation on light-regulated gene expression follows an organ-specific pattern. Therefore, we analyzed the effect of the *hy5* mutation on the expression of G-box–containing promoters in various organs by staining for GUS activity in both 6-day-old light-grown seedlings (Figures 6A to 6C) and 16-day-old light-grown plants (Figures 6D to 6J). At 6 days, the G/*NOS101* promoter displayed significantly decreased *GUS* expression in the hypocotyls, roots, and cotyledons in *hy5* seedlings compared with those of the wild type (Figure 6A). The arrows indicate the upper hypocotyl region of the seedlings. This region and the root were most drastically affected by the *hy5* mutation. G-GATA/*NOS101* expression was limited to the cotyledons, and reduced GUS staining was evident in the *hy5* mutants (Figure 6B). Similarly, the *RBCS-1A* seedlings exhibited an overall reduction in GUS staining intensity in *hy5* seedlings compared with wild-type seedlings (Figure 6C). It should be noted that in all cases, the effect of *hy5* mutations was fairly uniform in reducing the GUS staining of all cell types of different organs that express the promoters in wild-type plants (data not shown; see also Puente et al., 1996).

At 16 days, G-GATA/*NOS101* expression was confined to the adult leaves (Figure 6E), and the *hy5* mutants displayed significantly weaker staining than did the wild type with more than sixfold reduction in GUS activity (Figure 7A). No expression of G-GATA/*NOS101* was detected in the stems and roots of either wild-type plants or *hy5* mutants. The G/*NOS101* and *RBCS-1A* promoters were active in leaves, stems, and roots of 16-day-old plants (Figures 6D, 6G, and 6I for G/*NOS101*; and Figures 6F, 6H, and 6J for *RBCS-1A*). The effect of the *hy5* mutation was notable in the leaves (Figures 6D and 6F) and the stems (Figures 6G and 6H) but was most striking in the roots (Figures 6I and 6J). In fact, GUS activity in the roots of 16-day-old *hy5* plants containing the *RBCS-1A* promoter–*GUS* construct was close to the background level, whereas at least sevenfold higher GUS activity was found in their wild-type counterparts carrying the same transgene (Figure 7B). In summary, these results confirm that the *hy5* mutation caused a decrease in the light-elevated transcriptional activities of all three G-box–containing promoters compared with wild-type levels, and this reduction was observed in all of the organs and cell types in which the promoters were active.

HY5 Is Constitutively Nuclear Localized Regardless of Light Conditions and Cell Types

It has been reported that most of the G-box binding factors in cultured Arabidopsis and soybean cells are cytosolic, and some GBFs, particularly GBF2, could translocate to the nucleus when irradiated by light (Harter et al., 1994; Terzaghi et al., 1997). However, immunolocalization studies with iso-

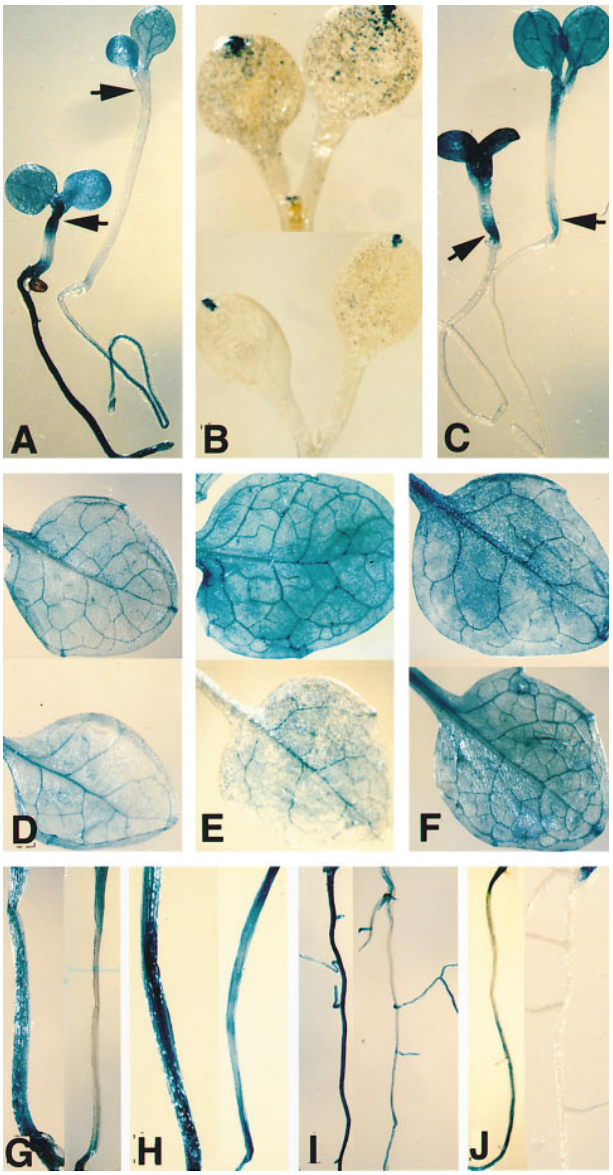

Figure 6. Effects of the *hy5* Mutation on Tissue-Specific Expression of G-Box–Containing Promoters in Light-Grown Plants.

In **(A)** to **(J)**, the wild type is shown to the left or at top and the *hy5* mutant is shown to the right or at bottom.

(A) to **(C)** Six-day-old seedlings carrying the G/*NOS101–GUS*, G-GATA/*NOS101–GUS*, and *RBCS-1A* promoter–*GUS* transgenes, respectively. Arrows indicate the dramatic GUS staining changes at the junctions of cotyledons and hypocotyls.

(D) to **(F)** Leaves of 16-day-old plants carrying the G/*NOS101*, G-GATA–*NOS101*, and *RBCS-1A* promoter–*GUS* transgenes, respectively.

(G) and **(H)** Stems of 16-day-old plants carrying the G/*NOS101* and *RBCS-1A* promoter–*GUS* transgenes, respectively.

(I) and **(J)** Roots of 16-day-old plants carrying the G/*NOS101* and *RBCS-1A* promoter–*GUS* transgenes, respectively.

lated root protoplasts suggested that HY5 is predominantly a nuclear protein (Oyama et al., 1997). To confirm further the subcellular localization pattern of HY5 in Arabidopsis and to investigate whether this localization is regulated by light or tissue-specific signals, a green fluorescent protein (GFP)–tagged full-length HY5 expression construct (S65TGFP–HY5) was stably introduced into Arabidopsis. Regardless of light conditions (dark, constant white, far-red, red, and blue light), the S65TGFP–HY5 fusion protein localized exclusively to the nuclei of all cell types in the cotyledons, hypocotyls, and roots of transgenic Arabidopsis seedlings. Because the seedlings grown in the dark and in far-red light have minimal background fluorescence, representative results with hypocotyl (Figures 8A and 8B) and root (Figures 8C and 8D) cells from selected dark- and far-red light-grown transgenic seedlings are presented. Our results indicate that HY5 is a nuclear protein and that its nuclear localization pattern is independent of light stimuli and cell type. Thus, light modulation of HY5 activity most likely occurs within the nucleus rather than by influencing its nucleocytoplasmic distribution, which is distinct from that reported for other G-box binding factors, such as GBF2 (Harter et al., 1994; Terzaghi et al., 1997).

DISCUSSION

Our results provide several lines of evidence for a direct role of HY5 in mediating light control of promoter activity in Arabidopsis. In the three G-box–containing promoters examined, including G/*NOS101*, G-GATA/*NOS101*, and *RBCS-1A*, the *hy5* mutation resulted in a significant reduction in expression levels in plants grown under continuous light (Figures 4A, 4B, 6, and 7). The *hy5* mutation also severely compromised the light inducibility of these promoters, as exemplified by the retarded response after transferring the dark-grown seedlings to white light (Figures 4C and 4D) and by a deficiency in low-fluence light pulse induction of G-GATA/*NOS101* and *RBCS-1A* promoters (Figures 5A and 5B). Therefore, our results suggest that HY5 is directly involved in phytochrome-mediated low-fluence red light induction of gene expression in addition to its role in phytochrome and blue light receptor-mediated inhibition of hypocotyl elongation, which are high-irradiance responses (Koornneef et al., 1980; Quail, 1994).

Because HY5 is able to bind specifically to the G-box in vitro, and because the G-box–containing promoters are selectively affected in the *hy5* mutant, it is likely that HY5 activates gene expression by directly binding to the G-box LRE in the promoter. This hypothesis may be further substantiated by in vivo analysis of HY5–DNA interactions in the future. However, it should be pointed out that HY5 may bind not only to the G-box motif. It is possible that HY5 may also bind to other distinct promoter element(s) with even higher affinity. This speculation is based on the fact that in our gel mobility and footprinting assays, relatively high concentrations

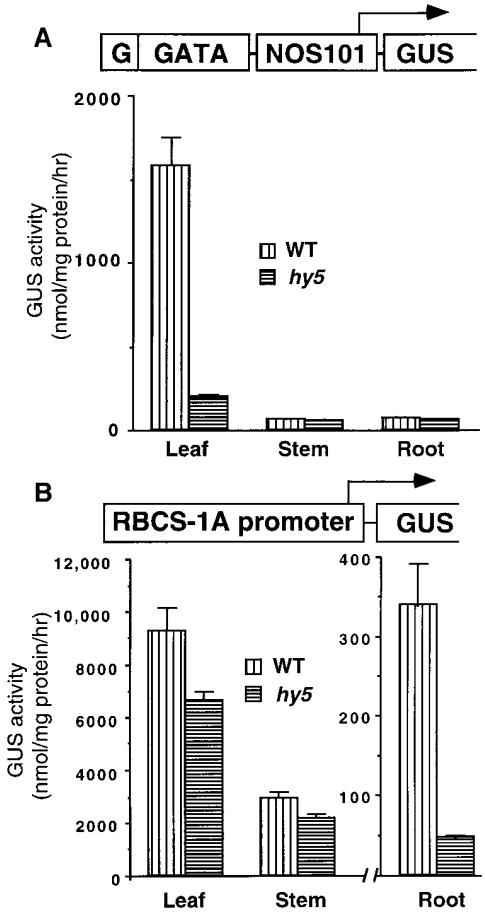

Figure 7. Comparison of GUS Activities in the Leaves, Stems, and Roots of 16-Day-Old Light-Grown Plants.

(A) Plants containing the G-GATA/*NOS101–GUS* transgene.
(B) Plants containing the *RBCS-1A* promoter–*GUS* transgene.
For further details, see the legend to Figure 4 and Methods.

of recombinant HY5 proteins were needed. Therefore, further research is needed to investigate what are the spectra of HY5 binding sites and also how the flanking sequences around the core motif may contribute to the binding specificity and affinity of HY5.

As a regulator of seedling photomorphogenesis, the activity of HY5 is likely to be modulated by light. We have shown that HY5 is a nuclear protein and that its localization is not regulated by light or cell-type specific signals. Thus, unlike GBF2, nuclear translocation is unlikely to be a light-regulatory mechanism for HY5 activity. It remains to be elucidated whether light directly regulates HY5–DNA interactions or modulates other aspects of HY5 activity and what factors link photoreceptors and HY5. It seems that components directly modulating the activity of HY5 would need to be localized within the nucleus. Previous genetic analyses of *hy5* and *cop*/*det*/*fus* mutations have implicated a possible functional interaction of HY5 with the pleiotropic COP/DET/FUS proteins, including COP1 (Ang and Deng, 1994) and DET1 (Chory, 1992; Pepper and Chory, 1997), which are the key nuclear repressors of photomorphogenesis. COP1 nucleocytoplasmic partitioning is regulated by light (von Arnim and Deng, 1994); thus, it may act as a potential link between cytosolic signaling components and nuclear-localized transcription factors such as HY5. The recent observation that COP1 is able to interact directly with and regulate HY5 in the nucleus (Ang et al., 1998), together with the data presented here, tempted us to speculate that HY5 represents a transcription factor that links the COP/DET/FUS proteins and the light-regulated genes via direct binding to the LRE (G-box) in the promoter. In future studies, it will be important to confirm this anticipated interaction in vivo and to determine how the interaction of HY5 with COP/DET/FUS proteins affects HY5's ability to regulate promoter activity. Further, the role of specific photoreceptors in regulating those regulatory interactions will be of great interest.

We have provided evidence that HY5, a genetically defined positive regulator of photomorphogenesis, acts as a specific DNA binding transcription factor and mediates the optimal expression of the gene in the light. However, HY5 is unlikely to be the only transcription factor that functions to mediate light-regulated gene expression and to promote photomorphogenic seedling development in the light. Redundancy of HY5-like transcription factors in plants, especially in green tissues, is implied by the following three main observations. (1) Light-grown *hy5* null mutant seedlings are only partially etiolated, suggesting the presence of additional activators for photomorphogenic development. (2) The negative effect of the null *hy5* mutation on light-activated gene expression is partial, because residual light activation is evident in all promoters tested. One exception is the *RBCS-1A* promoter in the roots of 16-day-old plants, in which the *hy5* mutation completely abolished its activity (Figures 6J and 7B). Thus, either there is little redundancy of HY5-like activities in roots or HY5 activity is dominant in the root relative to other factors. Redundancy of HY5 activity may also partially account for the earlier observation that *hy5* mutations do not affect total mRNA levels for *CAB* and *RBCS* genes when monitoring for the sum of multiple family members from mixed tissue types (Sun and Tobin, 1990; Chory, 1992; Ang and Deng, 1994). (3) There are many other LREs present in the promoters of light-regulated genes in addition to the G-box, and some light-regulated genes do not have a G-box in their promoters. Although it is possible that HY5 may regulate those promoters indirectly, it is reasonable to speculate that additional transcription factors, including CCA1 (Wang et al., 1997), which has a high affinity for other LREs, may play a role similar to that of HY5 and be directly involved in light regulation.

It is known that G-box–like sequences can also be bound by a large family of bZIP proteins, which includes GBFs and TGA1

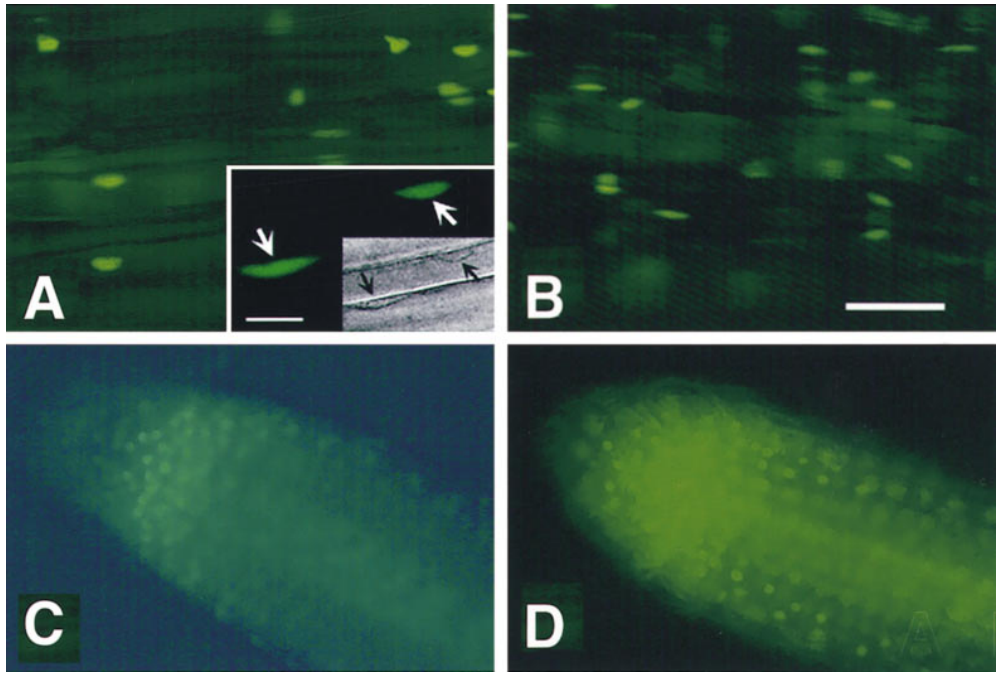

Figure 8. Nuclear Localization of GFP–HY5 in Arabidopsis.

(A) and **(B)** Dark- and far-red light–grown hypocotyl cells.
(C) and **(D)** Dark- and far-red light–grown root cells.
Fluorescence in the nuclei of 4-day-old dark- and far-red light–grown hypocotyl and root cells of transgenic lines expressing the S65TGFP full-length HY5 fusion protein is shown. In all cases, green fluorescence is located exclusively in the nuclei, as determined by a direct comparison of the confocal and Nomarski microscopic images of the same cells. For example, see the insert and the insert within the insert in **(A)**. The arrows indicate the nuclei. The bar in the insert in **(A)** = 50 μm. The bar in **(B)** = 0.2 mm for **(A)** to **(D)**.

(Schindler et al., 1992; Menkens et al., 1995; Feldbrugge et al., 1997). Therefore, HY5 most likely coordinates and competes with these transcription factors, and even possibly forms heterodimers with some of them, to bind to the G-box in a specific promoter. On the other hand, the G-box alone is insufficient to confer phytochrome-mediated low-fluence light regulation of gene expression (Puente et al., 1996). This implies that any one type of transcription factor, including HY5, is insufficient to make the promoter fully light responsive. Because distinct combinations of at least two LREs are necessary for mediating proper light control of promoter activity in a cell type and in a developmental-specific fashion (Degenhardt and Tobin, 1996; Puente et al., 1996; Feldbrugge et al., 1997), diverse coordinated binding and interaction of the involved transcription factors are necessary to achieve optimal light and developmental regulation of gene expression. In addition to light responses, detailed mutant phenotypic characterizations have revealed that HY5 may also participate in hormonal and gravitational responses (Oyama et al., 1997). It is possible that HY5 represents one of the integration points between light signaling pathways and hormonal signaling pathways in control of gene expression. This signal integration may be achieved in part through interactions between HY5 and other transcription factors involved in hormonal or gravitational responses.

METHODS

Plant Materials and Growth Conditions

The *Arabidopsis thaliana hy5-1* (*Ci88*) mutant was described by Koornneef et al. (1980), and all of the promoter–β glucuronidase (GUS) transgenic lines were described by Puente et al. (1996). After crossing the *hy5* mutant with a given transgenic line, we selected multiple *hy5* mutant and wild-type sibling lines homozygous for the transgene for initial examination. In all cases, similar results were obtained from those independent segregating lines, and only one representative wild type and one mutant line were selected for further analysis and presented. Seed sterilization and plant growth conditions were described previously (Ang and Deng, 1994; Puente et al., 1996). The light source in the growth chamber was a combination of cool-white fluorescent light and incandescent lights at 120 $\mu mol\ m^{-2}\ sec^{-1}$.

DNA Binding Assays and DNase I Footprinting

DNA binding assays were performed in a final volume of 20 μL containing a binding buffer of 15 mM Hepes, pH 7.5, 35 mM KCl, 1 mM EDTA, 6% glycerol, 1 mM DTT, and 2 μg of poly(dI-dC). One to 1.5 ng of 3′ end ^{32}P-labeled probes was used for binding assays. The glutathione *S*-transferase (GST)–HY5 and GST proteins were expressed in *Escherichia coli* and affinity purified according to the manufacturer's standard procedure (Biolab, Beverly, MA). The DNA fragment of the ribulose bisphosphate carboxylase small subunit (*RBCS*) *RBCS-1A* minimal promoter (from −320 to −125) was synthesized by polymerase chain reaction using the forward (5′-CCATCGATCCAGGCAAGTAAAATGAGCAAG-3′) and the reverse (5′-CGTTCTCGAGGATTTTGAGTGTGGATATGTGT-3′) primers. The polymerase chain reaction fragment was digested with ClaI and XhoI, cloned into pBluescript SK+ (Stratagene, La Jolla, CA) at the ClaI-XhoI sites, and confirmed by sequencing. For DNase I footprinting (de Lorenzo et al., 1991), the resulting plasmid DNA was digested with HindIII and KpnI, purified, and 3′ end labeled. Ten nanograms of labeled probe was used in a final volume of 100 μL in the binding buffer. Reactions were incubated for 20 min at 25°C with the GST–HY5 protein. Two nanograms of DNase I was added to each sample and incubated for 2 min. The DNA was precipitated and resuspended in 90% (v/v) formamide in Tris–borate–EDTA buffer (with tracking dye). The samples were run in a 7 M urea/6% polyacrylamide gel, and the resulting footprint was visualized by autoradiography.

GUS Staining and GUS Activity Measurement

The same procedures as described previously (Puente et al., 1996) were used for GUS histochemical staining and the quantitative GUS activity assay. For the quantitative GUS activity assay of young Arabidopsis seedlings, only the upper parts (hypocotyl and cotyledon) were used, unless otherwise specified. The wild-type plants and mutants carrying the same transgene were stained using the identical procedure for the same length of time.

The Green Fluorescent Protein–HY5 Fusion, Its Fluorescence, and Confocal Microscopy

The full-length *HY5* coding region was cloned into the pRTL2S65TGFP vector (Ang et al., 1998) to give S65TGFP–HY5, which contains the green fluorescent protein (GFP) with modified codons engineered to mutate the cryptic splicing sites in Arabidopsis (Haseloff and Amos, 1995). The fusion gene was under the control of the 35S cauliflower mosaic virus promoter and terminator. The resulting construct was cloned into binary plant transformation vector pPZP222 (Hajdukiewicz et al., 1994) and stably introduced into Arabidopsis (ecotype Columbia [Col-0]) via *Agrobacterium tumefaciens*–mediated vacuum infiltration. A total of 15 independent transgenic lines containing single transgene locus per haploid genome were generated, and similar cellular localization patterns were obtained. Fluorescent images of the GFP were taken with a ×20 objective on a Zeiss Axiophot (Oberkochen, Germany) microscope with the filter set of 480/40 excitation, 505LP dichroic, 535/50 emission (the numbers indicate midpoint wavelength/bandwidth in nanometers, respectively). Both the 4′,6-diamidino-2-phenylindole filter and the filter set for GFP were from Chroma Technology Corp. (Brattleboro, VT). Confocal images were taken on a Bio-Rad confocal microscope with a fluorescein isothiocynate filter set.

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