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Light regulated modulation of Z-box containing promoters by photoreceptors and downstream regulatory components, COP1 and HY5, in *Arabidopsis*

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Summary

The Z-box is one of the light-responsive elements (LREs) found in the promoters of light inducible genes. We have studied the light responsive characteristics of Z-box containing synthetic as well as native promoters. We show that promoters with Z-box as a single LRE or paired with another LRE can respond to a broad spectrum of light. The response is primarily mediated by phyA, phyB and CRY1 photoreceptors at their respective wavelengths of light. We have demonstrated that *CAB1* and *Z-GATA* containing promoters are down-regulated in *hy5* mutants in the light. On the other hand, a promoter with Z-box alone is down-regulated in *hy5* mutants both in dark and in light conditions, suggesting involvement of a similar regulatory system in the regulation of the promoter in two distinct developmental pathways: skotomorphogenesis and photomorphogenesis. Furthermore, similar to the *CAB1* promoter, a Z-GATA containing promoter is derepressed in *cop1* mutants in the dark. DNA–protein interaction studies reveal the presence of a DNA-binding activity that is specific to Z-box. These results provide insights into the regulation of the Z-box LRE mediated by various light signaling components.

Keywords: Z-box, ZBF, LRE, HY5, COP1, photoreceptor.

Introduction

Light is one of the most crucial factors involved in plant growth and development (Kendrick and Kronenberg, 1994). Depending on the presence or absence of light, higher plants, such as *Arabidopsis* thaliana, develop quite differently with respect to morphology, cellular and subcellular differentiation and gene expression. *Arabidopsis* seedlings are genetically capable of following two distinct developmental pathways: skotomorphogenesis in the dark and photomorphogenesis in the light (von Arnim and Deng, 1996). Whereas light-inducible genes are expressed at high levels in light-grown seedlings, dark-grown seedlings have low or no expression of light inducible genes. The expression of many light-inducible genes is influenced by the quality, quantity, periodicity, and direction of light sensed by a series of photoreceptors. Two major photoreceptor families have been characterized at the molecular level in *Arabidopsis*: the red/far-red responsive phytochromes, and blue light sensing cryptochromes (Ahmad and Cashmore, 1993; Deng and Quail, 1999; Fankhauser and Chory, 1997; Furuya, 1993; Quail, 1994). The phytochrome family of photoreceptors includes phyA to phyE, whereas CRY1 and CRY2 represent the family of cryptochrome. Another blue light receptor, which is known as NPH1, responds to phototropism (Christie *et al.*, 1998).

Genetic screens of *Arabidopsis* seedling development have identified several genes that function immediately downstream of phytochrome mediated signaling (Bolle *et al.*, 2000; Hoecker *et al.*, 1999; Hsieh *et al.*, 2000; Hudson *et al.*, 1999; Soh *et al.*, 2000). Additionally, several phytochrome interacting factors have recently been identified that are involved in both phyA and phyB-dependent light signaling (Choi *et al.*, 1999; Fankhauser *et al.*, 1999; Martinez-Garcia *et al.*, 2000; Ni *et al.*, 1998). Biochemical and pharmacological studies have revealed that G proteins, cGMP and Ca^{++}/calmodulin play an important role in phytochrome mediated signaling and in the regu-

lation of gene expression (Bowler *et al.*, 1994; Neuhaus *et al.*, 1993; Okamoto *et al.*, 2001).

A group of repressors of photomorphogenesis, COP/DET/FUS, acting downstream of photoreceptors have been identified and have been demonstrated to be down regulating the expression of several light-inducible genes in the dark. This class of genes (Fankhauser and Chory, 1997; Kwok *et al.*, 1996; Mise/ra *et al.*, 1994; von Arnim and Deng, 1996; Wei and Deng, 1999) has been found to be highly conserved in higher eukaryotes (Wang *et al.*, 1999; Wei and Deng, 1999). Among these, *COP1*, whose function has been studied in some detail, is considered to be a master regulator of photomorphogenic development (Deng and Quail, 1999; Holm and Deng, 1999; Osterlund *et al.*, 2000). Intracellular localization studies using a GUS-COP1 fusion protein reveal that COP1 is localized in the nucleus in the dark and that light reduces the COP1 level in the nucleus (Osterlund and Deng, 1998; Stacey *et al.*, 1999; von Arnim and Deng, 1996). Another class of regulatory proteins, which acts as positive regulators of photomorphogenesis, has been recently reported. Among these *HY5* has been genetically defined as a positive regulator based on its partially etiolated phenotype in light grown mutant seedlings (Ang and Deng, 1994; Koornneef *et al.*, 1980; Pepper and Chory, 1997). *HY5* encodes a bZIP protein that can physically interact with COP1 (Ang *et al.*, 1998; Oyama *et al.*, 1997). DNA–protein interaction studies have revealed that HY5 specifically interacts with the G-box and is required for the proper activation of G-box containing promoters in light (Ang *et al.*, 1998; Chattopadhyay *et al.*, 1998a). Two other COP1 interacting proteins that are present in the nucleus have been reported and their functions in light signaling have also been studied (Yamamoto *et al.*, 1998; Yamamoto *et al.*, 2001).

Regulation of transcription of specific genes is one of the important mechanisms by which light regulates plant growth and development (Millar and Kay, 1996; Terzaghi and Cahsmore, 1995; Tobin and Kehoe, 1994). Some of these genes, such as nuclear-encoded photosynthesis related genes for chlorophyll a/b binding proteins (*CAB*) and ribulose 1,5-bisphosphate carboxylase small subunit (*RBCS*), are induced by light. On the other hand, some genes, such as *PHYA*, NADPH-protochlorophyllide reductase and asparagine synthase are down-regulated by light (Donald and Cashmore, 1990; Gilmartin *et al.*, 1990; Ha and An, 1988; Quail, 1991; Silverthorne and Tobin, 1987; Sun and Tobin, 1990). The light responsive elements: G, GATA, GT1, and Z-box, which commonly occur in light regulated promoters have been demonstrated to be essential for light-controlled transcriptional activity (Millar and Kay, 1996; Terzaghi and Cashmore, 1995; Tobin and Kehoe, 1994). Recent studies have demonstrated that combinatorial interactions of distinct LREs is an important factor for light regulated promoter activities (Degenhardt and Tobin, 1996; Feldbrugge *et al.*, 1997; Puente *et al.*, 1996). Using synthetic promoter-reporter constructs with promoters containing single or paired LREs, it has been demonstrated that paired LRE-containing promoters can respond to phytochrome activating low-fluence light pulses and can mimic the responsiveness of native light regulated promoters. These promoters are also capable of responding to developmental signals such as for chloroplast development and tissue specificity (Puente *et al.*, 1996). Furthermore, it has been shown using G, GATA and GT1 LREs that paired element-containing promoters can respond to a broad spectrum of light, whereas single element-containing light inducible promoters can respond to specific wavelengths of light (Chattopadhyay *et al.*, 1998b). DNA–protein interaction studies have identified several *trans*-acting factors that interact with specific LREs. Some of these genes that specify such factors have been cloned and their roles in light signaling are also being investigated (Terzaghi and Cashmore, 1995; Tobin and Kehoe, 1994; Wang *et al.*, 1997).

Three putative Z-DNA forming sequences (ATACGTGT), the Z-box, have been reported to be present in the *Arabidopsis CAB1* promoter. Deletion analyses of this promoter have suggested that the Z-box, which is present in the light regulated minimal promoter region, is essential for the light dependent developmental expression of the *CAB1* gene (Ha and An, 1988). However, although G, GATA and GT1 LREs have been studied in detail, corresponding information about Z-box LRE is yet not available (Chattopadhyay *et al.*, 1998a; Foster *et al.*, 1994; Gilmartin *et al.*, 1992; Menkens *et al.*, 1995; Puente *et al.*, 1996; Terzaghi and Cashmore, 1995; Tobin and Kehoe, 1994). In this report, we have made an attempt to systematically study the regulation of the Z-box element mediated by several light signaling components.

Results

Promoters containing the Z-box alone or paired with another LRE respond to a broad spectrum of light

We used two stable transgenic lines: *Z/NOS101-GUS* and *Z-GATA/NOS101-GUS* for this study (Figure 1). The basal promoter used in these constructs was from the nopaline synthase gene (*NOS101*), which is from −101 to +4, contains the CAAT and the TATA boxes and is not active in transgenic plants (Mitra and An, 1989). Both these promoter-reporter constructs were individually introduced into photoreceptor null mutants (*phyA-1*, *phyB-B064* and *cry1/hy4–2.23 N*) by genetic crosses with the wild-type transgenic lines. Mutant lines homozygous for each transgene were then generated for further studies.

It was previously shown that the *Z/NOS101* promoter had higher activity in the dark compared with light in a

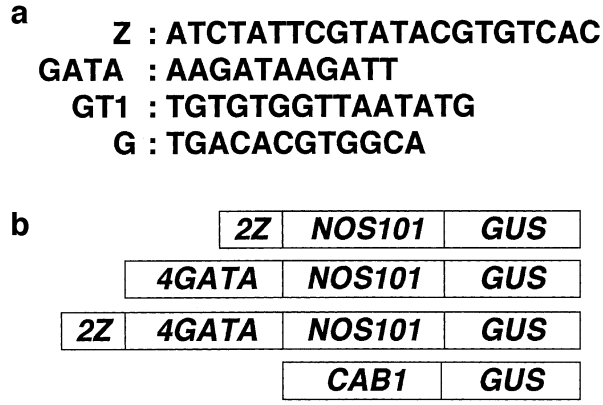

Figure 1. Light responsive elements (LREs) and promoter-reporter constructs used in this study.
(a) The consensus DNA sequences of four LREs (Z, GATA, GT1 and G) derived from different light responsive promoters.
(b) The synthetic and native promoter-GUS constructs. The *NOS101* promoter is the non-light regulated basal promoter from −101 to +4 of nopaline synthase gene.

wild-type background (Puente *et al.*, 1996). We therefore asked whether repression of the *Z/NOS101* promoter by light is mediated by certain specific photoreceptors. We first examined the repressive effect of various wavelengths of light on *Z/NOS101* promoter activity in wild type background. Four-day-old dark-grown seedlings were transferred to white light (WL), far-red light (FR), red light (RL) and blue light (BL) for 48 h and GUS activities were measured. As shown in Figure 2(a), all wavelengths of light tested reduced the activity of this promoter, suggesting that the activity of this promoter is reduced by a broad spectrum of light. To determine whether specific photoreceptors were involved in mediating the repression in specific wavelengths of light, we examined the activities of the *Z/NOS101* promoter in different photoreceptor mutant backgrounds. In *phyA* mutants, the repression of the promoter was significantly reduced in FR with practically no effect in other light conditions (Figure 2b), suggesting that phyA was specifically involved in FR-mediated repression of this promoter. In *phyB* mutants, promoter activity was increased in RL (Figure 2c) without major changes at other wavelengths of light, suggesting that RL-mediated repression may be caused primarily by the phyB photoreceptor. In the case of *cry1* mutants, the repression of *Z/NOS101* promoter caused by BL and WL was found to be significantly less effective (Figure 2d). These results together suggest that phyA, phyB and CRY1 are involved in mediating FR, RL, and BL repression of this promoter, respectively.

Previous studies had revealed that paired-element containing promoters, such as *Z-GATA/NOS101-GUS* were inducible by light (Puente *et al.*, 1996). To examine the pattern of induction of this promoter at various wavelengths of light in a wild-type background, we transferred 4-day-old dark-grown seedlings to FR, RL, and BL for 48 h and measured GUS activity. These assays revealed that the *Z-GATA/NOS101* promoter was induced more than four-fold in WL, RL and BL conditions and more than two-fold in FR (Figure 3a). To determine the involvement of specific photoreceptors in mediating light induction at various wavelengths of light, we monitored the activity of the promoter in different photoreceptor mutants. Induction was significantly reduced in FR in a *phyA* mutant background (Figure 3b). Additionally, we noted a substantial reduction in RL induction of this promoter in the same mutant background (Figure 3b). In *phyB* mutants, the induction level of *Z-GATA/NOS101* promoter was significantly compromised in both RL and FR conditions (Figure 3c). These results indicate that functional phyA and phyB are required for the proper induction of this promoter in either RL or FR conditions. However, in *cry1* mutants the BL induction was significantly reduced without affecting activity in other light conditions, suggesting that the CRY1 photoreceptor is primarily involved in mediating BL induction (Figure 3d). The WL induction of this promoter was not significantly affected in any of the photoreceptor mutants tested (Figure 3). These results probably indicate that phyA, phyB and CRY1 photoreceptors act redundantly to induce this promoter in WL.

Activation of Z-box containing promoters is regulated by downstream regulatory components, COP1 and HY5

HY5 specifically interacts with the G-box, and the G-box-containing promoters are down-regulated in *hy5* mutants in the light. However, any indirect role of HY5 in the regulation of other LREs remains to be studied. Furthermore, it is not clear whether HY5, which is exclusively present in a protein complex in both dark and light grown seedlings (Hardtke *et al.*, 2000), has any significant role in the regulation of gene expression in the dark. On the other hand, while many light inducible promoters are active in *cop1* mutants in the dark, the role of COP1 in the regulation of Z-box containing promoters is still unknown. We therefore asked whether the activity of the Z-box containing promoters is affected in the regulatory pathways defined by *cop1* and *hy5* mutations.

The Z-box containing synthetic (*Z/NOS101-GUS, Z-GATA/NOS101-GUS*) and native (*CAB1-GUS*) promoter-reporter constructs were introduced individually into *cop1–4* and *hy5-1* mutants by genetic crosses with the wild type transgenic lines (Puente *et al.*, 1996) and mutant lines homozygous for each transgene were generated. The *Z/NOS101-GUS* transgene was expressed in all the tissues in *cop1* mutants similar to wild-type seedlings in constant dark or light conditions (Figure 4a,b). Quantitative GUS activity measurements revealed that the activity of this

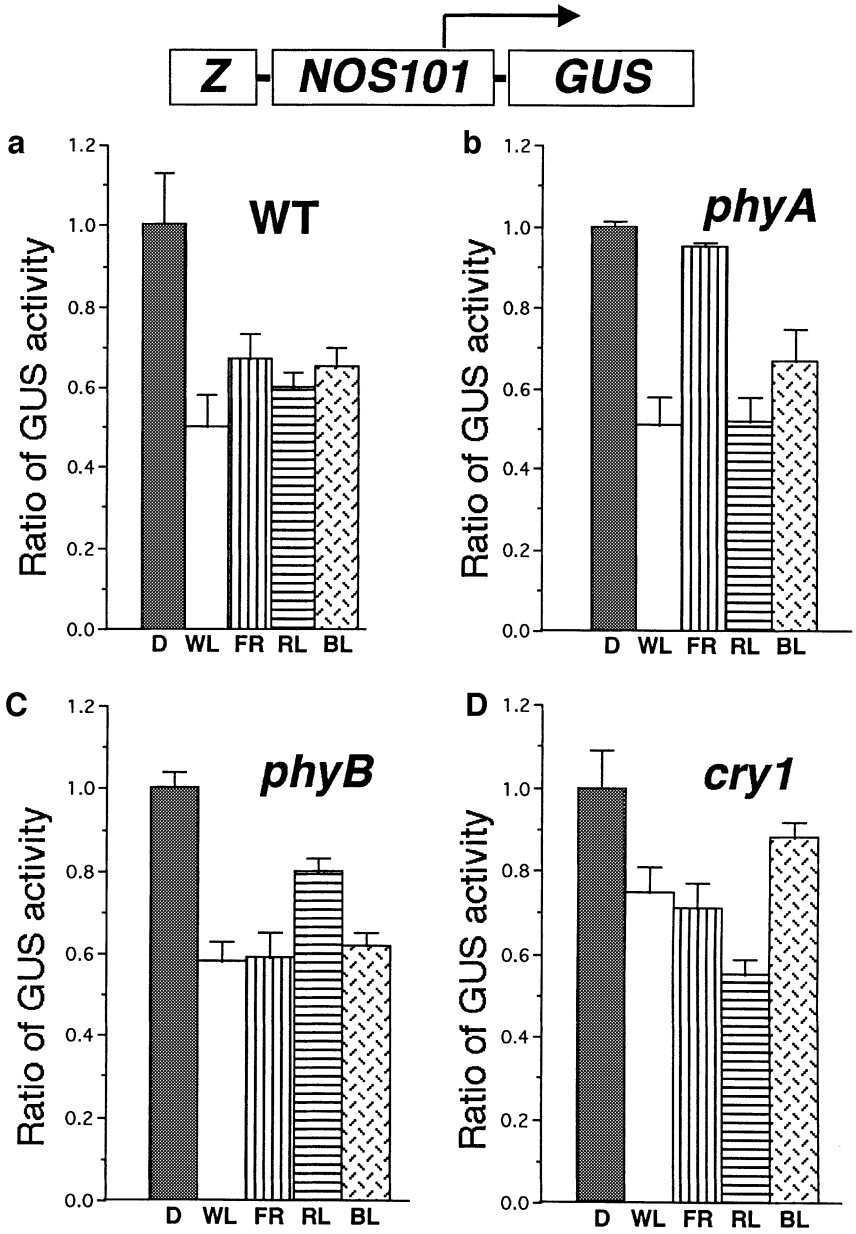

Figure 2. Involvement of different photoreceptors in light mediated repression of *Z/NOS101* promoter.
Four-day-old dark-grown seedlings of various genotypes containing the *Z/NOS101-GUS* transgene were exposed to different wavelengths of light: white light (WL), far-red light (FR), red light (RL), and blue light (BL) for 48 h and GUS activities were measured. The ratios of GUS activities (4 days dark plus 48 h in light versus 6 days in dark (D)) are shown in each panel. The error bars indicate standard deviation from at least three independent experiments; and the experiment was repeated for four times. The level of repression at various wavelengths of light in
(a) wild-type seedlings
(b) phyA mutants
(c) phyB mutants
(d) cry1 mutants

promoter in *cop1* mutants in dark- and light-grown conditions remained similar to the wild-type levels (Figure 4c), suggesting that there is very little effect of *cop1* mutation, if any, on the activity of the *Z/NOS101* promoter. In 6-day-old constant white light grown seedlings, the expression of the *Z-GATA/NOS101-GUS* transgene was confined to the cotyledons in wild-type and *cop1* mutants (Figure 4e). In *cop1* mutants, while the tissue specific expression pattern of the *Z-GATA/NOS101-GUS* transgene was largely maintained in dark- and light-grown seedlings, the level of expression of this transgene was significantly increased in the darkness compared with wild-type seedlings (Figure

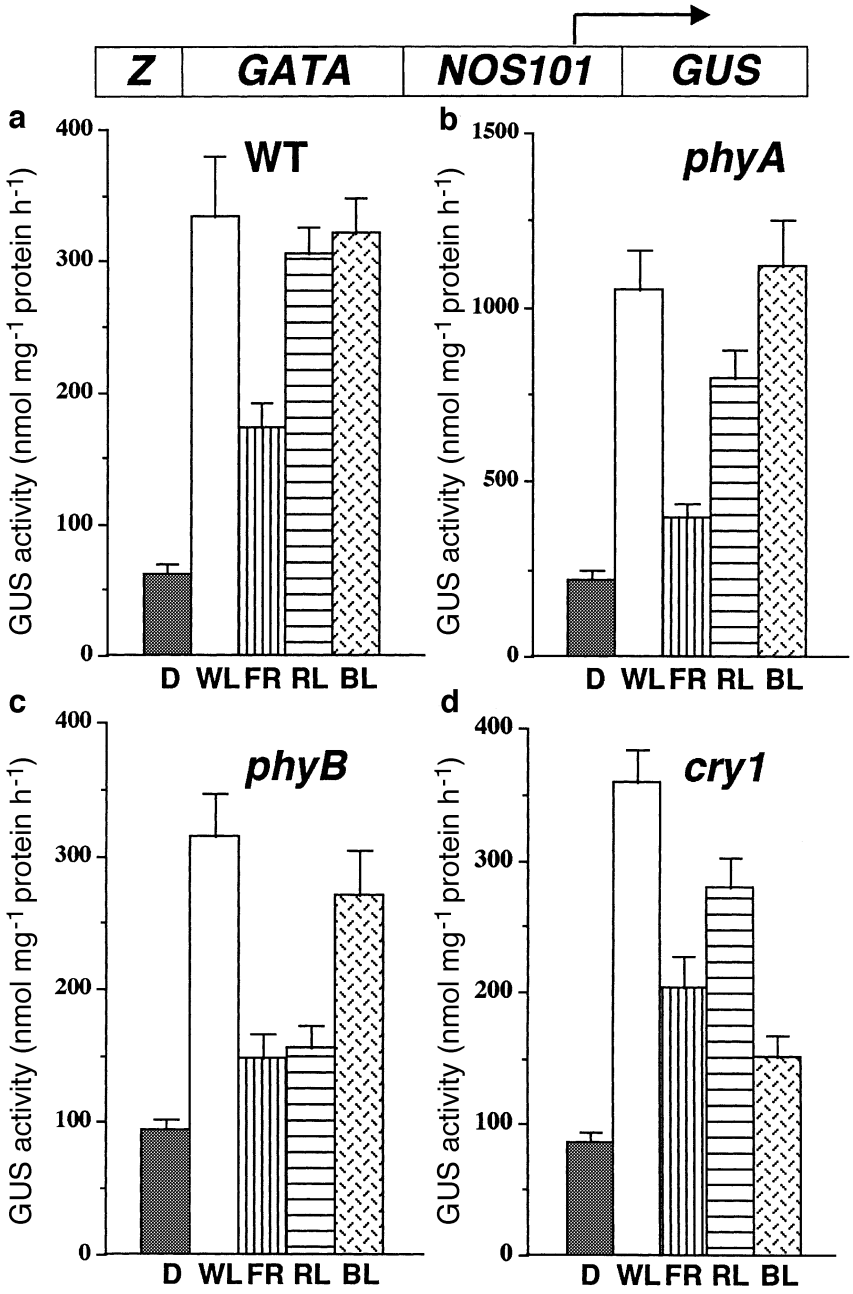

Figure 3. Involvement of photoreceptors in light mediated activation of *Z-GATA/NOS101* promoter. The level of activation at different wavelengths of light in comparison to six day old dark grown seedlings are shown in each panel. For experimental detail see legend to Figure 2. (a) wild-type seedlings, (b) *phyA* mutants, (c) *phyB* mutants, (d) *cry1* mutants.

4d). Quantitative GUS activity measurements revealed that similar to the *CAB1* promoter (data not shown), activity of the *Z-GATA/NOS101* promoter increased about three-fold in *cop1* mutants compared with wild-type seedlings in the dark. Activities of the promoter were almost equal in constant white light and dark conditions in *cop1* mutants (Figure 4f). These results indicate that similar to several native light regulated promoters, *Z-GATA/NOS101* is under the repressive control of COP1 in the darkness.

To determine the role of HY5 in the regulation of Z-box containing promoters, we used 6-day-old constant white light or dark-grown seedlings for GUS staining and activity measurements. While tissue specific expression was largely maintained, GUS activity staining of the *Z/NOS101-GUS*-expressing wild-type and *hy5* mutant seedlings showed significantly lower expression in *hy5* mutants compared with wild-type backgrounds both in dark and white light conditions (Figure 5a,b). GUS activity

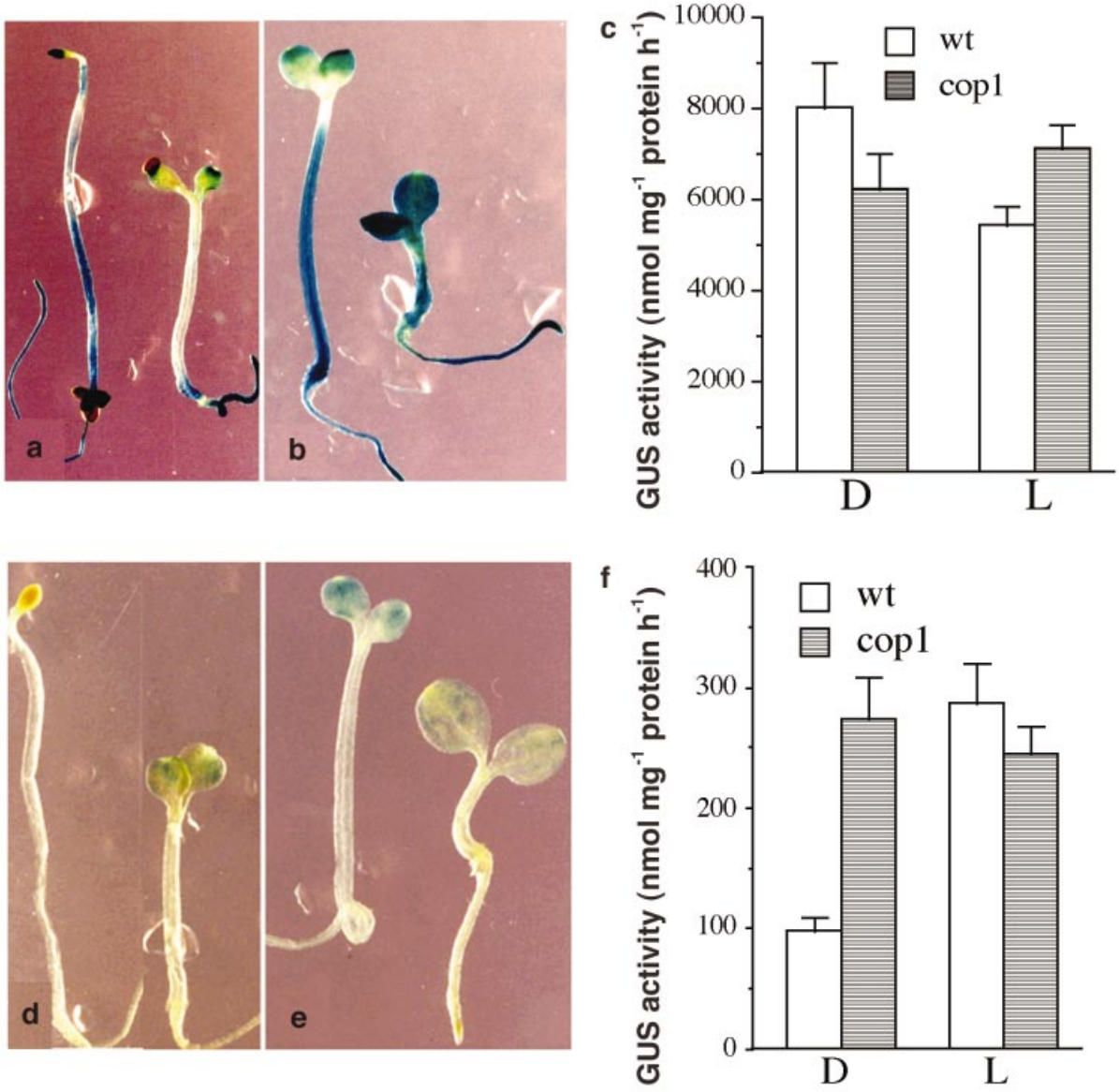

Figure 4. Effect of *cop1* mutation on the expression of *Z/NOS101-GUS* and *Z-GATA/NOS101-GUS* transgenes. In panels a, b, d and e wild-type seedlings are shown on the left and *cop1* mutant seedlings are on the right. All seedlings were grown either in constant dark or in constant white light conditions for 6 days.
(a,b) seedlings carrying *Z/NOS101-GUS* transgene were grown in dark or light, respectively, and used for GUS staining.
(c) Six-day-old constant dark- (D) or constant white light- (L) grown seedlings carrying *Z/NOS101-GUS* transgene were used for measurement of GUS activities.
(d,e) seedlings carrying *Z-GATA/NOS101-GUS* transgene were grown in dark or light, respectively, and used for GUS staining.
(f) Six-day-old constant dark- (D) or constant white light- (L) grown seedlings carrying *Z-GATA/NOS101-GUS* transgene were used for measurement of GUS activities.

measurement revealed that the activity of this promoter was about three-fold lower in *hy5* mutants compared with wild-type seedlings grown either in dark or in white light conditions (Figure 6a). No significant difference in GUS activity or GUS activity staining of the *GATA/NOS101-GUS* transgene (as control) in wild-type and *hy5* mutants was detected either in light or in dark conditions (Figure 5e and Chattopadhyay *et al.*, 1998a). The GUS activity staining of *Z-GATA/NOS101-GUS* and *CAB1-GUS* expressing wild-type and *hy5* mutant seedlings revealed that both transgenes were expressed mainly in the cotyledons in wild-type seedlings and that this tissue specificity was also maintained in *hy5* mutants. However, the level of expression of these transgenes was significantly compromised in *hy5* mutants compared with the wild-type background (Figure 5c,d). Quantitative GUS activity measurements

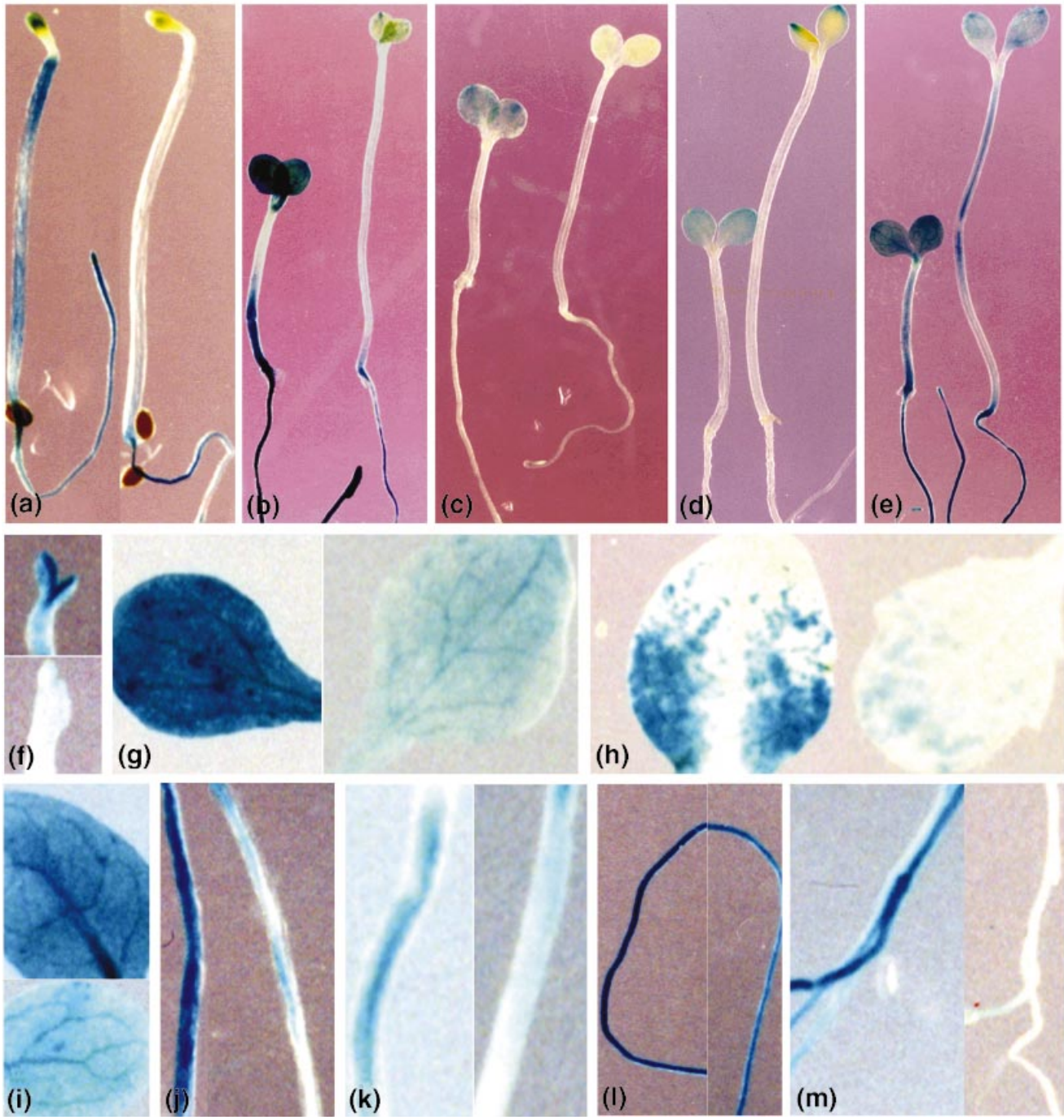

Figure 5. Effects of *hy5* mutation on the tissue specific expression of different transgenes.
In each panel, wild-type seedlings are shown on the left or top and the mutant seedlings are on the right or bottom. All the seedlings were grown either in constant dark or in constant white light conditions for six days (a–e) or 16 days (f–m).
(a,b) seedlings carrying *Z/NOS101-GUS* transgene were grown in dark and light, respectively.
(c–e) light grown seedlings carrying *Z-GATA/NOS101-GUS, CAB1-GUS* and *GATA/NOS101-GUS* transgenes, respctively.
(f,i) leaves of dark- and light-grown plants, respectively, carrying *Z/NOS101-GUS* transgene.
(g,h) leaves of light grown plants carrying *Z-GATA/NOS101-GUS* and *CAB1-GUS* transgenes, respectively.
(j,k) stems of dark- and light-grown plants, respectively, carrying *Z/NOS101-GUS* transgene.
(l,m) roots of dark- and light-grown plants, respectively, carrying *Z/NOS101-GUS* transgene.

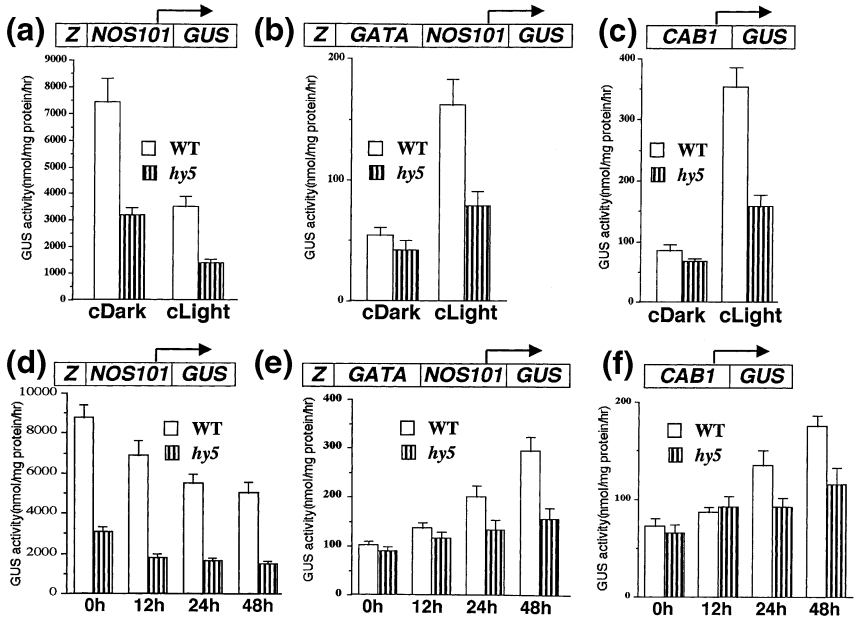

Figure 6. Effects of *hy5* mutation on the activities of Z-box-containing promoters. Promoter activities were monitored by measuring GUS activities of wild-type and *hy5* mutant seedlings carrying various transgenes. The experiments were repeated three times, and error bars indicate standard deviations. The promoter-reporter constructs are diagrammed on the top of each panel.
(a–c) GUS activities of 6-day-old constant dark or constant white light-grown seedlings of *Z/NOS101-GUS, Z-GATA/NOS101-GUS*, and *CAB1-GUS* transgenes, respectively.
(d–f) four-day-old dark-grown seedlings carrying *Z/NOS101-GUS*, *Z-GATA/NOS101-GUS* or *CAB1-GUS* transgene were exposed to white light for 0, 12, 24 and 48 h and GUS activities were measured.

revealed that the activity of *Z-GATA/NOS101* and *CAB1* promoters was reduced more than two-fold in *hy5* mutants compared with wild-type seedlings in constant white light. However, no significant difference in activity was detected between wild-type and *hy5* mutants in dark-grown seedlings (Figure 6b,c).

To further substantiate these results, we compared the light inducibility of *Z-GATA/NOS101* and *CAB1* promoters in wild-type and *hy5* mutant backgrounds. Although *Z/NOS101* promoter showed lower activities in light-grown seedlings compared with darkness in wild-type background, we used this promoter as a control for this study. For these experiments, four-day-old dark-grown seedlings were shifted to constant white light for 12, 24 and 48 h and GUS activities were measured. The activity of the *Z/NOS101* promoter was repressed to about three-fold in light both in wild-type and *hy5* mutant backgrounds, suggesting that HY5 is not involved in the repression of this promoter in light (Figure 6d). The *Z-GATA/NOS101* promoter, as shown in Figure 6(e), was induced more than three-fold in the wild-type background at 48 h, whereas there was very little induction, if any, in *hy5* mutants. Similarly, the light inducibility of the *CAB1* promoter was significantly compromised in *hy5* mutants compared with wild-type seedlings (Figure 6f). Therefore, these results together demonstrate that functional HY5 protein is required for the optimum activation of Z-box containing promoters.

HY5 is required for the tissue specific activation of Z-box containing promoters in adult plants

The *hy5* mutants have striking defects in hypocotyls, stems, and roots with less prominent defects in the cotyledons or leaves (Ang and Deng, 1994; Oyama *et al.*, 1997). In order to determine whether the effect of *hy5* mutation on the activation of Z-box containing promoters follows a tissue specific pattern in young adult plants, we examined the effect of the *hy5* mutation on the activation of Z-box containing promoters in different tissues by GUS activity staining measurements. We used 16-day-old-constant dark- or light-grown plants for this study. In the case of the *Z/NOS101-GUS* transgene, expression was significantly reduced in leaves of *hy5* mutants in comparison with wild-type (Figure 5f,i) with more than five-fold and three-fold reductions in dark- and light-grown plants, respectively (Figure 7a). The *Z/NOS101* promoter was also active in stems and roots of 16-day-old-plants grown either in dark or light conditions and the effect of the *hy5* mutation was clearly noticeable (Figure 5j,k,l,m), with the most drastic effect in the roots of light-grown plants (Figure 5m). While GUS activity in the roots of 16-day-old

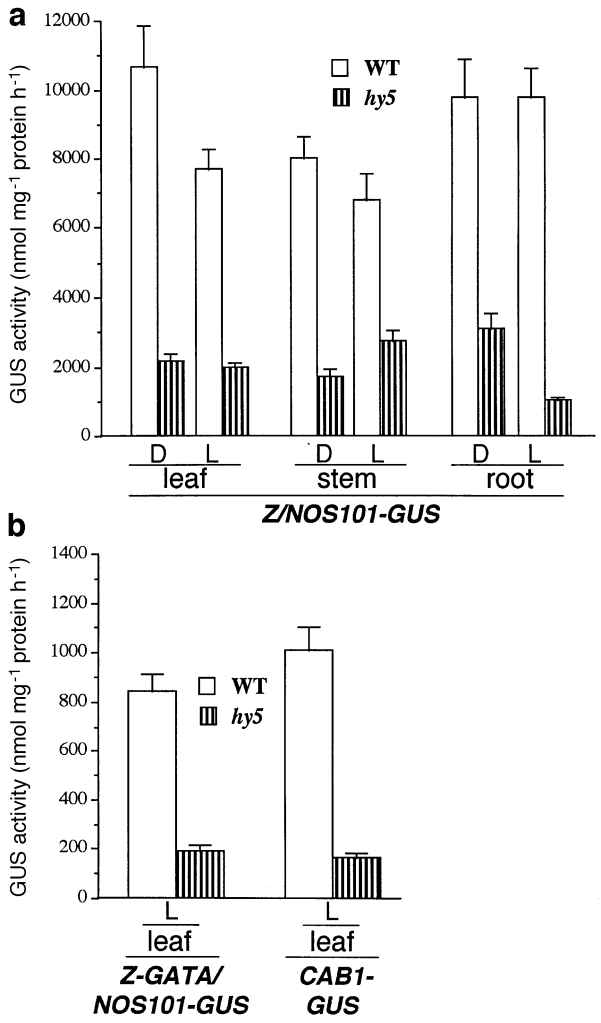

Figure 7. GUS activities in leaves, stems, and roots of 16-day-old light- or dark-grown wild-type and *hy5* mutant plants.
(a) Plants containing *Z/NOS101-GUS* transgene.
(b) Plants containing *Z-GATA/NOS101-GUS* or *CAB1-GUS* transgenes.

hy5 plants grown in constant light was close to the background level, more than nine-fold higher GUS activity was detected in wild-type plants carrying the same transgene (Figure 7a). Three- to five-fold lower GUS activity was also detected in *hy5* mutants compared with wild-type in stems and dark-grown roots (Figure 7a). At 16 days, the expression of *Z-GATA/NOS101-GUS* and *CAB1-GUS* transgenes in wild-type and *hy5* mutants was confined to the leaves (Figure 5g,h). Expression of these transgenes in *hy5* mutants was significantly reduced compared with wild-type (Figure 5g,h) with about four-fold and five-fold reductions in activity in *Z-GATA/NOS101* and *CAB1* promoters, respectively (Figure 7b). These results confirm that in comparison with wild-type plants the *hy5* mutation caused a striking decrease in the transcriptional activities of all three Z-box-containing promoters, and this reduction in activity was evident in all tissue-types where these promoters were active.

A Z-box binding factor (ZBF) activity is present in Arabidopsis

DNA–protein interaction studies using purified GST-HY5 fusion protein and different LREs have demonstrated that HY5 specifically binds to the G-box and does not interact with the Z-box (Ang *et al.*, 1998; Chattopadhyay *et al.*, 1998a; data not shown). Deletion analyses of the *CAB1* promoter have suggested that the Z-box is essential for light activation of this promoter (Ha and An, 1988). However, no *trans*-acting factor has been reported so far that specifically interacts with the Z-box. Therefore, to determine if there is any protein factor present that specifically interacts with the Z-box, we first used whole cell extracts of *Arabidopsis* seedlings and dimeric Z-box DNA (2Z) as a probe in gel shift assays. As shown in Figure 8(b), a strong low mobility DNA-protein complex (shifted band) could be detected along with the free probe (Figure 8b, lane 2). This shifted band was further intensified at the same position when twice the amount of whole cell extract was used (Figure 8b, lane 3). Furthermore, whereas a 50 or 100 molar excess of unlabeled 2Z DNA competed out the binding activity (Figure 8b, lanes 4 and 5), no competition was observed with a 100 molar excess of 4GT1 or 4G-box (Figure 8b, lanes 6 and 7), suggesting that the interaction was specific to Z-box. To confirm these results, we used mutated versions of the Z-box for gel shift assays. A 25 base pair DNA fragment containing three base pair substitutions in the Z-box (Zm-box) was used for gel shift assays (Figure 8a). While no DNA-protein complex was detected (Figure 8c, lane 2) with labeled Zm-box, a clear DNA-protein complex was formed with the labeled 2Z using the same extract (Figure 8c, lane 4). This complex was competed out by a 50 or 100 molar excess of unlabeled 2Z, but could not be competed out by a 100 molar excess of unlabeled Zm-box (Figure 8c, lanes 5, 6 and 7). These results together conclude the presence of a DNA-binding activity of ZBF that specifically interacts with the Z-box.

Discussion

Whereas light signaling pathways are becoming well characterized, understanding the regulation of individual LREs by light signaling components remains obscure. We have made an attempt to systematically study the function of photoreceptors and the downstream regulatory components in the regulation of one of the least studied LREs, the Z-box. In this study, we have demonstrated how high-irradiance light signals of different wavelengths can regulate activity of Z-box containing promoters. We

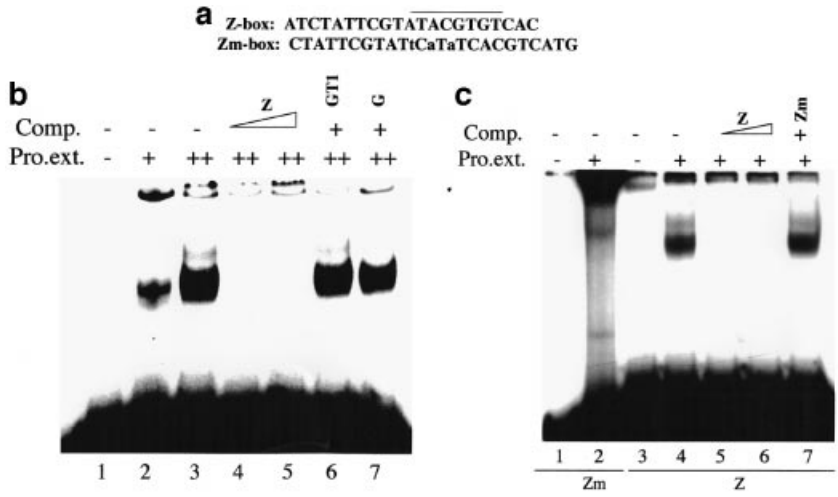

Figure 8. Identification of Z-box-specific DNA-binding activity.
(a) DNA sequences of Z-box and its mutated version (Zm).
(b) Electrophoretic mobility shift (gel shift) analysis using the whole cell extracts of 12-day-old constant light-grown seedlings and 42 base pair Z-box DNA as probe. No protein extract was added in lane 1. When 4 μg of extract was added in lane 2, 8 μg of whole cell extract was added in lanes 3 to lane 7. The amounts of competitors added in lanes 4, 5, 6 and 7 were 50 ng 2Z, 100 ng 2Z, 100 ng 4GT1 and 100 ng 4G, respectively. The increasing concentration of 2Z unlabelled DNA is shown by a triangle. Plus and minus signs indicate the presence and absence, respectively, of whole cell extracts (Pro. Ext.) or competitors (COMP).
(c) Electrophoretic mobility shift (gel shift) analysis using the whole cell extracts and 42 base pair Z-box DNA (lanes 3–7) or 25 base pair Zm-box DNA (lanes 1 and 2) as probe. No whole cell extract was added in lanes 1 and 3. Eight μg of whole cell extract was added in lanes 2, and 4–7. The amounts of competitors added in lanes 5, 6 and 7 were 50 ng 2Z, 100 ng 2Z, 100 ng Zm, respectively.

observed that repression or induction of the activities of the *Z/NOS101* or *Z-GATA/NOS101* promoter by light, respectively, was mediated primarily by specific photoreceptors responsive to their respective wavelengths of light. The distinct and overlapping functions of phytochromes A and B are generally accepted (Quail, 1994; Reed *et al.*, 1994). Besides their redundant and antagonistic functions, here we observed the interdependent functions of phyA and phyB in the regulation of gene expression. The *phyB* mutation not only almost completely abolished the induction of *Z-GATA/NOS101* promoter in RL, but also reduced significantly the induction in FR. Thus phyB is required for the optimum level of induction of this promoter in FR. Similarly, phyA is required for the proper induction of *Z-GATA/NOS101* promoter in RL. However, even though phyA and phyB are primarily involved in the repression of *Z/NOS101* promoter in FR and RL, respectively, they do not function interdependently in the regulation of *Z/NOS101* promoter. The overlapping functions of CRY1 and phyB in the regulation of G-box containing promoters have been previously demonstrated (Chattopadhyay *et al.*, 1998b). However, we did not observe any overlapping functions of phytochromes and cryptochromes in the regulation of any of the Z-box containing promoters. Therefore, the interdependent roles of photoreceptors in the regulation of gene expression are likely to be specific to light-responsive promoter determinants. The mechanism of significantly higher background activity of the *Z-GATA/NOS101* promoter in *phyA* mutants compared with wild-type, *phyB* and *cry1* mutant backgrounds (Figure 3) is not clear at this moment. The higher background activity was also detected in the *G-GATA/NOS101* but not in the *GT1-GATA/NOS101* promoter in *phyA* mutant background (Chattopadhyay *et al.*, 1998b). Therefore, one can speculate that phyA might be involved in repression of certain type of promoters in the darkness.

COP1 and HY5 are downstream regulatory components that function in a contrasting fashion in the regulation of light inducible gene expression. We examined the role of COP1 in the regulation of *Z/NOS101* and *Z-GATA/NOS101* promoters. We observed that while *cop1* mutation does not have any significant effect on the activity of *Z/NOS101* promoter, *Z-GATA/NOS101* promoter was activated in the dark in *cop1* mutants with almost the same level of activity in both dark- and light-grown seedlings. Therefore, repression of the *Z-GATA/NOS101* promoter is likely to be mediated by COP1 in the dark. Using G, GATA and GT1 LREs, it has been shown that paired-element-containing promoters are under COP1 regulation in the dark similar to several native light inducible promoters (Ang and Deng, 1994; Chattopadhyay *et al.*, 1998b). In agreement with these previous observations, we have demonstrated that Z-box paired with a GATA-box is also capable of responding to COP1 in the dark. Similar to *Z/NOS101* promoter, it has been demonstrated earlier that *cop1*

mutation does not have any repressive effect on *GATA/NOS101* promoter (Chattopadhyay *et al.*, 1998b).

HY5 specifically interacts with the G-box and is required for the activation of G-box-containing promoters in the light (Chattopadhyay *et al.*, 1998a). In all three Z-box-containing promoters tested in this study, *Z/NOS101, Z-GATA/NOS101* and *CAB1,* the *hy5* mutation caused a significant reduction in the activity of these promoters in light-grown seedlings. Furthermore, the activity of *Z/NOS101* promoter, which has higher activity in the dark compared with light-grown seedlings, was compromised in *hy5* mutants in the dark. The light induction kinetics with light inducible Z-box-containing promoters, such as *Z-GATA/NOS101* and *CAB1* showed a very low level of induction in the *hy5* mutants compared with wild-type seedlings. In young adult plants, while the tissue specific expression of *Z/NOS101-GUS*, *Z-GATA/NOS101-GUS* and *CAB1-GUS* transgenes was largely maintained in *hy5* mutants as in wild-type, the expression level in all tissue types tested was drastically reduced in *hy5* mutant plants. Therefore, taken together, our results firmly demonstrate that functional HY5 protein is required for the optimum transcriptional activities of the Z-box-containing promoters. It is interesting to note that probably two different modes of regulation seem to be operating on the *Z/NOS101* promoter in light. HY5, a positive regulator of photomorphogenesis and acting downstream to photoreceptors, is required for the optimum activation of *Z/NOS101* promoter in light (Figures 5b,i,k,m and 6a and 7a). On the other hand, the activity of the same promoter is repressed by light, mediated primarily by the specific photoreceptors at their respective wavelengths of light (Figure 2). The existence of branched and parallel pathways in the regulation of gene expression in light signaling cascades has been suggested (Barnes *et al.*, 1996; Deng and Quail, 1999). The regulation of the *Z/NOS101* promoter by HY5 and photoreceptors probably indicates the presence of parallel pathways, where the photoreceptor-mediated repression of the *Z/NOS101* promoter is operative in a different but parallel pathway than the HY5-mediated activation of this promoter in light signaling cascades.

We carried out gel shift assays to determine if there was any *trans*-acting factor present that could specifically bind to the Z-box. A strong DNA-binding activity was detected when the wild-type version of the Z-box was used. Competitive gel shift experiments with Z-box and other LREs clearly demonstrated that the DNA-binding activity was specific to the Z-box.

At least two possible modes of action for the HY5-mediated activation of Z-box containing promoters could be envisioned. Recently it has been demonstrated that HY5 is exclusively present in a 150-kDa protein complex as phosphorylated and unphosphorylated isoforms in dark- and light-grown seedlings (Hardtke *et al.*, 2000). Based on these findings, a post-translational modification of ZBF by HY5-protein-complex and thus activation of ZBF could be one possible mechanism by which HY5 regulates the Z-box-containing promoters. Alternatively, several G-box binding factors (GBFs) are known besides HY5, and it has been reported that GBF1, GBF2 and GBF3 proteins can heterodimerize *in vitro* (Menkens *et al.*, 1995). Furthermore, GBF1 and GBF2 are present both in dark- and in light-grown plants. Therefore, it could be envisioned that HY5 might heterodimerize with other transcription factors (including one of the GBFs) to interact with the Z-box and thus regulate Z-box containing promoters. In any case, further research on cloning and characterization of ZBF and *in vivo* and *in vitro* studies using ZBF and HY5 are required to test these possibilities.

Experimental procedures

Plant materials and growth conditions

All the promoter-reporter constructs used in this study have been described in Puente *et al.* (1996). Selected stable transgenes were individually introduced into *phyA-1* (Whitelam *et al.*, 1993), *phyB-B064* (Koornneef *et al.*, 1980), and *hy4–2.23 N* (Ahmad and Cashmore, 1993; Koornneef *et al.*, 1980) photoreceptor mutants by genetic crosses with the wild-type transgenic lines. The mutant lines homozygous for each transgene were obtained from the F3 generation for further studies. Similarly, the transgenes were also introduced into *cop1-4* and *hy5-1 (Ci88)* mutants by genetic crosses for further studies. For the growth of *Arabidopsis* seedlings, the white light intensity used was 100 μmol m^{-2} sec^{-1}. For the color light sources the intensities used (in LED chamber: Q-beam 3200-A; Quantum Devices, inc., WI 53507, USA) were far red light of 90 μmol m^{-2} sec^{-1}, red light of 96 μmol m^{-2} sec^{-1} and blue light of 36 μmol m^{-2} sec^{-1}.

GUS assays

GUS staining and GUS activity measurements (using about 40–50 seedlings in each sample) were carried out following the same procedure as mentioned in Chattopadhyay *et al.* (1998b). Wild-type and mutant plants (about 20–30 seedlings each) containing the same transgene were stained for the same length of time.

DNA-binding assays

Whole cell extracts were prepared from 12-day-old *Arabidopsis* seedlings grown in constant white light (100 μmol m^{-2} sec^{-1}). Whole cell extracts were prepared as described in Green *et al.* (1989). DNA-binding assays were performed at room temperature in 25 μl reaction volume with the binding buffer of 15 mM Hepes (pH 7.5), 35 mM KCl, 1 mM EDTA, 6% glycerol, 1 mM DTT, 1 mM $MgCl_2$ and 2 μg of Poly (dI-dC). After 15 min of incubation at room temperature, the samples were run on a 8% polyacrylamide gel at 12–15 mA, gels were then dried and autoradiographed.

DNA fragments and probes

The 42 base pair DNA fragment containing the Z-box dimer (Puente *et al.*, 1996) cloned in pBluescript (SK +) was digested with XhoI and HindIII, purified, and 3′ end labeled with α-P32 dCTP. The mutant complementary oligos of Zm-box (GGATCC-TATTCGTATtCaTaTCACGTCATGGAATTC) were annealed, and cloned into EcoRI and BamHI sites of pBluescript (SK +). This DNA fragment was purified and labeled for using as a probe for the DNA-binding assays. One ng of labeled DNA was used as a probe for each binding reactions.

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