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 and COP1 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; Heihe 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-
A group of repressors of photomorphogenesis, COP/Det/FRP, 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 Cashmore, 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 (Deggenhardt 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, 1998). 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 napaline 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-8064 and cry1/hy4-2.23 M) 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
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 2a, 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
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 4e).
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.

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, respectively.
(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.
(i,k) stems of dark- and light-grown plants, respectively, carrying Z/NOS101-GUS transgene.
(j,m) roots of dark- and light-grown plants, respectively, carrying Z/NOS101-GUS transgene.
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 6e, 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
the transcriptional activities of wild-type in stems and dark-grown roots (Figure 7a). At

compared with transgene (Figure 7a). Three- to five-fold activity was detected in wild-type

16 days, the expression of Arabidopsis seedlings and dimeric Z-box DNA (Z2) 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 Z2 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 Z2 using the same extract (Figure 8c, lane 4). This complex was competed out by a 50 or 100 molar excess of unlabeled Z2, 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 7. GUS activities in leaves, stems, and roots of 18-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 (Z2) 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 Z2 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 Z2 using the same extract (Figure 8c, lane 4). This complex was competed out by a 50 or 100 molar excess of unlabeled Z2, 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
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

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 32P-DCTP. The mutant complementary oligos of Z-box 5'-ATTTCCGATATCACTACGTCATGGAATTC-3' were annealed, and cloned into EcoRI and BamHI sites of pBluescript (SK+). This DNA fragment was purified and labeled for use 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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complement of ten essential and pleiotropic Arabidopsis COP/DET/FUS genes is necessary for repression of photomorphogenesis in darkness. Plant Physiol. 110, 731–742.


Light-mediated regulation defines a minimal promoter region of TOP2

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ABSTRACT

Light signaling has been demonstrated to be an important factor for plant growth and development; however, its role in the regulation of DNA replication and cell cycle has just started to be unraveled. In this work, we have demonstrated that the TOP2 promoter of Pisum sativum (pea) is activated by a broad spectrum of light including far-red light (FR), red light (RL) and blue light (BL). Deletions analyses of the TOP2 promoter in transformed plants, Arabidopsis thaliana and Nicotiana tabacum (tobacco), define a minimal promoter region that is induced by RL, FR and BL, and is essential and sufficient for light-mediated activation. The minimal promoter of TOP2 follows the phytochrome-mediated low-fluence response similar to complex light regulated promoters. DNA–protein interaction studies reveal the presence of a DNA binding activity specific to a 106 bp region of the minimal promoter that is crucial for light-mediated activation. These results altogether indicate a direct involvement of light signaling in the regulation of expression of TOP2, one of the components of the DNA replication/cell cycle machinery.

INTRODUCTION

DNA topoisomerases are a class of enzyme that alter the topology of DNA and are intimately involved in DNA replication. Depending on the mechanism of their action, topoisomerases are classified into two major groups: type I and type II enzymes. Whereas type I enzymes nick and seal one strand of DNA and change the linking number by one, type II enzymes nick and seal both strands of DNA and change the linking number by two (1–3). Nuclear topoisomerase II has been studied in detail in yeast and animal systems (4,5). Studies of topoisomerase II in mammalian systems have demonstrated that the activity of this enzyme is related to cell proliferation and suggested to be involved in cell cycle regulation (5–7). The cloning and functional analyses of TOP2 genes have been performed in a number of eukaryotes such as yeast (5,8), Drosophila (5,9,10) and mammals (5,11). However, very little information about TOP2 is available from higher plants (12). To our knowledge, the only reported clones of TOP2 in plants are from Arabidopsis (13,14) and pea (15). While studies in Arabidopsis have demonstrated the nuclear localization of topoisomerase II, studies in pea have suggested that the expression of this gene is increased by light and hormone (13–15).

Light is an important factor for plant growth and development (16). Higher plants, such as Arabidopsis thaliana, have developed a complex signaling network, which is modulated by light to optimize the photomorphogenic growth (17,18). Dark grown seedlings grow with long hypocotyls forming apical hooks, and cotyledons remain small and closed with largely undifferentiated cell types. In the presence of light, the hypocotyl growth is restricted and most of the energy of the plant is directed to cotyledon and leaf development with differentiated cell types (16,19). A number of genes are expressed at high level in light grown seedlings, however, the dark grown seedlings have very low or no expression of light inducible genes. Transcriptional regulation of specific genes is an important mechanism by which light regulates plant growth and development (17,20–25). A detailed analysis of the promoter of some of these genes, such as ribulose 1,5-bisphosphate carboxylase small subunit (RBCS) and nuclear-encoded photosynthesis related genes for chlorophyll a/b binding proteins (CAB) revealed the presence of several light responsive elements (LREs), such as G, GATA, GT1 and Z-box that are critical for light-controlled transcriptional activity (23–28).

Two major families of photoreceptors have been characterized in Arabidopsis that function at specific wavelengths of light to contribute to the plasticity of plant development (17,29–31). The phytochrome family of photoreceptors includes phylA to phylE that are specific to red (RL) and far-red (FR) light perception, whereas cryptochrome family is represented by cry1 and cry2 that specifically perceive blue (BL) and UV-A light (32,33). Several early and late signaling intermediates have been identified and demonstrated to be involved in light signal transduction from photoperception to transcription (34–42).

Light-mediated genome-wide gene expression during Arabidopsis seedling development has been recently investigated by DNA microarray technology (43–46). These studies have revealed that light controls the expression of many...
growth and developmental factors including DNA replication and cell cycle components (44,45). However, very little information of how light regulates the expression of these genes is available. Very recently, cloning of two intermediate genes of the brassinosteroid (BR) signaling pathways has been revealed to be BIN3/AtTOP6B and BIN5/AtSPO11-3, products of which constitute topoisomerase VI, a component of DNA replication machinery. Studies using bin3 and bin5 mutants suggest that topoisomerase VI is involved in plant growth and development (47). Plant steroid hormones, which are known as BR, modulate many growth and developmental processes including leaf, stem and root growth, xylem differentiation, apical dominance and senescence. Whereas the connection between BR signaling and light signaling pathways is still not clear, the dark grown BR mutant seedlings of Arabidopsis resemble light grown phenotypes with short hypocotyls and open and expanded cotyledons.

We previously reported that the pea TOP2 transcript level was increased in light during cell proliferation (15). In this report we have made an attempt to systematically study the role of light signaling in the regulation of the TOP2 promoter. We have determined the minimal TOP2 promoter region that is modulated by light and follows the low-fluence phytochrome response similar to complex light regulated promoters. Our results demonstrate that the expression of TOP2 is primarily confined to green tissues of light grown seedlings. DNA–protein interaction studies have revealed the presence of transacting factor(s) that shows DNA binding activity specific to AT1&1-box of TOP2 minimal promoter.

MATERIALS AND METHODS

Plant materials and growth conditions

Arabidopsis plants were grown at 22°C with a photoperiod of 16 h light and 8 h dark unless otherwise mentioned. The in vitro tobacco cultures as well as tobacco and pea plants in soil pots were maintained at 24–26°C. The white light and color light intensities and sources were the same as described by Yadav et al. (41).

Transcript analysis

Total RNA was isolated from the aerial part of 6-day-old (or as mentioned in the figure legends) pea seedlings using Trizol reagent (Gibco BRL) following the manufacturer’s instruction. We used a 1.8 kb DNA fragment of TOP2 of pea (15) for probe preparation using random priming kits (Megaprime™, Amersham) following the manufacturer’s instructions. The probe was purified through a Sephadex G-50 column. The same amount of total RNA (25 µg) was fractionated in 1% formaldehyde agarose gel. The hybridization procedures followed have been described previously (15). The membranes were exposed to X-ray film for autoradiography.

Generation of transgenic plants with promoter-reporter constructs

Different deletion versions of TOP2 promoter were generated by PCR amplification. The oligos used for UD, D1, D2, D3 and D4 promoter fragments are as follows: UD-forward: AACTGCAGCTCCACCGGTGGCGGCGC; UD-reverse: GCTCTAGACGGTAGATGGTGGGCTTGC; D1: CCC-
To determine whether TOP2 expression is regulated at different stages of development, we performed RNA gel blot analysis using WL grown pea plants. As shown in Figure 1C and F, the expression of TOP2 was detected to be at the highest level with ~10-fold more as compared to dark in 7-day-old plants. The transcript level significantly decreased in 14-day-old plants and showed only ~3-fold more expression than the dark in 21-day-old plants.

Deletion analyses of TOP2 promoter define a minimal promoter region that is induced by light

To determine the minimal light inducible promoter of TOP2, we made several promoter-reporter constructs (UD-TOP2-GUS, D1-TOP2-GUS, D2-TOP2-GUS, D3-TOP2-GUS and D4-TOP2-GUS) using various undeleted (UD) and deleted (D1 to D4) versions of the TOP2 promoter (15,54). These promoter reporter constructs (Fig. 2A) were individually introduced into Arabidopsis plants by stable transformation and several homozygous transgenic lines were generated for each transgene. Figure 2B–D shows the activity of three independent lines of each promoter-reporter construct. For this experiment we used 6-day-old constant dark or constant WL grown seedlings and measured the GUS activities. It is evident from Figure 2B that the activity of UD-TOP2 promoter was >6-fold higher in light as compared to the dark grown seedlings. D1-TOP2 promoter, which was derived from UD-TOP2 after deletion of 140 bp, had significantly reduced light-mediated activation as compared to UD-TOP2 promoter; however, it still showed ~5-fold-higher level of activity in light as compared to the dark grown seedlings (Fig. 2A and C). On the other hand, D2-TOP2 promoter, which was derived from D1-TOP2 after deletion of 106 bp, showed very little stimulation in GUS, if any, in light as compared to dark grown seedlings (Fig. 2A and D). A higher level of GUS activity in light as compared to darkness was not detected with D3-TOP2-GUS and D4-TOP2-GUS transgenes (data not shown).

To compare the light-mediated induction kinetics of the D1-TOP2 promoter with D2-TOP2, we transferred 4-day-old dark grown seedlings to light for 12, 24 and 48 h and measured GUS activity. As shown in Figure 2E, whereas the D1-TOP2 promoter was induced to ~5-fold, there was very little induction, if any, of D2-TOP2 promoter after 48 h of exposure to light. These results indicate that the inducibility of D2-TOP2 promoter was significantly compromised in light.
Figure 2. Minimal promoter region of TOP2 that is activated by light in transgenic Arabidopsis. (A) Schematics of deletion constructs of TOP2 promoter fused to GUS reporter. The arrow indicates the transcriptional start site, and the numbers indicate the length of each undeleted or deleted constructs from the transcriptional start site. (B) Three independent lines (UD1, UD2 and UD3) containing UD-TOP2-GUS transgene were used to determine the GUS activity. Six-day-old constant dark (D) or constant white light (WL) grown seedlings were used for GUS activity measurement. The error bars indicate standard deviation from at least three independent experiments; the experiment was repeated three times. (C) Three independent lines (D1–1, D1–2 and D1–3) containing D1-TOP2-GUS transgene were used to determine the GUS activity. For experimental detail see legend to (B). (D) Three independent lines (D2–1, D2–2 and D2–3) containing D2-TOP2-GUS transgene were used to determine the GUS activity. For experimental detail see legend to (B). (E) Four-day-old dark grown seedlings were transferred to WL for 12 (12h), 24 (24h), 48 h (48h) or kept in the dark for another 48 h (0h) and GUS activities were measured. D1 indicates D1-TOP2-GUS transgene and D2 indicates D2-TOP2-GUS transgene. The error bars indicate standard deviation from at least three independent experiments; the experiment was repeated four times.

grown seedlings. Taken together, these results suggest that the 468 bp region of TOP2 promoter is essential for light-mediated activation.

To determine whether D1-TOP2 promoter was also induced by other wavelengths of light, we examined the activity of D1-TOP2 promoter with UD-TOP2 and D2-TOP2 promoters as control under various wavelengths of light. For this experiment, 4-day-old dark grown seedlings were exposed to RL, FR, BL and WL for 48 h and GUS activities were measured. In the case of UD-TOP2 promoter, the induction was ~5-fold higher in all light conditions with the highest level of induction in WL (Fig. 3A). Whereas the level of activation
was ~4-fold in RL, FR and BL conditions, and ~5-fold in WL. In D1-TOP2 promoter, there was hardly any induction of D2-TOP2 promoter in similar conditions (Fig. 3B and C). These results suggest that D1-TOP2 promoter is the minimal promoter region that is essential and sufficient for activation mediated by a broad spectrum of light.

**D1-TOP2 promoter follows the phytochrome mediated low-fluence response**

Single RL pulse to dark grown seedlings followed by FR-mediated cancellation of gene expression is a characteristic of phytochrome-mediated low-fluence light induction (25, 30, 48). To determine whether TOP2 promoter can respond to the RL-induction and FR-mediated cancellation signaling and also to rule out the possibility that the light-mediated induction of TOP2 is not due to a secondary effect of light-mediated morphological changes, we studied the phytochrome-mediated low-fluence response of TOP2 promoter. We used CABI minimal promoter (CABI-GUS) as a control for this study (28, 41). As shown in Figure 4A, a single RL pulse to 5-day-old dark grown seedlings induced the expression of UD-TOP2-GUS transgene to 4-fold and a subsequent exposure to FR reduced the expression level to ~2-fold. In the case of D1-TOP2-GUS transgene, the RL pulse was also able to induce the expression to ~3-fold and this expression was cancelled to ~2-fold by subsequent FR light exposure (Fig. 4B). A similar result was obtained with the CABI-GUS transgene (Fig. 4C). These results suggest that the TOP2 promoter is able to respond to the phytochrome-mediated low-fluence response similar to complex light regulated promoters. These results further demonstrate that the D1-TOP2 minimal promoter is also capable of responding to phytochrome-mediated low-fluence response.

The expression of TOP2 is primarily confined to cotyledons and hypocotyls of light grown seedlings

To determine the tissue-specific expression pattern of TOP2 and also to determine whether D1-TOP2-GUS follows the same tissue specific expression pattern as the UD-TOP2-GUS, we analyzed GUS activity staining of different TOP2-GUS transgenes in various organs of light grown *Arabidopsis* seedlings. The expression of UD-TOP2-GUS transgene was confined to cotyledons and hypocotyls with no detectable expression in the roots (Fig. 5a). The expression of D1-TOP2-GUS transgene maintained the same tissue specific expression pattern as UD-TOP2-GUS; however, the level of expression was significantly reduced in both hypocotyls and in cotyledons (Fig. 5b). In the case of D2-TOP2-GUS transgene, the expression level was drastically reduced and the expression was only detectable in the cotyledons (Fig. 5c).

To test the above observations, we further introduced all five promoter–reporter constructs (UD-TOP2-GUS, D1-TOP2-GUS, D2-TOP2-GUS, D3-TOP2-GUS and D4-TOP2-GUS) individually into tobacco plants by leaf disc transformation method and obtained the transgenic plants. GUS staining activity measurements of different transgenic lines in tobacco background also mimicked the expression pattern observed in *Arabidopsis*. Furthermore, in the case of UD-TOP2-GUS transgene, as observed by transverse sectioning of the stem, the expression was clearly concentrated in the vascular cylinder (Fig. 5d). While the tissue-specific expression pattern was largely maintained in D1-TOP2-GUS and D2-TOP2-GUS transgenes, the level of expression decreased significantly in D1-TOP2-GUS with very little expression in D2-TOP2-GUS transgene (Fig. 5e and f). No GUS activity staining of D3-TOP2-GUS and D4-TOP2-GUS transgenes was detected either in *Arabidopsis* or in tobacco background (data not shown).

Vascular tissues form a pattern in the stem that reflects the developmental connection between the stem and the leaves. GUS activity staining of tobacco seedlings, as revealed by the serial transverse section of the stem (Fig. 5g), showed that the expression of the D1-TOP2-GUS transgene had extended gradually from the vascular cylinder of the stem toward the formation of the leaf, and the expression is concentrated in rapidly dividing cells of leaf traces (Fig. 5h–l).
Figure 4. RL pulse-mediated induction and its cancellation by FR light of dark grown seedlings containing UD-TOP2-GUS, D1-TOP2-GUS or CAB1-GUS transgenes. Five-day-old dark grown seedlings (D) were exposed for 2 min to RL or followed by 10 min exposure of FR light (RL+FR). After the light treatments the seedlings were kept in dark for an optimum period of 20 h before the seedlings were harvested for GUS activity measurements. (A) UD-TOP2-GUS transgene. (B) D1-TOP2-GUS transgene. (C) CAB1-GUS transgene.

Figure 5. Tissue specific expression of UD-TOP2-GUS, D1-TOP2-GUS or D2-TOP2-GUS transgenes in Arabidopsis or tobacco light grown seedlings. (a-c) Six-day-old Arabidopsis seedlings containing UD-TOP2-GUS, D1-TOP2-GUS or D2-TOP2-GUS transgenes, respectively. (d-f) Tobacco seedlings containing UD-TOP2-GUS, D1-TOP2-GUS or D2-TOP2-GUS transgenes, respectively, were used for transverse section of the stem and GUS staining. (g) The tobacco seedling used for serial transverse section and GUS staining. (h-l) Tobacco seedling as shown in (g) was used for serial transverse section of the stem and staining. The arrows indicate the leaf traces.

The minimal promoter region of TOP2 has DNA binding activity

GUS activity measurement and staining of different deletion versions of the TOP2 promoter thus far revealed that the activity of the promoter was detectable at a very low level in the D2-TOP2 promoter with no detectable activity in the D3-TOP2 promoter in light grown seedlings (Figs 2, 3 and 5, and data not shown). Computer analysis of −468 to −262 DNA sequence (D1–D3) revealed several putative cis-acting elements (Fig. 6A) to be present within this region (15) (website: http://oberon.rug.ac.be:8080/PlantCARE/index.html). For example, as shown in Figure 6A, there are at least I, AT1 and GA motifs in the D1–D3 promoter region. Trans-acting factors specific to I box (also known as GATA box) and AT1 motif have already been demonstrated to be present and involved in light-regulated gene expression (24). We ask whether any of these cis-acting elements in the TOP2 minimal promoter region are recognized by specific trans-acting factor(s). To test this possibility, we performed electrophoretic mobility shift (gel shift) assays using 207 bp (D1–D3) DNA fragment (Fig. 6A) of the TOP2 promoter as a probe and whole cell extracts made from 6-day-old light grown pea seedlings. Figure 6B shows a strong low mobility DNA-protein complex formed (Fig. 6B, lane 2) and the complex became more intense at the same position when twice the amount of protein was used (Fig. 6B, lane 3). Whereas a 50 and 100 molar excess of unlabeled 01-D3 could compete out the binding activity (Fig. 6B, lanes 4 and 5), no competition was observed with a 100 molar excess of MCS (Fig. 6A and B, lane 6), suggesting that this DNA binding activity was specific to D1–D3 DNA fragment.

To further substantiate and narrow down the DNA binding activity region in D1–D3, we performed similar gel shift assays and competed with unlabelled D1–D2 and D2–D3 DNA fragments (Fig. 6A). As shown in Figure 6C, whereas unlabelled D1–D2 was able to compete the DNA binding activity at 50 and 100 molar excess, unlabelled D2–D3 fragment was unable to compete the binding activity at even 100 molar excess (Fig. 6C, lanes 4–7). These results confirm that the D1–D2 region of TOP2 promoter, which is essential and sufficient for light-mediated activation, has a specific DNA binding activity.

Since computer analyses reveal several cis-acting elements within D1–D3 region, we ask whether the DNA binding
competed out by binding activity was specific to AT1&I-box. To rule out the shown) . The DNA-protein complex was competed out by a extracts made from RL and BL grown pea seedlings (data not shown) . The DNA-protein complex was detected with the whole cell extract of the dark grown seedlings (Fig. 8B, lane 2), a clear DNA­ box] was used as competitor (Fig. 8A) . While no DNA­promoter. For these light grown seedlings (Fig. 8B, lane 3) and also with the gel shift assays using the whole cell extracts of 6-day-old light grown pea seedlings and 207 bp D1-D3 DNA as probe. No protein extract was added in lane 1. Four micrograms of extract were added in lane 2, 8 µg of whole cell extract were added in lanes 3–6. The amount of competitors added in lanes 4, 5 and 6 was 50 ng D1-D3, 100 ng D1-D3 and 100 ng MCS, respectively. The increasing concentration of D1-D3 unlabeled DNA is shown by triangles. Plus and minus signs indicate the presence and absence, respectively, of whole cell extracts (Pro. Ext.) or competitors (Comp.). (B) Electrophoretic mobility shift (gel shift) analysis using the whole cell extracts of 6-day-old light grown pea seedlings and 207 bp D1-D3 DNA as probe. No whole cell extract was added in lanes 1. Four micrograms of whole cell extract were added in lane 2, and 8 µg in lanes 3–7. The amount of competitors added in lanes 4–7 was 50 ng D2-D3, 100 ng D2-D3, 50 ng D1-D2 and 100 ng D1-D2, respectively. The increasing concentration of D1-D2 or D2-D3 unlabeled DNA is shown by triangles. Plus and minus signs indicate the presence and absence, respectively, of whole cell extracts (Pro. Ext.) or competitors (Comp.).

activity of D1–D2 is specific to any of these cis-acting elements. To determine the specificity of the DNA binding activity, we performed DNase I footprinting analyses of D1--D3 DNA fragment. As shown in Figure 7, the DNA binding activity protected at least a 6 bp region centered around one I box and its overlapping AT1 box from DNase I cleavage suggesting that the DNA binding activity was likely to be specific to this region.

To confirm the above observations, we further studied the gel shift assays using the 106 bp D1–D2 region of the TOP2 promoter. For these studies, a 27 bp DNA fragment containing three base pair substitutions in the AT1&I-box [AT1&I(m)-box] was used as competitor (Fig. 8A). While no DNA–protein complex was formed with the whole cell extract of the dark grown seedlings (Fig. 8B, lane 2), a clear DNA–protein complex was formed with the extracts made from the light grown seedlings (Fig. 8B, lane 3) and also with the extracts made from RL and BL grown pea seedlings (data not shown). The DNA–protein complex was competed out by a 50 or 100 molar excess of unlabeled AT1&I-box, but could not be competed out by 50 or 100 molar excess of unlabeled AT1&I(m)-box (Fig. 8B, lanes 4–7), suggesting that the DNA binding activity was specific to AT1&I-box. To rule out the possibility that the absence of a shifted band with dark grown extracts (Fig. 8B, lane 2) is due to the inhibitory activity present in the extract, we mixed the extracts from dark and light grown seedlings and used for DNA binding assays. A DNA–protein complex was formed with the mixed extracts as was observed with the extracts from light grown seedlings (data not shown). These results together conclude that an AT1&I-box-specific DNA-binding activity is present in the light grown seedlings.

DISCUSSION

Information about light-regulated expression of genes that are involved in DNA replication or cell cycle is rarely available. Light has been shown to stimulate cell division rates in pea apical nodes (49) and also enhances mRNA levels of nucleolin, which was reported to be a cell-cycle-regulated protein (50). Earlier we have shown that the expression of one of the components of DNA replication machinery, topoisomerase II, is regulated by light. However, the mechanism of light-mediated induction was not investigated. In this study, we have examined in detail the steady state mRNA level of TOP2 in dark and light grown pea seedlings and have
Figure 7. DNase I foot printing analysis of 207 bp D1–D3 fragment (top strand) of TOP2 promoter. Lane 1 shows the A+G Maxam and Gilbert sequencing ladder. Lane 2 is the control lane (cont.) without any protein. Lanes 3 and 4 show the ladder caused by DNase I cleavage with 10 and 20 μg of whole cell extract (Pro. Ext.), respectively. The triangle indicates the increasing concentrations of whole cell extracts. The hypersensitive nucleotides are marked as open circles and protected nucleotides as stars.

demonstrated that the expression of this gene was up-regulated by light. Furthermore, we have demonstrated that the light-mediated up-regulation of TOP2 is not specific to a particular wavelength of light rather it is mediated by a broad spectrum of light including RL, FR and BL.

The minimal promoter regions of several light-regulated genes have been deciphered by deletion analysis and generally found to be ~250 bp long from the transcriptional start site (24). However, there are several examples where the minimal promoter regions are significantly longer than 250 bp, and

there are also examples of as short as 52 bp long light responsive minimal promoters (51,52). In close agreement with these previous observations, deletion analyses of the TOP2 promoter reveal that a 468 bp region from the transcriptional start site is essential and sufficient for light-mediated activation of the TOP2 promoter. Whereas deletion from -608 to -469 reduced the activity of the TOP2 promoter, it was still inducible by RL, FR, BL and WL to ~4-fold. However, further 106 bp deletion from -468 almost abolished the light-mediated induction of this promoter, suggesting that the 106 bp region between D1-TOP2 and D2-TOP2 is crucial for light-mediated activation of the promoter.

Arabidopsis seedlings grow with contrasting morphologies in light and dark conditions. A single RL pulse can rapidly change the dark grown morphology and programmed the seedlings to grow photomorphogenically. This rapid change in developmental processes mediated by phytochrome signaling is likely to accompany the DNA replication and cell cycle processes. We have demonstrated that TOP2 promoter, similar to CABI promoter, indeed responds to RL and FR reversible induction and cancellation, respectively, mediated by phytochromes. These results probably suggest that light-mediated activation of TOP2 promoter is not a secondary effect rather its activation is under the control of phytochrome-mediated signaling. The D1-TOP2 minimal promoter also equally responded to this phytochrome-mediated low-fluence response and thereby suggests that this minimal promoter region contains the essential light-responsive promoter determinants.
The expression of UD-TOP2-GUS transgene was confined to the cotyledons and hypocotyls where the photomorphogenic development is evident. This expression pattern was also maintained by D1-TOP2-GUS transgene, which is driven by the minimal promoter, D1-TOP2. Our results show that the expression of D1-TOP2-GUS transgenes was highly concentrated in the leaf tracts of vascular cylinder that contains actively dividing cells, consistent with the notion that topoisomerase II should be more active in the rapidly dividing cells. It has been reported that topoisomerase II in animals has different isoforms; however, detailed work in plants in this aspect is not available. It could be envisioned that there may also be different isoforms of topoisomerase II in plants as we have found in tobacco (Singh, B.N. and Sopory, S.K., unpublished data) that might account for activities specific to roots. Alternatively, the root-specific cis-acting elements involved in the expression of TOP2 in roots are outside the length of the promoter used in this study.

We carried out electrophoretic mobility shift (gel shift) assays to determine whether there was any trans-acting factor present that shows DNA binding activity specific to D1–D3 promoter fragment. The gel shift assays altogether reveal the presence of a DNA binding activity specific to D1–D2 promoter fragment (106 bp), which is crucial for light-mediated activation. Furthermore, the DNA foot printing analyses with D1–D3 (Fig. 7) and gel shift analysis with D1–D2 (Fig. 8) promoter fragments confirm a DNA binding activity specific to AT1&1-box. It is interesting to note that 3AFI site of pea RBCS3A promoter also contains an AT1 motif combining a GATA motif (or I box), which has been demonstrated to have a specific DNA binding activity by gel shift and foot printing analyses and is probably involved in light-regulated activation of the promoter (53). In our case, further detailed research for functional evidence through transgenic plants is required to establish the involvement of AT1&1-box in light-mediated regulation of D1-TOP2 promoter. Additionally, cloning and characterization of trans-acting factor(s) specific to AT1 and I boxes will help to analyze the light-mediated regulation of the TOP2 promoter. Nonetheless, this work firmly demonstrates for the first time the direct involvement of light signaling in the regulation of expression of TOP2, an important component of the DNA replication/cell cycle machinery.

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