5256–5265 *Nucleic Acids Research, 2003, Vol. 31, No. 18*
DOI: 10.1093/nar/gkg744

Light-mediated regulation defines a minimal promoter region of *TOP2*

G. H. C. M. Hettiarachchi[1,2], Vandana Yadav[1], M. K. Reddy[2], Sudip Chattopadhyay[1,*] and Sudhir K. Sopory[2]

[1]National Center for Plant Genome Research, PO Box 10531, JNU Campus, New Delhi 110067, India and
[2]International Center for Genetic Engineering and Biotechnology, Aruna Asaf Ali Marg, New Delhi 110067, India

Received July 3, 2003; Revised and Accepted July 30, 2003

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). Deletion analyses of the *TOP2* promoter in transformed plants, *Arabidopsis thaliana* and *Nicotiana tobaccum* (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 phyA to phyE 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

*To whom correspondence should be addressed. Tel: +91 11 2670 4531; Fax: +91 11 2616 7394; Email: sudipchatto@yahoo.com

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&I-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. 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: AACTGCAGCTCCACCGCGGTGGCGGCGC; UD-reverse: GCTCTAGACGGTAGA TGGTGGGCTTGC; D1: CCCAAGCTTCTACGTCTTTGTTTCAGTT; D2: CCCAAGCTTAATAACCCTAGTTTGACAC; D3 CCCAAGCTTCACTCTCCACCTA CCAACA; and D4: CCCAAGCTTCACCATCCTCCTCACCCTCCA. The PCR products were digested with HindIII and XbaI and cloned into the HindIII + XbaI site of pBI101.2 (Stratagene) binary vector. *Agrobacterium* strain *GV3101* was transformed with different versions of the promoter–reporter constructs, and wild-type *Arabidopsis* (ws) plants were transformed using *Agrobacterium* carrying various recombinant pBI101.2 constructs individually by vacuum infiltration method. Transgenic plants were screened on 20 µg/ml kanamycin Murashige and Skoog plates and several lines of homozygous transgenic plants containing each transgene were generated. All the promoter–reporter constructs were also individually introduced into tobacco plants by *Agrobacterium*-mediated co-cultivated tissue culture method.

GUS assay

GUS staining and GUS activity measurements were performed following the same procedure as described by Yadav *et al.* (41). Transgenic seedlings containing various transgenes were stained for the same period of time. Free hand transverse section of tobacco stem was subjected to GUS staining following the same procedure.

Electrophoretic mobility shift and foot printing analyses

Whole cell extracts were prepared from 6-day-old light grown pea seedlings (41). 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 10 min incubation at room temperature, the samples were run on 6% polyacrylamide gel, dried and autoradiographed. The D1–D3 (Fig. 6) or D1–D2 (Fig. 8) DNA fragments, which were cloned into pBluescript (SK+), were digested with HindIII + NotI and 3′-end labeled with [α-^{32}P]dCTP for marking probes. For AT1&I-box and AT1&I(m)-box competitor DNA fragments preparation, first the complementary oligos were annealed and cloned into EcoRI and BamHI sites of pBlueScript (SK+) and then digested and purified from polyacrylamide gels. For DNase I foot printing, the D1–D3 DNA fragment in pBluescript was digested with HindIII and KpnI, purified from gel and labeled at the 3′ end. Approximately 15 µg of labeled DNA was used in 60 µl of total volume in binding buffer with various concentrations of total extract and incubated for 15 min. DNase I (0.05 U) was added to samples and incubated for 1 min. The DNA was precipitated, and resuspended in 90% formamide in Tris–borate–EDTA buffer. The samples were run in 6% polyacrylamide gel with 7 M urea, dried and autoradiographed.

RESULTS

The expression of *TOP2* is induced by a broad spectrum of light

To determine the effect of light on the expression of *TOP2* during early seedling development, we used 6-day-old constant dark (D) and constant white light (WL) grown pea

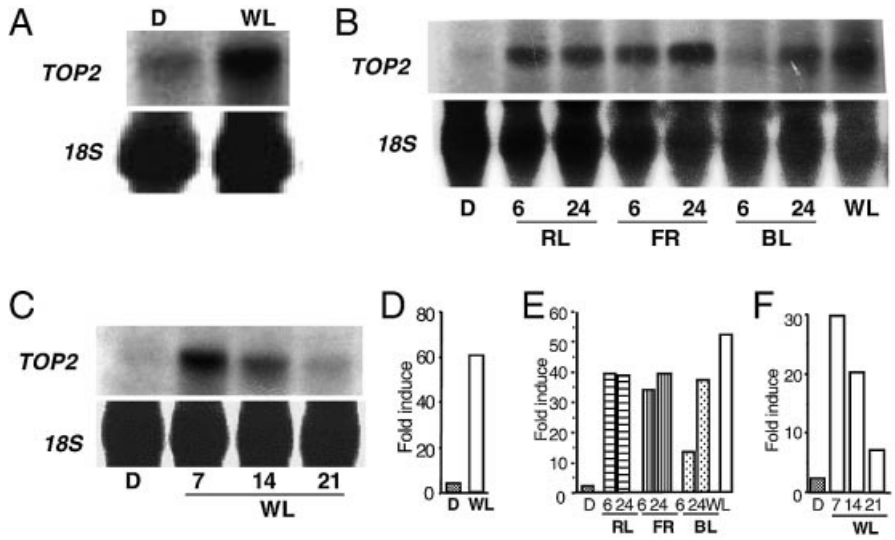

Figure 1. Light-regulated expression of *TOP2* gene in pea. (**A**) Seedlings were grown for 6 days in either constant dark (D) or in constant white light (WL) for RNA gel blot analysis. Total RNA (25µg) was loaded in each lane; 18S rRNA (18S) was shown as a loading control. A representative autorad from three independent experiments was shown. (**B**) Five-day-old dark grown seedlings were transferred to red light (RL), far-red light (FR) or blue light (BL) for 6 or 24 h for transcript analysis. Total RNA (25µg) was loaded in each lane and 18S rRNA (18S) was used as a loading control. A representative autorad from four independent experiments was shown. (**C**) Seedlings were grown for 7 days in constant dark (D), or grown for 7, 14 or 21 days in constant white light (WL) for RNA gel blot analysis. Total RNA (25µg) was loaded in each lane; 18S rRNA (18S) was shown as a loading control. A representative autorad from three independent experiments was shown. (**D**) Quantification of the data in (A) by Fluor-S-MultiImager (Bio-Rad) (**E**) Quantification of the data in (B) by Fluor-S-MultiImager (Bio-Rad) (**F**) Quantification of the data in (C) by Fluor-S-MultiImager (Bio-Rad).

seedlings for RNA gel blot analysis. As shown in Figure 1A and D, the expression of *TOP2* is >10-fold higher in constant WL grown seedlings as compared to the darkness.

Since it has been demonstrated that higher plants are able to receive various wavelengths of light such as RL, FR and BL through specific photoreceptors, we ask if the expression of *TOP2* is also induced by different wavelengths of light. We transferred 5-day-old dark grown seedlings to RL, FR, BL and WL for 6 and 24 h and monitored the transcript level by RNA gel blot analysis. As shown in Figure 1B and E, the expression of *TOP2* was induced by all wavelengths of light including RL, FR and BL. Whereas there was an ~3-fold induction in BL after 6 h exposure, RL and FR induced the level of expression to ~8-fold. However, after 24 h the BL-mediated induction was detected to be ~8-fold, which was similar to the level of induction in RL and FR conditions (Fig. 1B and E). Taken together these results suggest that the expression of *TOP2* is induced by a broad spectrum of light, and that the rate of BL-mediated induction of *TOP2* is slower when compared to the RL- and FR-mediated inductions.

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

Since the expression of *TOP2* is induced by a broad spectrum of light, we were interested to determine and study the light-mediated regulation of the minimal promoter region of *TOP2*. 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

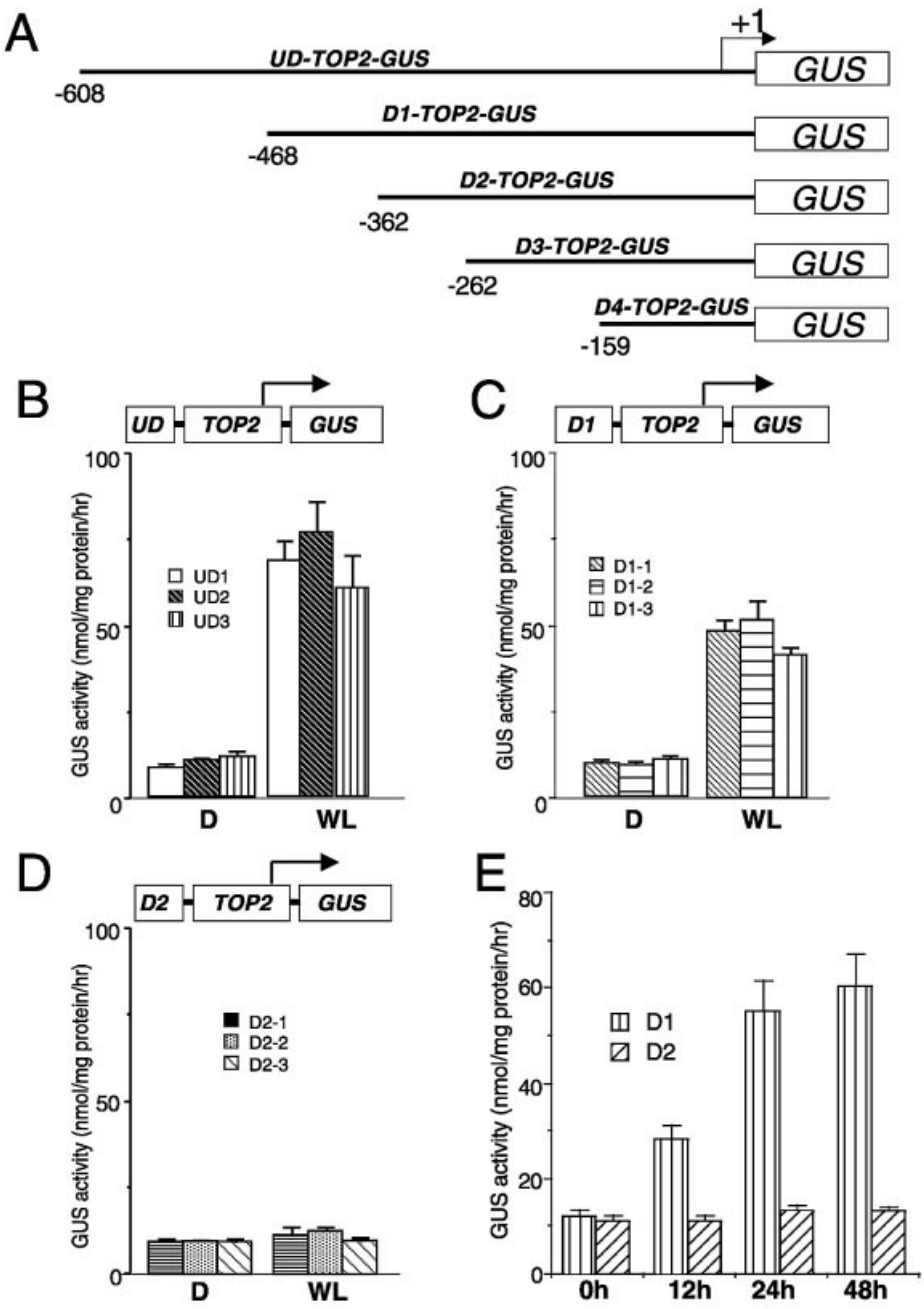

Figure 2. Minimal promoter region of *TOP2* that is activated by light in transgenic *Arabidopsis*. (**A**) Schematics of deletion constructs of *TOP2* promoter fused to GUS reporter. The arrow indicates the transcriptional start site, and the numbers indicate the length of each undeleted or deleted 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

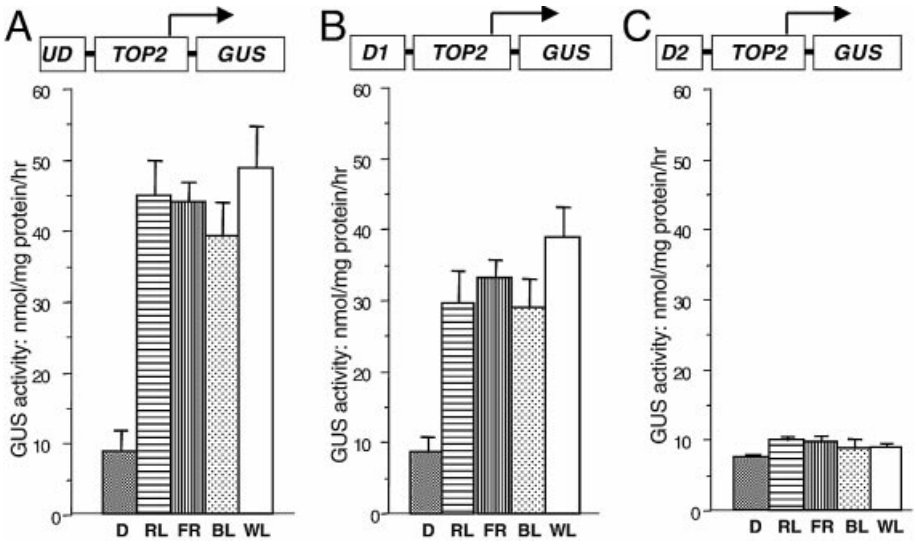

Figure 3. Activation of *TOP2* minimal promoter by various wavelengths of light. Four-day-old dark grown seedlings were transferred to red light (RL), far-red light (FR), blue light (BL) and white light (WL) for 48 h and GUS activities were measured. Six-day-old dark grown seedlings were used as dark (D) control. The level of activation at various wavelengths of light of (**A**) UD-TOP2-GUS transgene, (**B**) *D1-TOP2-GUS* transgene and (**C**) *D2-TOP2-GUS* transgene.

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 *CAB1* minimal promoter (*CAB1-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 *CAB1-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).

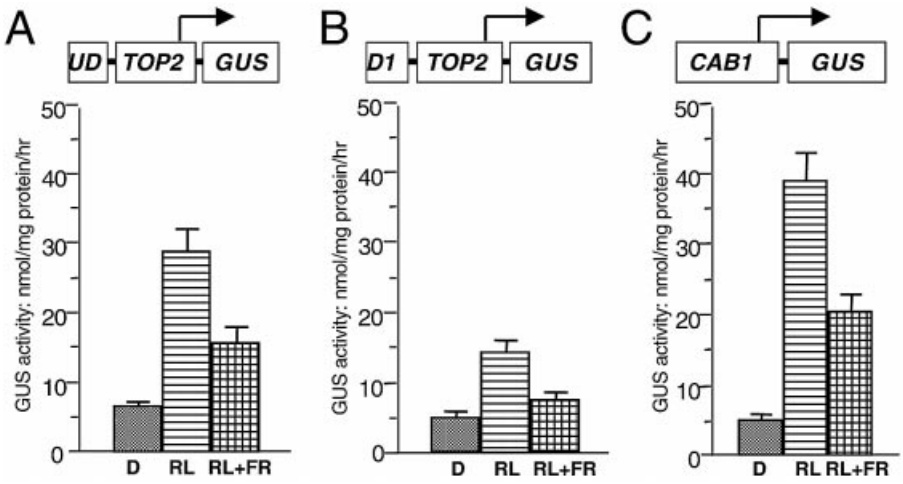

Figure 4. RL pulse-mediated induction and its cancellation by FR light of dark grown seedlings containing *UD-TOP2-GUS*, *D1-TOP2-GUS* or *CAB1-GUS* transgenes. Five-day-old dark grown seedlings (D) were exposed for 2 min to RL or followed by 10 min exposure of FR light (RL+FR). After the light treatments 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.

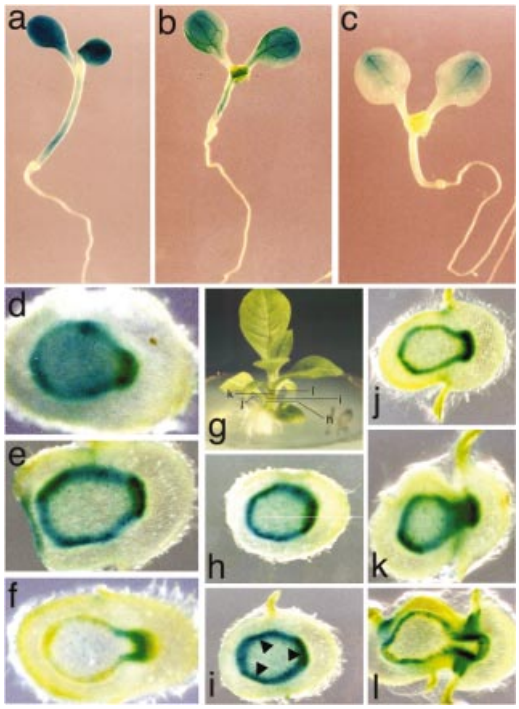

Figure 5. Tissue specific expression of *UD-TOP2-GUS*, *D1-TOP2-GUS* or *D2-TOP2-GUS* transgenes in *Arabidopsis* or tobacco light grown seedlings. (**a**–**c**) Six-day-old *Arabidopsis* seedlings containing *UD-TOP2-GUS*, *D1-TOP2-GUS* or *D2-TOP2-GUS* transgenes, respectively. (**d**–**f**) Tobacco 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 D1–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

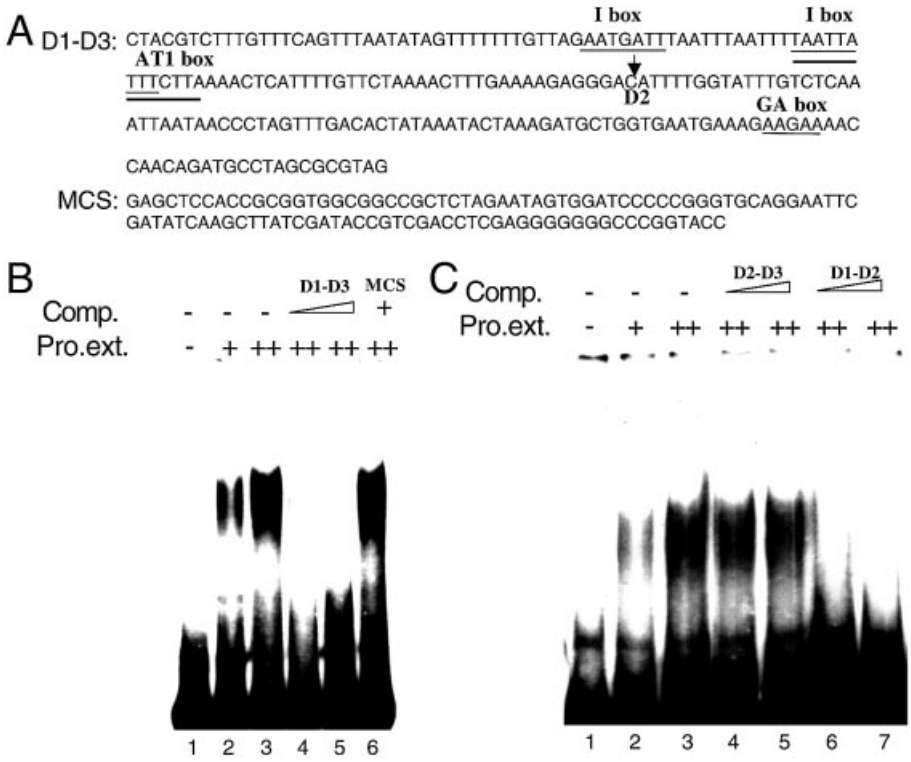

Figure 6. Identification of *TOP2* minimal promoter-specific DNA-binding activity. (**A**) DNA sequences of *TOP2* minimal promoter region from –468 to –262 bp (D1–D3), and the multiple cloning site (MCS) of pBluescript from 653 to 759 bp. The arrow indicates the start of D2–D3 promoter fragment. The *cis*-acting elements are underlined: two I boxes and GA box by thin line and AT1 box by thick line. (**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 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.). (**C**) 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 foot printing 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 detected 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

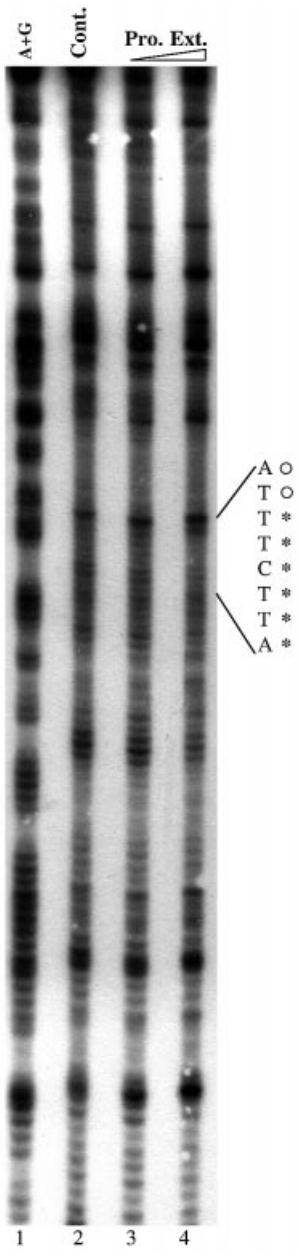

Figure 7. DNase I foot printing analysis of 207 bp D1–D3 fragment (top strand) of *TOP2* promoter. Lane 1 shows the A+G Maxam and Gilbert sequencing ladder. Lane 2 is the control lane (cont.) without any protein. Lanes 3 and 4 show the ladder caused by DNase I cleavage with 10 and 20 µg of whole cell 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.

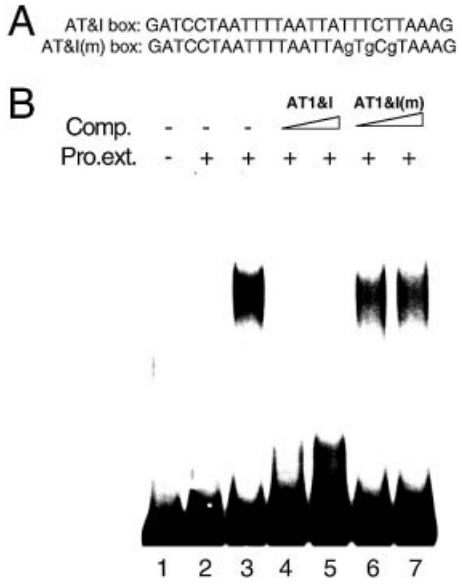

Figure 8. Electrophoretic mobility shift (gel shift) analysis using the whole cell extracts of 6-day-old dark or light grown pea seedlings and D1–D2 DNA as probe. (**A**) The DNA sequence of AT1&I box and its mutated version, AT1&I (m). (**B**) No protein extract was added in lane 1. Eight micrograms of protein extract were added in lane 2 from dark grown seedlings. Eight micrograms of protein extract from light grown seedlings were added in lanes 3–7. The amount of AT1&I box DNA fragment added in lanes 4 and 5 was 50 and 100 ng, respectively. The amount of AT1&I (m) box DNA fragment added in lanes 6 and 7 was 50 and 100 ng, respectively. The increasing concentration of unlabelled competitor DNA is shown by triangles. Plus and minus signs indicate the presence and absence, respectively, of whole cell extracts (Pro. Ext.) or competitors (Comp.).

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 *CAB1* 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 photomorphogeneic 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 traces 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&I-box. It is interesting to note that 3AF1 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&I-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.

ACKNOWLEDGEMENTS

We thank Dr Sushil Kumar for critically reading and commenting on the manuscript. We thank Dr Baishnab Tripathy, School of Life Sciences, JNU, for providing us with the facility of color light sources. This work was supported by the block grant of NCPGR and a DBT grant to S.C., and internal grant of ICGEB to S.K.S. V.Y. is a recipient of CSIR fellowship, Government of India.

REFERENCES

1. Champoux,J.J. (1990) Mechanistic aspect of type I topoisomerases. In Cozzarelli,N.R. and Wang,J.C. (eds), *DNA Topology and its Biological Effects*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 217–242.
2. Hsieh,T.S. (1990) Mechanistic aspects of type II topoisomerases. In Cozzarelli,N.R. and Wang,J.C. (eds), *DNA Topology and its Biological Effects*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 243–263.
3. Wang,J.C. (1996) DNA topoisomerases. *Annu. Rev. Biochem.*, **65**, 635–692.
4. Madhusudan,K. and Nagaraja,V. (1996) Alignment and phylogenetic analysis of type II topoisomerases. Alignment and phylogenetic analysis of type II topoisomerases. *J. Biosci.*, **21**, 613–629.
5. Wang,J.C. (2002) Cellular roles of DNA topoisomerases: a molecular perspective. *Nature Rev.*, **3**, 430–440.
6. Adachi,N., Nomoto,M., Kohno,K. and Koyama,H. (2000) Cell cycle regulation of the DNA topoisomerase II alpha promoter is mediated by proximal CCAAT boxes possible involvement of acetylation. *Gene*, **245**, 49–57.
7. Smith,A.P., Gimenoz-Abian J.F. and Clarke,D.J. (2002) DNA damage independent checkpoints: yeast and higher eukaryotes. *Cell Cycle*, **1**, 16–33.
8. Voelkel-Meiman,K., Dinardo,S. and Sternglanz,R. (1986) Molecular cloning and genetic mapping of the DNA topoisomerases II gene of *Saccharomyces cerevisiae*. *Gene*, **42**, 193–199.
9. Gemkow,M.J., Diehter,J. and Arndf Jovin,D.J. (2001) Developmental regulation of DNA topoisomerases during *Drosophila* embryogenesis. *Exp. Cell Res.*, **262**, 114–121.
10. Wyckoff,E., Natalia,D., Nolan,J.M., Lee,M. and Hsieh,T.S. (1988) Structure of a *Drosophila* DNA topoisomerase II gene. Nucleotide sequence and homology among topoisomerases II. *J. Mol. Biol.*, **205**, 1–13.
11. Jenkins,J.R., Ayton,P., Jones,T., Davies,S.L., Simmons,D.L., Harris,A.L., Sheer,D. and Hickson,I.D. (1992) Isolation of cDNA clones encoding the beta isozyme of human DNA topoisomerase II and location of the gene to chromosome 3p24. *Nucleic Acids Res.*, **20**, 5587–5592.
12. Bakshi,R.P., Galande,S. and Muniyappa,K. (2001) Functional and regulatory characteristics of eucariotic type II DNA topoisomerase. *Crit. Rev. Biochem. Mol. Biol.*, **36**, 1–37.
13. Xie,S. and Lam,E. (1994) Abundance of nuclear DNA topoisomerase II is correlated with proliferation in *Arabidopsis thaliana*. *Nucleic Acids Res.*, **22**, 5729–5736.
14. Xie,S. and Lam,E. (1994) Characterization of a DNA topoisomerase II cDNA from Arabidopsis thaliana. *Plant Physiol.*, **106**, 1701–1702.
15. Reddy,M.K., Nair,S., Tewari,K.K., Mudgil,Y., Yadav,B.S. and Sopory,S.K. (1999) Cloning and characterization of cDNA encoding topoisomerase II in pea and analysis of its expression in relation to cell proliferation. *Plant Mol. Biol.*, **41**, 125–137.
16. Kendrick,R.E. and Kronenberg,G.H.M. (1994) *Photomorphogenesis in Plants*, 2nd Edn. Kluwer Academic Publishers, Dordrecht.
17. Deng,X.-W. and Quail,P.H. (1999) Signalling in light-controlled development. *Semin. Cell Dev. Biol.*, **10**, 121–129.
18. Fankhauser,C. and Chory,J. (1997) Light control of plant development. *Annu. Rev. Cell. Dev. Biol.*, **13**, 203–229.
19. vonArnim,A.G. and Deng,X.-W. (1996) Light control of seedling development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 215–243.
20. Chattopadhyay,S., Ang,L.-H., Puente,P., Deng,X.-W. and Wei,N. (1998) *Arabidopsis* bZIP protein HY5 directly interacts with light-responsive promoters in mediating light control of gene expression. *Plant Cell*, **10**, 673–683.
21. Degenhardt,J. and Tobin,E.M. (1996) A DNA-binding activity for one of two closely defined phytochrome regulatory elements in an *Lhcb* promoter is more abundant in etiolated than in green plants. *Plant Cell*, **8**, 31–41.
22. Martinez-Garcia,J.F., Huq,E. and Quail,P.H. (2000) Direct targeting of light signals to a promoter element-bound transcription factor. *Science*, **288**, 859–863.
23. Millar,A.J. and Kay,S.A. (1996) Integration of circadian and phototransduction pathways in the network controlling *CAB* gene transcription in *Arabidopsis*. *Proc. Natl Acad. Sci. USA*, **93**, 15491–15496.
24. Terzaghi,W.B. and Cashmore,A.R. (1995) Light-regulated transcription. *Plant Mol. Biol.*, **46**, 445–474.
25. Tobin,E.M. and Kehoe,D.M. (1994) Phytochrome regulated gene expression. *Semin. Cell Biol.*, **5**, 335–346.
26. Chattopadhyay,S., Puente,P., Deng,X.-W. and Wei,N. (1998) Combinatorial interaction of light-responsive elements play a critical role in determining the response characteristics of light-regulated promoters in *Arabidopsis*. *Plant J.*, **15**, 69–77.

27. Kuno,N. and Furuya,M. (2000) Phytochrome regulation of nuclear gene expression in plants. *Semin. Cell Dev. Biol.*, **11**, 485–493.
28. Puente,P., Wei,N. and Deng,X.W. (1996) Combinational interplay of promoter elements constitutes the minimal determinants for light and developmental control of gene expression in *Arabidopsis*. *EMBO J.*, **15**, 3732–3743
29. Quail,P.H. (2002) Photosensory perception and signaling in plant cells: new paradigms? *Curr. Opin. Cell Biol.*, **14**, 180–188.
30. McNellis,T.W. and Deng,X.-W. (1995) Light control of seedling morphogenetic pattern. *Plant Cell*, **7**, 1749–1761.
31. Neff,M.M., Fanhauser,C. and Chory,J. (2000) Light: an indicator of time and place. *Genes Dev.*, **14**, 257–271.
32. Quail,P.H. (2002) Phytochrome photosensory signaling networks. *Nature Rev. Mol. Cell Biol.*, **3**, 85–93.
33. Ahmad,M. and Cashmore,A.R. (1993) HY4 gene of *A.thaliana* encodes a protein with characteristics of a blue-light photoreceptor. *Nature*, **366**, 162–166.
34. Ang,L.-H., Chattopadhyay,S., Wei,N., Oyama,T., Okada, K, Batschauer,A. and Deng,X.-W. (1998) Molecular interaction between COP1 and HY5 defines a regulatory switch for light control of *Arabidopsis* development. *Mol. Cell*, **1**, 213–222.
35. Barnes,S.A., Quaggio,R.B., Whitelam,G.C. and Chua,N.H. (1996) *fhy1* defines a branch point in phytochrome A signal transduction pathways for gene expression. *Plant J.*, **10**, 1155–1161.
36. Bowler,C., Neuhaus,G., Yamagata,H. and Chua,N.-H. (1994) Cyclic GMP and calcium mediate phytochrome phototransduction. *Cell*, **77**, 73–81.
37. Fankhauser,C., Yeh,K.C., Lagarias,J.C., Zhang,H., Elich,T.D. and Chory,J. (1999) PKS1, a substrate phosphorylated by phytochrome that modulates light signaling in *Arabidopsis*. *Science*, **284**, 1539–1541.
38. Holm,M. and Deng,X.-W. (1999) Structural organization and interactions of COP1, a light-regulated developmental switch. *Plant Mol. Biol.*, **41**, 151–158
39. Ni,M., Halliday,K.J., Tepperman,J.M. and Quail,P.H. (1998) PIF3, a phytochrome interacting factor necessary for normal photoinduced signal transduction, is a novel basic helix–loop–helix protein. *Cell*, **95**, 657–667.
40. Soh,M.S., Kim,Y.-M., Han,S.-J. and Song,P.-S. (2000) REP1, a basic helix–loop–helix protein, is required for a branch pathway of phytochrome A signaling in *Arabidopsis*. *Plant Cell*, **12**, 2061–2073.
41. Yadav,V., Kundu,S., Chattopadhyay,D., Negi,P., Wei,N, Deng,X.-W. and Chattopadhyay,S. (2002) Light regulated modulation of Z-box containing promoters by photoreceptors and downstream regulatory components, COP1 and HY5, in *Arabidopsis*. *Plant J.*, **31**, 741–753.
42. Yang,H.-Q., Tang,R.-H. and Cashmore,A. (2001) The signaling mechanism of *Arabidopsis* CRY1 involves direct interaction with COP1. *Plant Cell*, **13**, 2573–2587.
43. Harmer,S.L., Hogenesch,J.B., Straume,M., Chang,H.S., Han,B., Wang,X., Kreps,J.A. and Kay,S.A. (2000) Orchestrated trascription of key pathways in *Arabidopsis* by the circadian clock. *Science*, **290**, 2110–2113.
44. Ma,L., Gao,Y., Qu,L., Chen,Z., Li,J., Zhao,H. and Deng,X.W. (2002) Genomic evidence for COP1 as a repressor of light-regulated gene expression and development in *Arabidopsis*. *Plant Cell*, **14**, 2383–2398.
45. Ma,L., Li,J., Qu,L., Hager,J., Chen,Z., Zhao,H. and Deng,X.W. (2001) Light control of *Arabidopsis* development entails coordinated regulation of genome expression and cellular pathways. *Plant Cell*, **13**, 2589–2607.
46. Schaffer,R., Landgraf,J., Accerbi,M., Simon,V., Larson,M. and Wisman,E. (2001) Microarray analysis of diurnal and circadian-regulated genes in *Arabidopsis*. *Plant Cell*, **13**, 113–123.
47. Yin,Y., Cheong,H., Friedrichsen,D., Zhao,Y., Hu,J., Mora-Garcia,S. and Chory,J. (2002) A crucial role for the putative *Arabidopsis* topoisomerase VI in plant growth and development. *Proc. Natl Acad. Sci. USA*, **99**, 10191–10196.
48. Sun,L. and Tobin,E.M. (1990) Phytochrome-regulated expression of genes encoding light-harvesting chlorophyll a/b-protein in two long hypocotyl mutants and wild type plants of *Arabidopsis thaliana*. *Photochem. Photobiol.*, **15**, 51–56.
49. Thampson,B.F. (1959) Far-red reversal of internode stimulating effect of red light on peas. *Am. J. Bot.*, **48**, 256–261.
50. Tong,C.G., Reichles,S., Blumenthal,S., Balh,J., Hsich,H.L. and Roux,S.J. (1997) Light regulation of the abundance of mRNA encoding a nucleolin-like protein localized in the nucleoli of pea. *Plant Physiol.*, **114**, 643–652.
51. Rocholl,M., Talke-Messerer,C., Kaiser,T. and Batschuer,A. (1994) Unit I of the mustard chalcone synthase promoter is sufficient to mediate light responses from different photoreceptors. *Plant Sci.*, **97**, 189–98.
52. Ueda,T., Pichersky,E., Malik,V.S. and Cashmore,A.R. (1989) The level of expression of the tomato *rbcS-3A* gene is modulated by a far-upstream promoter element in a developmentally regulated manner. *Plant Cell*, **1**, 217–227.
53. Lam,E., Kano-Murakami,Y., Gilmartin,P., Niner,B. and Chua,N.-H. (1990) A metal-dependent DNA-binding protein interacts with a constitutive element of a light-responsive promoter. *Plant Cell*, **2**, 857–866.
54. Hettiarachchi,G.H.C.M. (2003) Analysis of light and stress responsive DNA topoisomerase II promoter and interacting trans-acting factors from *Pisum sativum*. PhD thesis, School of Life Science, JNU, New Delhi, India.