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 tabaccum* (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 photoreception 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/AtSP011-3, 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: AACTGAGCTTCACTCAGGCCCCTGGAACCA; UD-reverse: GCTCATAGACCTATTGTTCTCTGACCA; D1: CCC-
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
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 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 constan1t 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.
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).
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 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
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 detected with the whole cell extract of the dark grown seedlings (Fig. 8B, lane 2), a clear DNA–protein complex was formed with the whole cell extract of 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 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.).

**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
demonstrated that the expression of this gene was up-regulated by light. Furthermore, we have demonstrated that the light-mediated up-regulation of \( \text{TOP2} \) 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 \( \text{TOP2} \) promoter reveal that a 468 bp region from the transcriptional start site is essential and sufficient for light-mediated activation of the \( \text{TOP2} \) promoter. Whereas deletion from -608 to -469 reduced the activity of the \( \text{TOP2} \) 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 \( \text{D1-TOP2} \) and \( \text{D2-TOP2} \) is crucial for light-mediated activation of the promoter.

\textit{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 \( \text{TOP2} \) promoter, similar to \( \text{CABI} \) promoter, indeed responds to RL and FR reversible induction and cancellation, respectively, mediated by phytochromes. These results probably suggest that light-mediated activation of \( \text{TOP2} \) promoter is not a secondary effect rather its activation is under the control of phytochrome-mediated signaling. The \( \text{D1-TOP2} \) 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.

**Figure 7.** DNase I foot printing analysis of 207 bp D1–D3 fragment (top strand) of \( \text{TOP2} \) 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 \( \mu \text{g} \) 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.).
The expression of UD-TOP2-GUS transgene was confined to the cotyledons and hypocotyls where the photomorphogenetic 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.

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