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Short Communication

Regulation of *TOP2* by Various Abiotic Stresses Including Cold and Salinity in Pea and Transgenic Tobacco Plants

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Although abiotic stress affects plant growth and development, demonstration of its direct effect on regulation of the components of the DNA replication machinery is largely unknown. In this work, we demonstrate that the expression of *TOP2* (encoding topoisomerase II) is up-regulated by various abiotic stresses including salinity and low temperature, and phytohormones such as ABA (abscisic acid) and SA (salicylic acid). Transgenic studies with different deletion versions of the *TOP2* promoter in tobacco define several promoter determinants responsible for specific abiotic stress responsiveness. Taken together, these results demonstrate a direct involvement of stress in the transcriptional regulation of *TOP2*.

Keywords: *TOP2* — Promoter — Stress — Gene expression — Pea — Tobacco.

Abbreviations: ABA, abscisic acid; GUS, β-glucuronidase; SA, salicylic acid.

Since plants are sessile, they withstand stress by activating multiple defense mechanisms through synthesizing a large number of stress proteins that are known to function in stress tolerance (Shinozaki and Yamaguchi-Shinozaki 1997, Zhu 2002). Many stress-responsive genes show strong association in their expression as they are induced by multiple stresses, whereas several others show specific induction in response to a particular stimulus (Seki et al. 2002, Rabbani et al. 2003). The *cis*- and *trans*-acting elements involved in low temperature-regulated gene expression have been well studied (Baker et al. 1994, Dunne et al. 1998, Medina et al. 1999). Several phytohormones have been demonstrated to be involved in the stress response. Abscisic acid (ABA) is produced under such environmental stresses and plays an important role in stress tolerance in plants (Yamaguchi-Shinozaki and Shinozaki 1994, Shinozaki and Yamaguchi-Shinozaki 1997, Zhu 2002).

DNA topoisomerases change the topology of DNA and are intimately involved in DNA replication. Topoisomerases are generally classified into two major groups: type I and type II, depending on the mechanism of their actions (Wang 2002). Although the *TOP2* gene which encodes topoisomerase II has been cloned and functionally investigated in a number of eukaryotes, very little information of *TOP2* is available from higher plants (Wang 2002, Singh et al. 2004). The only reported clones of *TOP2* in plants are from *Arabidopsis* (Xie and Lam 1994) and pea (Reddy et al. 1999). Although a large number of genes have been regulated in response to abiotic stress (Seki et al. 2002, Takahashi et al. 2004), the connection between DNA replication/cell cycle regulation and abiotic stresses has just started to be unraveled. Cell cycle genes such as some of the cyclin-dependent kinases have been reported to be regulated under salinity stress (Burssens et al. 2000, Hirt 2000).

It has been demonstrated recently that *TOP2* is regulated by phytochrome-mediated light signaling pathways (Hettiarachchi et al. 2003). Here we show that *TOP2* is also regulated by various abiotic stresses including cold, salinity, ABA and SA. The transgenic studies have revealed specific promoter determinants in the *TOP2* promoter responsible for specific stress responsiveness.

To examine the regulation of *TOP2* in response to abiotic stresses such as salinity, cold and drought, we carried out transcript analyses using 10-day-old pea seedlings. Total RNA was isolated from roots and shoots separately at various time points after cold treatment at 4°C and relative transcript levels were monitored. A transcript of ~4.4 kb, which corresponded to the expected size of pea *TOP2* mRNA, was detected. As shown in Fig. 1A, and E, 2.5-fold higher expression of *TOP2* was detected in shoots after 2 h of cold treatment and this increased level remained the same up to 12 h exposure to cold. Although the cold-mediated induction of *TOP2* in roots was slower, >6-fold induction was observed in roots after 12 h exposure to cold (Fig. 1B, F). These results suggest that the expression of *TOP2* is up-regulated under low temperature, which is perceived by shoots faster than by roots.

To examine the expression of *TOP2* under salinity stress, 10-day-old pea seedlings were treated with different concentrations of sodium chloride, and shoots and roots were harvested separately after 4 h of treatment. Analyses of the steady-state mRNA level revealed that the expression of *TOP2* was strongly induced by salinity stress (Fig. 1C, D). Whereas the maximum

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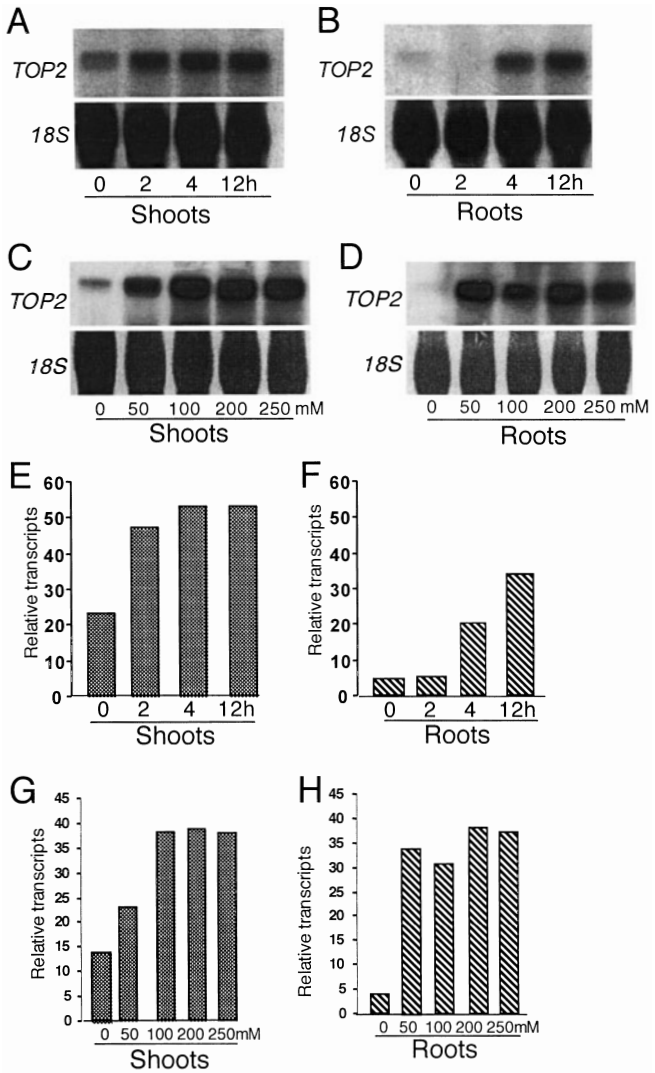

Fig. 1 Cold and salinity stress-regulated expression of *TOP2*. Ten-day-old light-grown pea seedlings were exposed to 4°C (light intensity: 100 μmol m^{-2} s^{-1}) for 0 (control), 2, 4 or 12 h, or treated with 0 (control), 50, 100, 200 or 250 mM sodium chloride for 4 h. Total RNA was isolated from shoots or roots separately. *18S* rRNA is shown as a loading control. A 1.8 kb DNA fragment of *TOP2* (Hettiarachchi et al. 2003) was used as probe. (A) Induction of *TOP2* expression in shoot tissues by cold. (B) Induction of *TOP2* expression in root tissues by cold. (C) Induction of *TOP2* expression in shoot tissues by salinity. (D) Induction of *TOP2* expression in root tissues by salinity. (E) Quantification of the data in (A) by Fluor-S-MultiImager (Biorad). (F) Quantification of the data in (B) by Fluor-S-MultiImager (Biorad). (G) Quantification of the data in (C) by Fluor-S-MultiImager (Biorad). (H) Quantification of the data in (D) by Fluor-S-MultiImager (Biorad).

level of induction was detected at 100 mM sodium chloride in shoots, 50 mM sodium chloride was sufficient to induce the highest level of expression of *TOP2* in roots. As shown in Fig. 1G and H, whereas >7-fold induction was detected in roots, the expression of *TOP2* was induced to about 3-fold in shoots. Taken together, these results suggest that the expression of

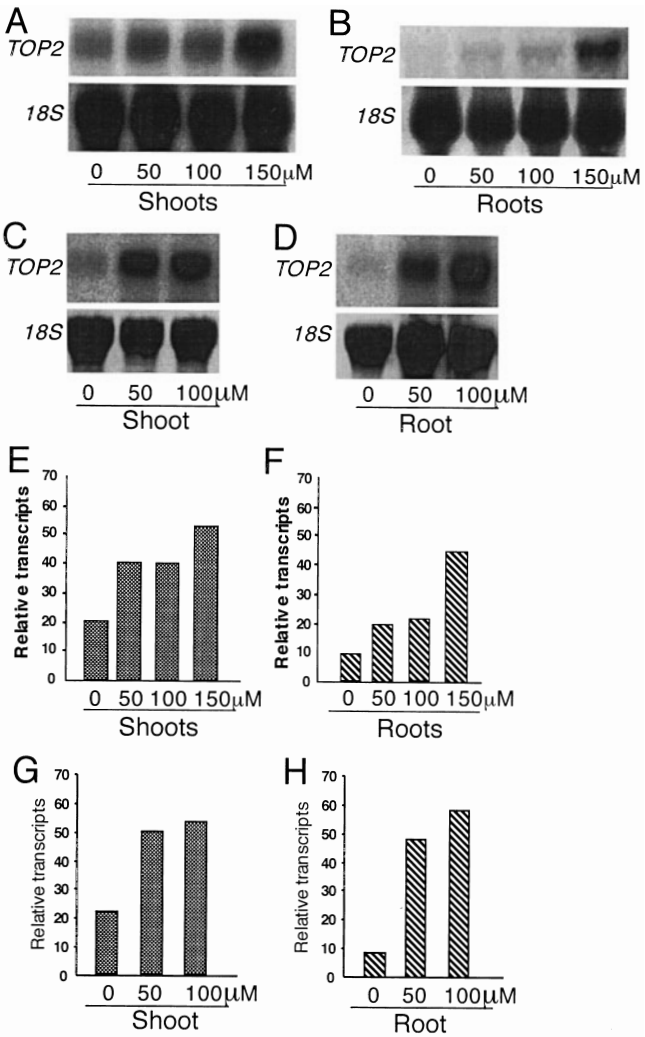

Fig. 2 ABA- and SA-mediated regulation of *TOP2* expression. Ten-day-old light-grown pea seedlings were treated with 0 (control), 50, 100 or 150 μM ABA (A, B), or with 0 (control), 50 or 100 μM SA (C, D) for 4 h and total RNA was isolated from shoots or roots. For experimental details, see the legend to Fig. 1. (A) Induction of *TOP2* expression in shoot tissues. (B) Induction of *TOP2* expression in root tissues. (C) Induction of *TOP2* expression in shoot tissues. (D) Induction of *TOP2* expression in root tissues. (E) Quantification of the data in (A) by Fluor-S-MultiImager (Biorad). (F) Quantification of the data in (B) by Fluor-S-MultiImager (Biorad). (G) Quantification of the data in (C) by Fluor-S-MultiImager (Biorad). (H) Quantification of the data in (D) by Fluor-S-MultiImager (Biorad).

TOP2 is up-regulated by salinity stress and the response was stronger in roots compared with shoots. To determine the expression of *TOP2* under dehydration stress, water was withheld from 10-day-old pea seedlings for various times up to 12 h. However, no significant change in the expression of *TOP2* was detected (data not shown).

Several abiotic stress responses are mediated by ABA- or SA-induced signaling pathways. To determine whether ABA and SA play any role in the regulation of *TOP2*, we examined

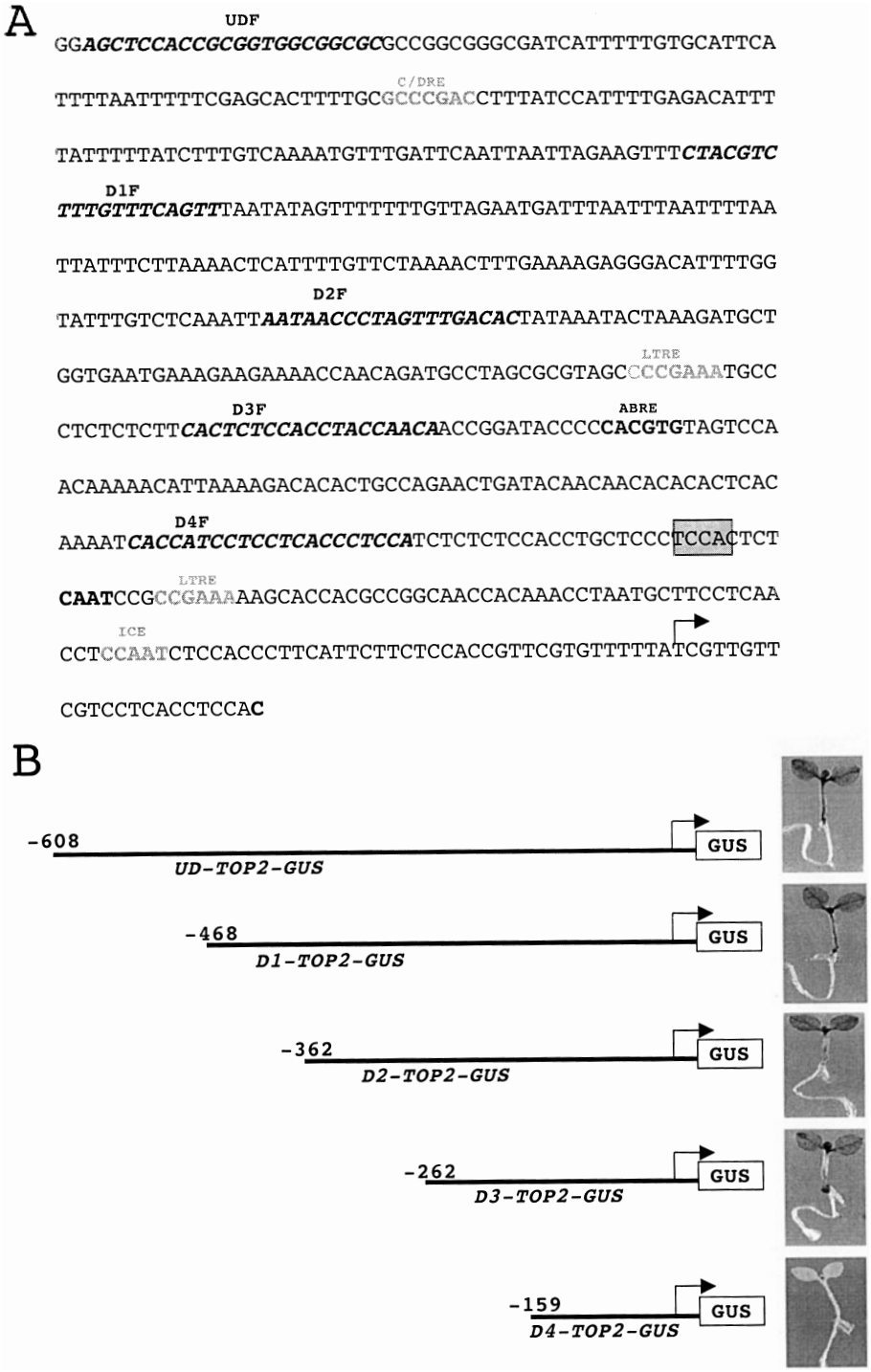

Fig. 3 Minimal promoter regions of *TOP2* responsive to cold, salinity, ABA and SA in transgenic tobacco plants. (A) DNA sequence of the *TOP2* promoter. The arrow shows the transcriptional start site (Hettiarachchi et al. 2003). The CAAT box and various stress-responsive promoter elements present in this promoter region are shown. The forward PCR primers used for generating various deleted versions of the *TOP2* promoter are shown as UDF, D1F, D2F, D3F and D4F (Hettiarachchi et al. 2003). (B) Schematics of deletion constructs of the *TOP2* promoter fused to the GUS reporter. The arrow indicates the transcriptional start site, and the numbers indicate the length of each undeleted or deleted promoter from the transcriptional start site. Five independent transgenic lines of each promoter–*GUS* transgene were used to determine the GUS activity staining. GUS activity staining of one representative transgenic line from each construct is shown on the right-hand panel.

the transcript level of *TOP2* following treatment of 10-day-old pea seedlings with various concentrations of ABA or SA. As shown in Fig. 2 (A, B, and E, F), the expression of *TOP2* was up-regulated in response to exogenous ABA and the maximum level of induction was observed at 150 μM ABA in both root and shoot tissues. However, the induction was much faster in shoots compared with roots. Next, we examined whether the expression of *TOP2* was modulated by SA. Ten-day-old seedlings were exposed to various concentrations of SA, and the analysis of steady-state mRNA levels revealed that SA could induce the maximum level of expression of *TOP2* at 100 μM in both shoot and root tissues (Fig. 2C, D, G, H). Taken together these results suggest that ABA and SA are able to induce the expression of *TOP2*.

To determine the minimal promoter regions responsive to specific abiotic stresses tested, we generated transgenic tobacco plants containing undeleted (*UD-TOP2*) or various deleted versions (*D1-TOP2*, *D2-TOP2*, *D3-TOP2* and *D4-TOP2*) of *TOP2-promoter-GUS* transgenes (Fig. 3). As shown in Fig. 3B, the β-glucuronidase (GUS) activity staining of the transgenes gradually decreased from *UD-TOP2-GUS* to *D3-TOP2-GUS*, with practically no activity staining of *D4-TOP2-GUS* in 10-day-old constant light-grown seedlings.

To determine the cold-mediated induction kinetics of those promoters, we transferred 10-day-old transgenic seedlings to 4°C for 2, 4 and 12 h and measured the GUS activities. The activity of the *UD-TOP2* promoter was induced with time and showed about 3-fold induction after 12 h cold treatment as compared with the normal growth temperature (Fig. 4A). The *D1-TOP2* promoter, which was derived from *UD-TOP2* after deletion of 160 bp (Fig. 4B), had significantly reduced low temperature-mediated activation compared with the *UD-TOP2* promoter. However, the *D1-TOP2* promoter still showed about a 2-fold higher level of activity at 12 h, with very little induction, if any, up to 4 h of cold treatment (Fig. 4B). Although the *D2-TOP2* promoter was derived after deletion of 128 bp from the *D1-TOP2* promoter, it showed a similar level and pattern of activities to the *D1-TOP2* promoter (Fig. 4C). Further deletions of the *TOP2* promoter resulting in the *D3-TOP2* or *D4-TOP2* promoter severely compromised the activity of the promoters with no detectable induction by cold treatment (Fig. 4D and data not shown).

To determine the activity of various undeleted and deleted versions of *TOP2* promoters under salinity stress, 10-day-old transgenic tobacco seedlings were exposed to 100 and 200 mM sodium chloride for 4 h and GUS activities were measured. As shown in Fig. 4E–H, whereas the *UD-TOP2* promoter was induced to >2 fold at 200 mM sodium chloride, no induction was detected with any of the deleted versions of the promoters up to 200 mM sodium chloride. These results together suggest that whereas the *D2-TOP2* promoter is the minimal promoter region responsive to cold stress, the 670 bp containing *UD-TOP2* is required for the induction by salinity stress.

To determine the minimal promoter regions of *TOP2* responsive to ABA and SA, GUS activities of transgenic seedlings treated with various concentrations of ABA or SA were measured. Ten-day-old tobacco seedlings carrying various transgene were treated with 100 or 150 μM ABA for 4 h and GUS activities were measured. The *UD-TOP2* promoter showed about a 3-fold induction at 100 μM ABA, and this induction level was increased further to about 7-fold at 150 μM ABA (Fig. 5A). The *D1-TOP2* promoter showed about 3-fold induction at 100 μM ABA and this activity was again increased to 6-fold at 150 μM ABA (Fig. 5B). On the other hand, *D2-TOP2* and *D3-TOP2* promoters showed very little induction, if any, at 100 μM ABA. However, both these promoters showed about 2-fold induction at 150 μM ABA (Fig. 5C, D). The *D4-*

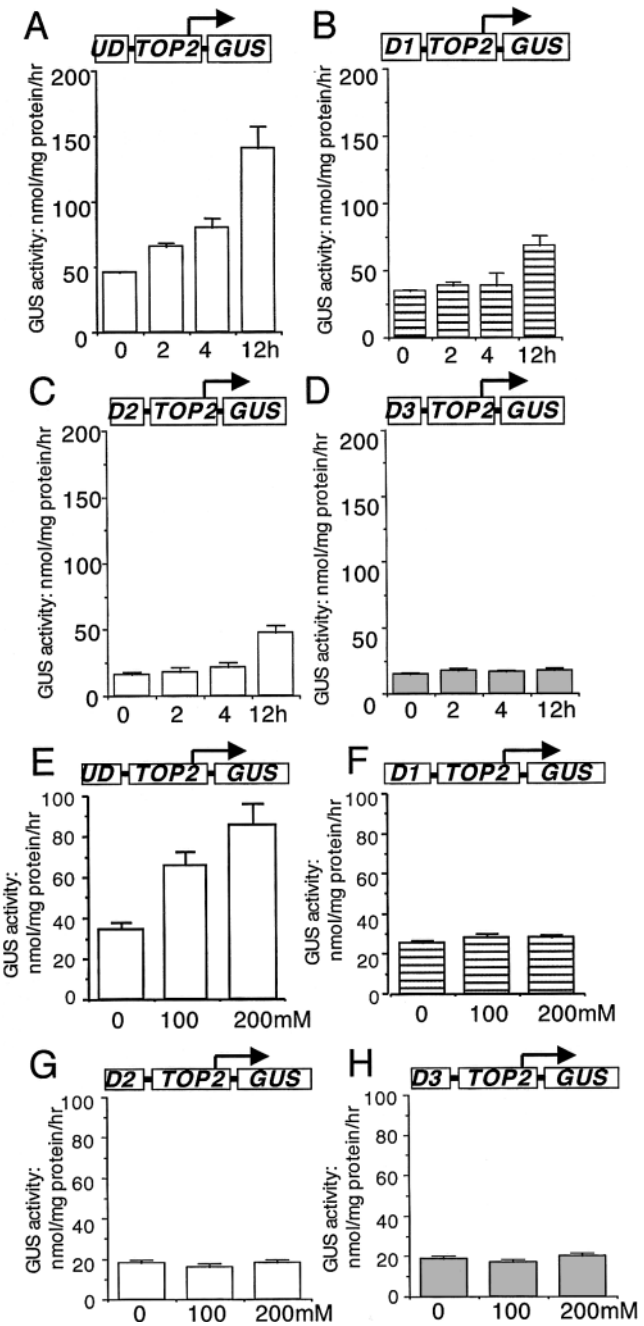

Fig. 4 Activation of various deleted versions of the *TOP2* promoter by cold and salinity. Ten-day-old light-grown tobacco transgenic seedlings were exposed to 4°C (light intensity: 100 μmol m^{-2} s^{-1}) for 0 (control), 2, 4 or 12 h (A–D), or were exposed to 0 (control), 100 or 200 mM sodium chloride for 4 h (E–H) and GUS activities were measured. About 25 seedlings of each transgenic line were used for the measurement of GUS activities. The error bars indicate the SD from four independent experiments. The transgenic seedlings with specific promoter–GUS constructs are indicated on the top of each panel.

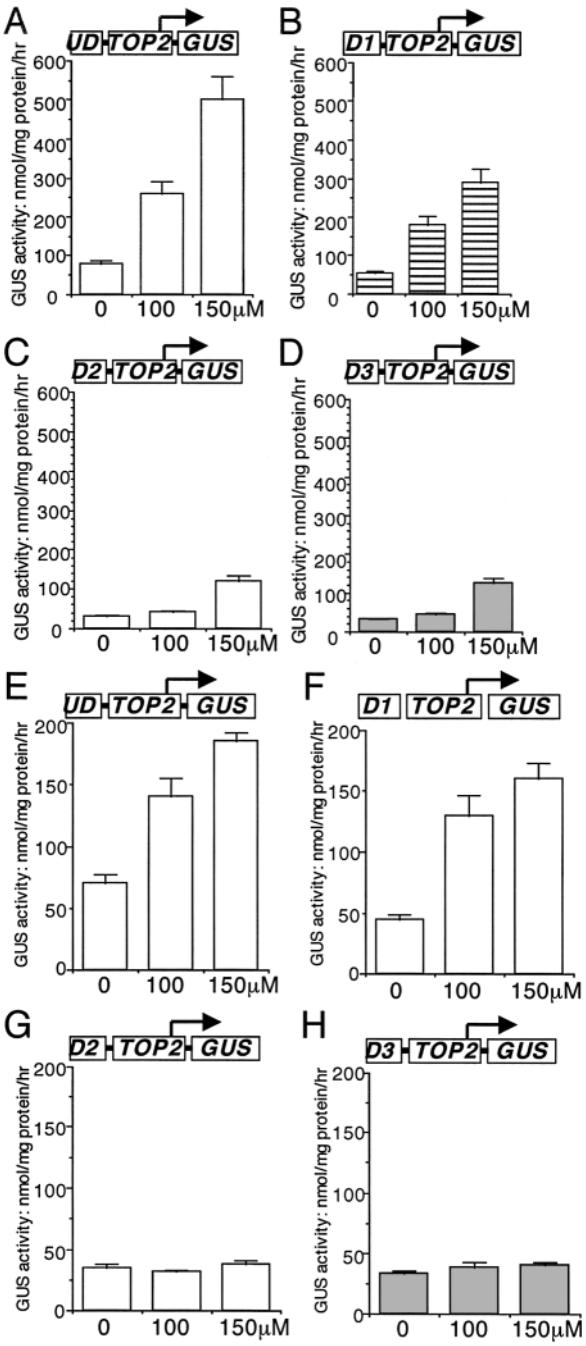

Fig. 5 Activation of various deleted versions of the *TOP2* promoter by ABA and SA. Ten-day-old light-grown tobacco transgenic seedlings were exposed to 0 (control), 100 or 150 μM ABA for 4 h (A–D) or were exposed to 0 (control), 100 or 150 μM SA (E–H) and GUS activities were measured. For experimental details, see the legend to Fig. 4.

TOP2 promoter was, however, unable to display any induction up to 150 μM ABA treatment (data not shown).

To determine the effect of SA on the activity of the *TOP2* promoter, 10-day-old seedlings carrying various transgenes were individually treated with 100 or 150 μM SA for 4 h and GUS activities were monitored. As shown in Fig. 5E and F, whereas 2-fold induction was observed in the case of the *UD-TOP2* promoter, >3-fold induction was detected with the *D1-TOP2* promoter. These results suggest that promoter element(s) present in between the *UD-TOP2* and *D1-TOP2* promoters may be responsible for the inhibition of optimal induction of the *UD-TOP2* promoter. Further deletion of *D1-TOP2*, resulting in *D2-TOP2*, *D3-TOP2* or *D3-TOP2*, completely abolished the induction by SA (Fig. 5G, H, and data not shown). Taken together, these results suggest that *D3-TOP2* and *D1-TOP2* are the minimal promoter regions of the *TOP2* gene responsive to ABA and SA, respectively.

This study demonstrates that abiotic stress mediates transcriptional regulation of *TOP2*, an important component of the DNA replication machinery, and thus establishes a functional relationship between these pathways. Our transcript analyses data show that *TOP2* responds to low temperature and salinity stresses, and also to ABA- and SA-mediated transcriptional regulation. In this study, we have observed that ABA mediates up-regulation of *TOP2* transcription, and this effect is dependent on the ABA concentration and tissue type. SA in general has been shown to have an important role in the defense response of many plant species to pathogen attack. However, recent studies also support a major role for SA in modulating the plant response to many abiotic stresses (Borsani et al. 2001). In this study, we find that pea *TOP2* expression is induced in response to SA.

To determine the minimal promoter regions responsive to ABA or SA, and also to individual stresses such as cold and salinity, we have monitored the activities of various deleted versions of the *TOP2* promoter in tobacco transgenic plants. Comparisons of GUS activities have revealed that the maximum level of induction by low temperature is obtained with *UD-TOP2*. However, the *D2-TOP2* promoter is the minimal promoter region that is induced by cold. On the other hand, the minimal promoter region responsive to salinity stress is *UD-TOP2*. No deleted versions of the *TOP2* promoter show any induction with salinity stress. The minimal promoter regions that are responsive to ABA- and SA-mediated induction are *D3-TOP2* and *D1-TOP2*, respectively. It is interesting to note that the minimal promoter region responsive to light (Hettiarachchi et al. 2003) and SA has been found to be the *D1-TOP2* promoter. It will be interesting to investigate in the future whether light and SA signaling pathways cross-talk through a common *trans*-acting factor in *TOP2* regulation.

The DNA sequence analysis of the pea *TOP2* promoter revealed the presence of various abiotic stress-responsive elements including the C/DRE consensus, two LTRE motifs, a WUN motif and an ABRE element (Fig. 3A). These elements are known to be present in the promoters of many abiotic stress-responsive genes (Baker et al. 1994, Busk and Pages 1998, Dunne et al. 1998, Jaglo-Ottosen et al. 1998, Medina et al. 1999). Interestingly, with SA, the maximum induction has

been found in *D1-TOP2*, suggesting that some promoter elements may be present in between *UD-TOP2* and *D1-TOP2* that reduce the maximium activity of the *UD-TOP2* promoter. The pea *TOP2* promoter contains a C/DRE element in between *UD-TOP2* and *D1-TOP2* that is known to be involved in cold, dehydration and salinity stress (Shinozaki and Yamaguchi-Shinozaki 1997, Zhu 2002). Since the *UD-TOP2* promoter shows maximum induction in cold and salinity as compared with the *D1-TOP2* promoter, the C/DRE element may play an important role in this regulation. Our results altogether suggest that several stress-responsive elements are involved in the regulation of the *TOP2* promoter. Further studies with point mutations in these elements are required to determine the specific and overlapping functions of each of these elements in specific stress responsiveness of the *TOP2* promoter.

The major question here is why *TOP2* expression is up-regulated when DNA replication and cellular activities are reduced under stressed conditions. Although the reason is still unknown, it is possible that *TOP2* may play a role in chromatin remodeling and maintaining the appropriate DNA topology and thus affects the expression of several stress-regulated genes. In fact, the chromatin accessibility complex in *Drosophila* has been shown to have ISWI and topoisomerase II as two of its five subunits (Varga-Weisz et al. 1997). However, very little information is available, if at all, in plants in this regard. It is worth mentioning here that overexpression of TOP2 in tobacco plants caused the transgenic plants to be salt and cold tolerant (Singh and Sopory, unpublished data).

Materials and Methods

Surface-sterilized transgenic seeds were sown on Murashige and Skoog (MS) plates and transferred to light at 24°C. The intensity of the continuous white light source used in this study was 100 μmol m^{-2} s^{-1}. The in vitro growing tobacco cultures as well as tobacco (*Nicotiana tabacum*) and pea (*Pisum sativum*) plants growing in soil pots were maintained at 24°C with 60% humidity unless otherwise mentioned. All the phytohormone stocks were prepared in dimethylsulfoxide (DMSO) and stored at 4°C. The filter-sterilized solution of heat-labile antibiotics and phyhormones was added to the autoclaved medium pre-cooled to 45°C.

For the imposition of low temperatures on the expression of *TOP2*, 10-day-old whole seedlings were exposed to 4°C for various times. To apply the salinity stress, 10-day-old whole seedlings were exposed to 0 (control), 50, 100, 200 or 250 mM sodium chloride for 4 h and the shoots and roots were separated for total RNA isolation. The ABA and SA treatments were given by keeping the 10-day-old whole seedlings on various concentrations such as 0 (control), 50, 100 or 150 μM ABA or SA for 4 h. Total RNA was isolated from shoots or roots separately after each stress treatment. In each case, some stress-treated plants were transferred to soil to test whether they were able to recover and grow.

For Northern blot analysis, about 0.1 g of tissue was used for each sample for isolation of RNA by the Trizol method. Total RNA (~25–30 μg) from shoot or root tissue of stress-treated plants was fractionated on 1% denaturing formaldehyde gels. A random primed [α-^{32}P]dCTP-labeled (~1.8 kb) DNA (random labeling kits, Amersham) fragment of *TOP2* was used as probe (Hettiarachchi et al. 2003). The hybridization was carried out as described in Hettiarachchi et al. (2003).

The transgenic tobacco plants used in this study have been described in Hettiarachchi et al. (2003). Transgenic plants with a single locus for the T-DNA for each construct were identified by kanamycin segregation ratios (3 : 1 as resistant vs. sensitive) and homozygous lines were generated. After screening the transformed plants for the presence of *TOP2-GUS* by polymerase chain reaction (PCR) and GUS assays, one representative line from each construct was selected for further studies.

GUS activity and activity staining were carried out as described in Puente et al. (1996).

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