Chapter 6: Expression of TOP2 Under Various Stress Conditions
6.1 Introduction

Plants are frequently exposed to unfavourable environmental conditions such as drought, high salinity, high temperature and freezing which greatly affect their productivity. Plants have also developed mechanism to respond and adapt to these stresses to survive under these conditions. In general plant responses to environmental stresses consist of three main events, perception of stimulus, generation and transmission of signals and subsequent induction of genes leading to changes in biochemical and metabolic processes.

Low temperature including freezing is one of the most common environmental conditions that has a major impact on plant survival. Plant species exhibit extensive diversity in their response to low temperature and many cold inducible genes have been isolated from a range of species (Thomashow, 1999). The cis and trans-acting elements involved in low temperature regulated gene expression have also been well studied (Baker et al., 1994; Dunne et al., 1998; Jiang et al., 1996; Medina et al., 1999). Heat stress responses have also been well documented in wide range of organisms. In all species studied, high temperature results in the production of specific families of proteins known as heat shock proteins (Larkindale and Knight, 2002). It has been reported that some of these proteins play a major role in cell cycle control in yeast (Li and Cai, 1999; Rowley et al., 1993). Understanding of salt and drought stress signalling pathways in plants is still at an early stage. Although the importance of salt and drought stress signalling was recognized long ago, few molecular components were known until recently (see Zhu, 2002). ABA plays an important role to regulate plant water balance and osmotic stress tolerance during stress conditions. There are two separate regulatory systems that control gene expression during cold and drought stress. One is ABA independent and the other is ABA dependent (Yamaguchi- Shinozaki and Shinozaki, 1994). ABA responsive elements (ABRE) and their trans-acting factors which regulate the expression of the genes mediated by ABA dependent pathway have been extensively analysed (Leung and Giraudat, 1998) and several components involved in the signal transduction pathway have been identified at the molecular level (Finkelstein and Lynch, 2000).
Although large number of genes have been shown to be regulated in response to abiotic stresses (Seki et al., 2001, 2002), there are few reports on the regulation of cell cycle regulated gene in response to abiotic stresses. Mudgil et al. (2002) have shown that tobacco TOP1 expression is cell cycle specific and stimulated by light, ABA and cold. Cell cycle genes like some of the cyclin dependent kinases have been reported to be regulated under salinity stress (Burssens et al., 2000; Hirt, 2000). An Arabidopsis helicase mutant has been shown to impaired in the cold regulated expression of CBF genes and their down stream target is sensitive to chilling stress (Gong et al., 2002). Recently involvement of phytochrome in cold induced expression through the C/DRE elements (Kim et al., 2002) and the interaction of phytochrome signalling with the SA signal transduction using single and multiple mutants affected in light perception in Arabidopsis has been shown (Genoud et al., 2002). Weatherwax (1996, 1998) has earlier reported on an interaction of light and ABA in the regulation of plant gene expression in Lemna gibba. These facts reveal a connection between abiotic stress and light signalling pathway mediated by phytochrome. Therefore to understand the gene regulation in better way, the cross talk between different pathways need to be understood in a more intricate manner.

The pea TOP2 promoter contains several light regulatory and stress regulatory cis-acting elements such as GATA, GT1, I box, C/DRE, HSE, ABRE, WUN motif and LTRE. In the third chapter we have discussed the light regulation of pea TOP2. In this chapter we have attempted to study the expression of pea TOP2 under different abiotic stress conditions and to check whether the putative stress regulatory cis-acting elements in TOP2 promoter are functional and whether there is a cross talk between light and stress signalling pathway.
6.2 Results

6.2.1 Pea TOP2 expression is regulated under various abiotic stress conditions

To study the expression pattern of TOP2 gene in response to abiotic stresses such as salinity, cold, heat, ABA, SA and drought, we carried out transcript analysis using six days old pea seedlings.

To determine the effect of low temperature stress on the expression of pea TOP2, seedlings which were grown under normal green house conditions at 25 °C were transferred to low temperature at 4 °C for 2, 4, and 12 hrs. Total RNA was isolated from roots and shoots separately at each time point and RNA gel blot analysis was carried out. A transcript of ~ 4.4 kb was detected which corresponded to the expected size of pea TOP2 gene. The level of transcript increased in response to cold stress under our experimental conditions. As shown in Figure 1A, the expression of TOP2 was higher in shoots than roots. Compared to control, where seedlings were not exposed to cold, a 2.5 fold higher expression was seen after 2 hrs of cold treatment in shoots and this increased level remained stable until 12 hrs. The TOP2 induction in roots was slower (Figure 1A and B). There was no induction till 2 hrs, however, a 6 fold induction was seen in roots after 4 hrs. These data suggested that TOP2 gene is upregulated under low temperature and cold signal could be perceived by shoots much faster than by roots.

To check the effect of high temperature stress, seedlings which were grown under normal green house condition (25 °C), were transferred to 37 °C for different time points. Total RNA was isolated separately from shoots and roots exposed to 2, 4 and 12 hrs of heat treatment and RNA gel blot analysis was carried out. As shown in Figure 2A and B, shoots and roots followed completely different induction pattern. Compared to shoots kept at normal temperature, a 2 fold higher induction was observed at 2 hrs and the transcript level declined at 12 hrs. However, in roots, there was an 8 fold stimulation at 2
hrs which reduced to 3 fold by 4 hrs and then increased again at 12 hrs (Figure 2B). This pattern of induction in roots under high temperature stress was repeatable in three replicates. These results suggest that TOP2 is also upregulated in response to high temperature.

To check the expression of TOP2 under salinity stress, seedlings were treated with different concentrations (50, 100, 200, and 250 mM) of sodium chloride. As mentioned in previous experiments, shoots and roots were separately harvested after 4 hrs of treatment. Transcript analysis showed that there is upregulation of TOP2 under salinity stress and the maximum level of induction was achieved under 200 mM NaCl in both roots and shoots (Figure 3A and B). To determine the induction kinetics under salinity stress, the seedlings were transferred to 200 mM NaCl and shoots and roots were collected separately after 2, 4, and 12 hrs of treatment. Northern blot analysis (Figure 3C and D) revealed that as compared to shoots, 6 fold higher induction was seen in roots at 2 hrs and the transcript level reached a maximum at 4 hrs and then gradually decreased until 12 hrs. In shoots, higher transcript level was seen at 4 hrs. These results suggest that the TOP2 is upregulated under salinity stress and the response was faster in roots.

To determine the expression of TOP2 under dehydration stress, water was withheld from 6 days old pea seedlings for 12 hrs and 24 hrs. Under these conditions TOP2 expression was not upregulated in response to dehydration stress. Infact with increase in time, TOP2 expression was downregulated under dehydration stress (Figure 4A and B).

Many of the abiotic stress responses are mediated via ABA induced signalling pathway. To find out if this is so in the present system too, we checked the transcript level of TOP2 following ABA treatments. As shown in figure 5A and B, TOP2 was upregulated in response to ABA stress and the maximum induction was seen at 150 μM concentration. However, it did not show significant induction compared to seedlings which were not treated with ABA.
To check if SA has any effect, pea seedlings were transferred to 50, 100 and 150 μM SA for 10 hrs. The transcript analysis showed that the maximum expression was achieved at the 100 μM concentration in both shoots and roots (Figure 6A and B). To check the kinetics of this response, pea seedlings were treated with 100 μM of SA for 2, 4 and 12 hrs and northern blot analysis was carried out. Total RNA was isolated at each time point from shoots and roots separately. Results showed there was about 3 fold higher induction in shoots at 2 hrs as compared to the untreated shoots and the increased level was maintained until 12 hrs. In roots an increase of more than 10 fold was observed at 4 hrs and then declined (Figure 6C and D).

6.2.2 Expression of TOP2 gene in response to different hormonal stress

It was found earlier that internodal stem which showed very low TOP2 transcripts levels, when cultured in vitro on B5 medium supplemented with 2,4 dichloro phenoxy acetic acid (2,4 D) and benzylamino purine (BAP), that induced callusing and cell proliferation, the expression of TOP2 was significantly stimulated and correlated well with callus formation upon hormone treatment (Reddy et al., 1999). To check if the expression of TOP2 under different hormonal conditions is affected in planta situation. pea seedlings were treated with different concentrations (1, 10 and 100 p mol) of 2,4-D. These treatment did not show significant upregulation at the transcript level (Figure 7).

6.2.3 Promoter Analysis using transgenic tobacco seedlings

Pea TOP2 promoter contains putative C/DRE element, ABRE element, two LTRE elements and two HSE elements (Figure 8). To check whether these elements are functional we analysed T1 generation tobacco transgenic seedlings harboring full length (UD) and deleted versions (D1, D2, D3 and D4) of the promoter under various stress conditions. As mentioned in UD, D1, D2, D3 and D4 vector promoter constructs were made and introduced into tobacco by Agrobacterium mediated transformation method. Transgenic plants were confirmed for the transgene with genomic DNA by PCR method (Figure 12, Chapter 4). The PCR positive transformed plants were screened by GUS
hitochemical assay. Plants, showed blue colour development in GUS histochemical staining (Figure 14, Chapter 4) were selected for further studies. After screening the transformed plants for the presence of TOP2-GUS by PCR and by GUS assay, five plants from each construct were selected for further analysis.

Seeds collected from the selected plants (T1 generation) were plated on medium containing kanamycin (50 μg/ml) to stop germination and growth of seedlings which were recessive for the trans gene. Three representative lines of each transgene were selected for further studies (UD- 9, 16, 21, D1- 10, 22, 36, D2- 2, 20, 30, D3- 0, b, 27 and D4- 7, 33, 38). All the experiments related to stress (low and high temperature stress, salinity, ABA and SA) were carried out with these three representative lines. Since T1 generation seedlings segregate for its transgene, mixed population was obtained in each case. Though we could avoid recessive transgene by plating on kanamycin, there was no way to distinguish between homozygous and heterozygous seedlings. Therefore results we obtained in each case showed high variation among three representative lines. A representation of the variation, for example, with respect to ABA treatment is shown in Table 3. However, when we compared the results under all the stress treatments, the maximum induction was seen among UD for UD-16, among D1 for D1-22 among D2 for D2-20, among D3 for D3-27, and among D4 for D4-7. These selected lines UD-16 (UD), D1-22 (D1), D2-20, (D2), D3-27 (D3) and D4-7 (D4) were used for stress treatments and the experiments were repeated twice.

6.2.4 Effect of low temperature on the activity of pea TOP2 promoter

To determine the cold mediated induction kinetics of UD, D1, D2, D3 and D4-TOP2 promoters, we transferred 10 days old transgenic tobacco seedlings to 4 °C for 2, 4, 12 and 24 hrs and measured the GUS activity as described in materials and methods. Except D4-TOP2-GUS, all other promoter reporter constructs showed upregulation under low temperature treatment. The higher level of activity was detected after 4 hrs of treatment under cold and it reached the maximum level at 24 hrs (Figure 9). It is evident that the activity of UD-TOP2 promoter was more than 10 fold higher at 24 hrs compared to
normal growth temperature (Figure 9). \textit{D1-TOP2} promoter which was derived from \textit{UD-TOP2} after deletion of 160 bp, had significantly reduced low temperature mediated activation as compared to \textit{UD-TOP2} promoter, however, it still showed about 6 fold higher level of activity compared to 0 hr treatment. Compared to 0 hr treatment, D2 and D3 also showed significantly higher level at 24 hrs treatment, however, as compared to the activities of UD and D1, the activity of D2 and D3 was quite low. Figure 10 shows the histochemical staining of 10 days old transgenic tobacco seedlings, subjected to low temperature stress. Similar to the GUS activity results, it showed maximum staining with \textit{UD-TOP2-GUS} (Figure 10a) and with the deletions the expression went down and \textit{D3-TOP2-GUS} staining was restricted only in cotyledons (Figure 10b,c and d). Staining was seen in \textit{D4-TOP2-GUS} (Figure 10e). These results suggest that pea \textit{TOP2} promoter can respond to cold stress minimally with \textit{D3-TOP2-GUS} promoter and with further deletions the promoter activity goes down. It also indicated that the LTRE elements present in this promoter are functional.

\textbf{6.2.5 \textit{TOP2} promoter is induced in dark under low temperature stress}

Since \textit{TOP2} is upregulated under light, to determine whether there is any interaction of light signalling pathway and abiotic stress signalling pathway, \textit{UD-TOP2-GUS} and \textit{D1-TOP2-GUS} homozygous \textit{Arabidopsis} seedlings were grown for 6 days under white light and dark. These were then transferred to 4 °C and 23 °C for 48 hrs under white light and darkness. The expression of pea \textit{TOP2} promoter was checked by histochemical GUS staining method and flurometric method. The results showed that the maximum expression was seen in \textit{UD-TOP2-GUS} under light and cold (Figure 11). Under dark the seedlings grown at 23 °C for 48 hrs shows basal level of expression only in cotyledons (Figure 11e) however, seedlings grown under 4 °C for 48 hrs showed expression in hypocotyl (Figure 11f). GUS activity results were in good agreement with histochemical staining (Figure 12). Interestingly, dark grown seedlings also showed 3 fold induction under cold stress compared with dark grown seedlings under room temperature (23 °C) revealing that LTRE elements present in the promoter region are functional even in the absence of any light mediated signalling event.
6.2.6 Expression of pea TOP2 promoter under high temperature stress

Pea TOP2 promoter contains two HSE elements. Such elements have been shown to respond to high temperature stress (Rieping and Schoffl, 1992). To determine the involvement of these elements in TOP2 promoter activity, we carried out MUG activity assays on transgenic tobacco lines subjected to high temperature stress. Ten days old T1 generation tobacco seedlings with UD, D1, D2, D3 and D4 promoter reporter constructs were transferred to 42 °C for 4, 12 and 24 hrs. Except D4-TOP2 promoter, the activity of UD, D1, D2 and D3 promoter reporter constructs were strongly induced within 4 hrs after transferring these seedlings to heat stress at 42 °C. As shown in figure 13, UD and D1-TOP2 promoter showed the same level of activity until 12 hrs but at 24 hrs D1 promoter activity was slightly reduced. These results suggest that the HSE element present with in D1 and D2 region may play an important role in the activity of TOP2 promoter under high temperature stress.

6.2.7 Pea TOP2 promoter activity is stimulated under ABA treatment

ABA controls large number of functions in plant growth and development. Since it was demonstrated at transcript levels that TOP2 is upregulated under ABA stress, we were interested to study its effect on transgenic tobacco seedlings. Ten days old tobacco seedlings, that are carrying UD, D1, D2, D3 and D4-TOP2-GUS were treated with 100 and 150 μM of ABA concentration for 10 hrs. As compared to control, UD-TOP2 promoter showed about 5 fold and D1-TOP2 showed 2 fold higher GUS activity with 150 μM ABA concentration (Figure 14). Though D1, D2, D3 promoters showed less activity compared to UD, yet they responded to ABA treatment suggesting at least D3-TOP2-GUS may have requisite cis-elements for ABA mediated induction.

6.2.8 Response of transgenic tobacco seedlings to SA

Pea TOP2 gene showed increasing transcript level following treatment with SA. To check the pea TOP2 promoter region responding to SA, ten days old T1 generation
tobacco seedlings were transferred to 100 and 150 μM of SA concentration and GUS activity was measured. As compared to control, UD-TOP2-GUS promoter showed about 50 % induction with 100 μM whereas D1-TOP2-GUS showed about 3 fold induction (Figure 15). The results showed that inducibility of D1 promoter is 3 fold more than UD promoter. As compared to control, D2, D3 and D4 promoters did not show an enhanced expression.

6.2.9 Expression of pea TOP2 under salinity stress

To determine the activity of UD, D1, D2, D3 and D4-TOP2-GUS promoters under salinity stress, we transferred ten days old T1 generation tobacco seedling to 100, 200 and 300 mM of NaCl solution for 10 hrs and GUS activity was measured. Figure 16 shows, that as compared to control, only UD-TOP2-GUS promoter was induced under 200 mM NaCl concentration after 10 hrs and showed about 50 % induction.
6.3 Discussion

Computer analysis of pea TOP2 promoter revealed the presence of abiotic stress responsive elements such as C/DRE consensus, two LTRE motifs, two HSE consensus sequences, WUN motif and ABRE element (Figure 8). These elements are known to be present in the promoters of many abiotic stress responsive genes (Bakers et al., 1994; Busk and Pages, 1998; Dunn et al., 1998; Jaglo-Ottosen et al., 1998; Jiang et al., 1996; Medina et al., 1999; Rieping and Schoffl, 1992). Our transcript analysis data showed that TOP2 responds to low temperature, high temperature, salinity, ABA and SA stresses. Since pea TOP2 did not show any induction under dehydration stress it looks like that it may not be responding to general osmotic stress. On comparing the transcript data it was found that maximum response was obtained with low temperature treatment and the transcript level was stably maintained even with increasing time under low temperature. The level of induction was found to be dependent on the organ perceiving the stress. Though in general all stresses showed a higher increase in shoots, the increase in the transcript level was more in roots in response to NaCl and heat stresses. Even the kinetics of response was different in shoots and roots in response to different stresses. The cold (Figure 1) and heat stress (Figure 2) was perceived faster in shoots whereas the salinity stress was perceived faster in roots (Figure 3).

The phytohormone ABA plays an important role in many physiological processes. This hormone is necessary for regulation of several events during seed development and for the response to environmental stresses such as dessiccation, salinity and cold (Busk and Pages, 1998). In the present study it was found that ABA increased pea TOP2 transcript levels. This effect was concentration, tissue and time dependent. Among other signaling molecules SA in general has been shown to have an important role in the defense in many plant species to pathogen attack. However, several studies also support a major role of SA in modulating the plant response to many abiotic stresses (Yalpani et al., 1994; Senaratna et al., 2000). Borsani et al. (2001) have shown that SA is involved in the plant response to salt and osmotic stress by playing a role in the ROS-mediated pathway. In the present study also we find that pea TOP2 expression is marginally induced in response to...
SA. Gene expression patterns of 8,200 genes representing one-third of the *Arabidopsis* genome were analysed by cDNA microarray analysis and expression profiling revealed novel interactions between wounding, pathogen, abiotic stress and hormonal responses (Cheong et al., 2002). There are few reports in literature that the genes involved in cell cycle and DNA replication are regulated under abiotic stresses and in response to ABA and SA. Earlier cyclin dependent kinases were found to be regulated under stress conditions like salinity stress (Burssens et al., 2000; Hirt, 2000). Tobacco *TOP1*, one of the important enzyme involved in DNA replication showed increased transcript level under abiotic stress conditions (Mudgil et al., 2002).

As mentioned in the beginning, there are several cis-elements present in the promoter region of pea *TOP2*. To find out the function of such elements we checked the expression of GUS as induced under the influence of different fragments of pea *TOP2* promoter in T1 generation tobacco and *Arabidopsis* seedlings in response to different stress conditions. A comparison of the expression of GUS activity revealed that maximum level of induction was obtained with *UD-TOP2-GUS*. The promoter was maximally stimulated in response to low temperature and ABA treatment. In fact the overall activity was higher in ABA (see Table 4). SA treatment showed much less stimulation in comparison to ABA even with UD promoter. Interestingly with SA the maximum induction was in *D1-TOP2-GUS* where WUN motif is present. This also shows that some elements may be present in between UD and D1 which may inhibit the optimal activity. Pea *TOP2* promoter contains C/DRE element in between *UD-TOP2-GUS* and *D1-TOP2-GUS* which is known to be involved in cold, dehydration and salinity stress. Since *TOP2* showed maximum promoter activity in several stress conditions with *UD-TOP2-GUS*, the C/DRE element present there may play an important role.

There are two LTRE elements present in pea *TOP2* promoter in between D2-D3 and *D4-TOP2-GUS* promoter. Results suggest that the LTRE element present in *D4-TOP2-GUS* is not sufficient to give induction alone. Similarly though ABRE element is present in *D3-TOP2-GUS* construct yet the activity obtained with this fragment in response to ABA was not equivalent to UD, D1 or D2. This suggests that single ABRE element alone is not
enough to induce maximum promoter activity and to achieve higher activity some elements in the upstream region may be needed (see Table 4 and Figure 8). There are few studies which have shown interaction between light and abiotic stresses (Kim et al., 2002; Weatherwax, 1998; Genoud et al., 2002). In an experiment where cold treatment was given to the seedlings grown either in light or in darkness, we found that light was not needed for cold mediated upregulation of the pea \textit{TOP2} promoter. In fact in the absence of light, the specific induction seen in few cell types was not noticed and the GUS activity was distributed all along the hypocotyl. These results suggest that there may be cross talk between light and cold signalling in regulating the expression of pea \textit{TOP2} in a cell specific manner (Figure 10 and 11). The intricacies and components that interact in these pathways need to be worked out in future studies.

Compared to induction under low or high temperature and ABA, under salinity stress we could detect low level of expression with \textit{UD-TOP2-GUS} and \textit{DI-TOP2-GUS} and it decreased drastically in \textit{D2-TOP2-GUS} (Table 4). This low level of induction was not highly comparable to the transcript data that was obtained in pea seedlings exposed to salinity treatment. We know that this promoter does not get expressed in roots under normal conditions. We have seen this earlier for both tobacco and \textit{Arabidopsis} (see Fig. 11, and 14, Chapter 4). From promoter analysis data it follows that this promoter does not get induced in response to salinity stress also. This means that the \textit{TOP2} that gets expressed in response to NaCl in pea roots could be a different isoform, yet its transcript size may be of the same size as the shoot form. It would be interesting to look for such an isoform in future studies. The other reason could be that one may still need some other far upstream elements, not present in the promoter that we have isolated, for expression in root tissue.

In addition to the present studies, the only other report on tobacco \textit{TOP1} revealed that the gene is upregulated under light, cold, ABA, SA and salinity (Mudgil et al., 2001). The exact reason why genes involved in DNA metabolism and cell cycle are regulated directly in response abiotic stress cannot be provided from the work done so far in this area. Recently three different mechanism have been suggested to explain the tolerance of
plants to abiotic stresses: ion homeostasis, cell division and detoxification and cellular repair (Zhu, 2002). The present study gives strong support to the mechanisms that may be related to cell division and DNA metabolism in relation to abiotic stresses. It is possible that TOP2 may play a role in chromatin remodeling and maintaining the appropriate DNA topology that influence the expression levels of several genes under stress conditions. Under physiological stress conditions its higher expression level may be required to maintain an active chromatin structure to carry out various cell processes. The role of topoisomerases and other related proteins in global gene expression in response to abiotic stresses, if any, needs further investigation.
Figure 1: Expression of TOP2 in response to cold stress. (A) Pea seedlings were subjected to cold stress at 4 °C for 2, 4, and 12 hrs. About 30 µg of total RNA was isolated separately from both shoots and roots of treated seedlings and total RNA was probed with ~1.8 kb TOP2. (B) Quantification of the data in Figure A by Fluor-S-Multi Image (Biorad)
Figure 2: Expression of TOP2 in response to heat stress. (A) Six days old pea seedlings were subjected to heat stress at 37 °C for 2, 4 and 12 hrs. About 30μg of total RNA was isolated separately from both shoots and roots of treated pea seedlings. The total RNA was probed with ~1.8 kb TOP2. (B) Quantification of the data by Fluor-S-Multi Image (Biorad)
Figure 3: Expression of TOP2 in response to salt stress. (A) Six days old pea seedlings were treated with 50, 100, 200 and 250 mM NaCl for 4 hrs. (C) The 6 days old pea seedlings were treated with 200 mM NaCl for 2, 4 and 12 hrs. About 30 μg of total RNA isolated from treated pea seedlings were probed with 1.8 kb of TOP2. (B) & (D) Quantification of the data by Fluor-S-Multi Image (Biorad)
Figure 4: Expression of TOP2 in response to dehydration stress. (A) The 6 days old pea seedlings were subjected to dehydration stress without providing water for 12 and 24 hrs. About 30 μg of total RNA isolated from treated pea seedlings were probed with 1.8 kb pea TOP2. (B) Quantification of the data in figure by Fluro-S-Multi Image (Biorad)
Figure 5: Expression of \textit{TOP2} in response to ABA stress. (A) Six days old pea seedlings were treated with different concentrations of ABA (50, 100 and 150 μM) for 10 hrs. About 30 μg of total RNA were probed with 1.8 kb \textit{TOP2}. (B) Quantification of the data by Fluor-S- Multi Image (Biorad)
Figure 6: Expression of TOP2 in response to SA stress. (A) Six days old pea seedlings were treated with different concentrations of SA (50, 100 and 150 µM) for 10 h. (C) The 6 days old pea seedlings were treated with 100 µM of SA for 2, 4 and 12 hrs. About 30 µg of total RNA was probed with 1.8 kb TOP2. (B) & D) Quantification of the data (by Fluor-S-Multi Image (Biorad))
Figure 7: Expression of TOP2 in response to different hormonal stress. Six days old pea seedlings were treated with different concentrations of (1, 10 and 100 p mol) of (A) BAP and (B) 2.4 D for 12 hrs. About 30 µg of total RNA were probed with 8 kb of TOP2. (C) & (D) Quantification of the data of (A) & (B) by Fluor-S-Multi Image (Biorad)
Figure 8: Sequence analysis of **TOP2** promoter. The sequence of various putative stress regulated cis-acting elements identified are shown in different colours. The different oligos UDF, D1F, D2F, D3F, D4F and R, used for the promoter deletion analysis are shown in blue colour.
Table 3. MUG Activity (n mol/ mg of protein/ hr$^{-1}$) of three representative lines in each transgene under ABA stress

<table>
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<th>Transgenic Line</th>
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Figure 9: The cold inducibility of UD and deletion versions (D1, D2, D3 and D4) of TOP2 promoter. Ten days old T1 generation tobacco seedlings were exposed to 4 °C for 2hrs, 4hrs, 12 hrs and 24 hrs and GUS activities were measured. UD indicates UD-TOP2-GUS, D1 indicates D1-TOP2-GUS, D2 indicates D2-TOP2-GUS, D3 indicates D3-TOP2-GUS and D4 indicates D4-TOP2-GUS transgene.
Figure 10: Histochemical GUS staining of tobacco transgenic in response to cold stress. Tobacco seedlings (a) UD-TOP2-GUS (b) D1-TOP2-GUS (c) D2-TOP2-GUS (d) D3-TOP2-GUS (e) D4-TOP2-GUS were grown for 10 days and transferred 4°C for 24 hrs. GUS staining was performed by using X-gluc.
Figure 11: Histochemical GUS staining of transgenic Arabidopsis. Transgenic Arabidopsis seedlings containing UD-TOP2-GUS & D1-TOP2-GUS were grown under white light and dark separately, subjected to 23 °C and 4 °C temperature for 48 hrs under the same conditions and promoter activity was assayed by visualizing GUS expression histochemically. (a) Light grown UD-TOP2-GUS under 23 °C (b) light grown seedling treated 48 hrs under 4 °C (c) Light grown D1-TOP2-GUS under 23 °C (d) light grown seedling treated 48 hrs under 4 °C (e) Dark grown UD-TOP2-GUS under 23 °C (f) dark grown seedling treated under 4 °C for 48 hrs. (g) Dark grown D1-TOP2-GUS under 23 °C (h) dark grown seedling treated under 4 °C (right).
Figure 12: Cold inducibility of light and dark grown transgenics *Arabidopsis*. Six days old UD and D1-TOP2-GUS containing *Arabidopsis* seedlings were transferred to 23 °C and 4 °C temperature for 48 hrs and kept under dark or light and GUS activity was measured.
Figure 13: The heat inducibility of UD and different deletion versions of TOP2 promoter. Ten days old T1 generation tobacco seedlings were exposed to 42 °C heat for 4 hrs, 12 hrs and 24 hrs and GUS activity was measured. UD, D1, D2, D3 and D4 indicate their respective transgene as mentioned in Figure 8.
Figure 14: The ABA inducibility of TOP2 promoter and its deleted versions. Ten days old T1 generation tobacco seedlings were transferred to 100, 150 µM of ABA for 10 hrs and GUS activities were measured. UD, D1, D2, D3 and D4 indicate their representative transgenes as mentioned in Figure 8.
Figure 15: The SA inducibility of TOP2 promoter and deleted versions. Ten days old T1 generation tobacco seedlings were transferred to 100 and 150 μM SA for 10 hrs and GUS activities were measured. UD, D1, D2, D3 and D4 indicate their representative transgenes as mentioned in Figure 8.
Figure 16: The salinity inducibility of TOP2 promoter and deleted versions. Ten days old T1 generation tobacco seedlings were transferred to 100, 200 and 300 mM of NaCl for 10 hrs and GUS activities were measured. UD, D1, D2, D3 and D4 indicate their representative transgenes as mentioned in Figure 8.
### Table 4. Optimal GUS Activity of various TOP2 deletions under different stress conditions

<table>
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<th>Promoter construct</th>
<th>GUS Activity nmol/ mg protein/ hr&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>Cold (24 hrs)</th>
<th>Heat (4 hrs)</th>
<th>ABA (150μM)</th>
<th>SA (100 μM)</th>
<th>NaCl (200 mM)</th>
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