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Promoter Analysis of a DNA Topology and Morphogenesis Involved Gene-*TopI* Reveals Novel Stress Related Functions in *Nicotiana tabacum*

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ABSTRACT

DNA topoisomerases type I (TopI) are enzymes that relieve torsional stress in DNA by inducing ATP dependent single strand break and change the linking number in steps of one. In plants like wheat, pea and tobacco, topoisomerases are reported to play essential roles in plant morphogenesis and development. Studies using *Arabidopsis* have established their role in plant development via stem cell regulation in shoots and floral meristems, and plant response to light stimulus and flowering time. Transcript levels of tobacco *TopI* during different stages of cell cycle had revealed stage-specific expression. There are a few reports related to the roles of topoisomerases in different plant abiotic stress responses. The regulation of *Top1* is not well understood. To investigate this aspect, we investigated the *TopI* promoter from *N. tabacum* and cloned and sequenced the promoter region, ~500 bp upstream of the initiation codon. Detailed in silico and in planta characterization of cis-regulatory elements present in *TopI* promoter revealed the presence of elements involved in cold, dehydration and salinity within this putative promoter sequence. We performed transcription start site mapping along with electrophoretic mobility shift assay with tobacco nuclear extract to validate the functionality of the isolated promoter in planta. Functional characterization of cis-regulatory elements was carried out using transient expression of promoter-GUS construct in *N. benthamiana*. Our results show that in silico motif analysis and stress-responsiveness of *TopI* promoter reveal the stress responsive nature of the isolated promoter. These findings are novel as they show the involvement of *TopI* gene in stress responses, besides previously reported involvement in regulation of DNA topology and plant morphogenesis. This study opens the possibility for the use of *TopI* promoter in basic research as well as in crop breeding strategies.

Key words: Electrophoretic mobility shift assay assay (EMSA), *Nicotiana tabacum,* Primer extension, Stress responsive cis-elements, *TopI* promoter, Transient expression

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Introduction

The genetic material in a cell is constantly subjected to DNA damaging agents that may be external (ultraviolet sunlight, ionizing radiation and chemicals) or internal (reactive oxygen or nitrogen species, ROS or NOS), leading to modifications and deleterious cellular outcomes like disease and aging. All these damaging agents are associated with the generation of single strand breaks, produced either directly or as intermediates of DNA repair events. In order to maintain genomic integrity through evolution, organisms contain robust DNA repair and recombination pathways to repair/remove or tolerate lesions (Chatterjee and Walker, 2017). The intertwining of complementary strands, which forms the DNA duplex, provides an elegant structural basis for storage and transmission of genetic information. However, the processes of DNA transactions, which include protein tracking on DNA, unwinding and rewinding of the duplex and coiling of DNA to form higher-order structures, can lead to topological entanglements causing genome instability if left unresolved. The cell has evolved a DNA damage response system, which includes repair mechanisms, specific to damage type, to constantly monitor genomic integrity for faithful transmission of genetic material to the next generation. DNA topoisomerases are essential enzymes that control the topology of the DNA, play critical roles in the fundamental biological processes of replication, transcription, recombination, repair, and chromatin remodelling.

DNA topoisomerases are ubiquitous and essential enzymes which regulate the structures of DNA/chromosomes and their associated cellular functions, thereby acting as nature's tools to resolve the problems of

DNA entanglements through topological transformations (Pommier et al., 2006; Schoeffler and Berger, 2008). DNA topoisomerases have been classified into two classes type I and type II, depending on whether they mediate relaxation of DNA supercoils by creating either single- or double-strand breaks (TopI and TopII, respectively) in the phosphodiester backbone of DNA (Champoux, 2002; Ashour et al., 2015). Both type I and II topoisomerases have been extensively studied in bacteria, viruses, yeast and animals. In eukaryotes, TopI is a key nuclear enzyme that regulates the topology of DNA during replication, transcription, and chromatin remodelling (Bush et al., 2015; Liu & Wang, 1987). The crystal structures of human TopI and TopI-DNA complexes have been determined by X-ray crystallography (Redinbo et al., 1998). However, in animal systems, the study of developmental function is difficult because *TopI* knockouts are embryonic lethal (Lee et al., 2001; Morham et al., 1996). On the other hand, in *S. cerevisiae*, one type II and two type I topoisomerases have been identified and functionally characterized (Goto et al., 1984; Goto & Wang, 1984; Goto & Wang, 1985). In *C. elegans*, *TopI* possible functions in morphogenesis, stem cell niche specification, life span, and growth control have been indicated (Lee et al., 2001; Lee et al., 2014).

Plants being sessile are more prone to environmental cues such as light, temperature, salinity etc. Their genomic integrity is more at risk due to these environmental factors, which may induce spontaneous changes in genetic material, hence impeding the normal functioning of DNA molecule. The potential of topoisomerases in stress responses has so far been poorly explored. Topoisomerases are reported from wheat, *Arabidopsis*, pea, tobacco and have been demonstrated to play essential role for plant morphogenesis and abiotic stress responses (Dynan et al., 1981; John et al., 2016; Kieber et al., 1992; Mudgil et al., 2002; Reddy et al., 1998; Singh et al., 2015). In *Arabidopsis,* disruption of the two *TopI* homologs encoding genes, *Top1α* and *Top1β* resulted in seedling lethality (Takahashi et al., 2002). Interestingly, *Top1β* mutant showed no visible defects whereas *Top1α* mutation is associated with obvious defects related to organization of shoot, floral, seed size and root meristems resulting in abnormal plant growth suggesting that Top1α has specific developmental functions (Laufs et al., 1998; Takahashi et al., 2002; Zhang et al., 2016; Gao et al., 2017; Li et al., 2020). Over-expression of *TopoII* in *N. tabacum* has been found to enhance tolerance to salt stress (John et al., 2016). In pea and tobacco, transcript levels of *TopoII* are regulated in response to salt, cold and drought stress (Hettiarachchi et al., 2005). *Top1α* in *Arabidopsis* plays an important role in stem cell maintenance and also regulates adaptive plant response to light (Kupriyanova et al., 2017).

There are several stress-related genes which have inducible promoters with stress specific cis-regulatory elements playing vital role in the gene expression and regulation. Currently, in silico analysis of sequenced genomes is routine to predict presence of these elements in DNA. Demonstration of promoter activity in response to abiotic and biotic stresses is essential in order to understand functions of these putative cis-elements as well as to explore their potential importance for engineering stress tolerance to have their biotechnological applications (Hernandez-Garcia & Finer, 2014). Therefore, the functional validation of the upstream regulatory regions of *N. tabacum TopI* gene was deemed important.

Isolation and functional characterization of *N. tabacum TopI* was performed. Primer extension to map the transcription initiation site and binding of nuclear proteins in vivo using electrophoretic mobility shift assay (EMSA) was done to establish functioning of isolated ~500 bp fragment as promoter. In silico comparisons of the *TopI* promoter sequence with different stress-specific promoters revealed several conserved motifs. A promoter GUS chimeric construct was used to study response under abiotic stress conditions. All experiments in toto established stress-responsive nature of *TopI* promoter. Therefore we conclude that *TopI* promoter has potential to serve as an important candidate for stress specific promoter in genetic engineering of plants under stress conditions.

Materials and Methods

Cloning of the 5' Flanking Region of the N. tabacum TopI

High quality genomic DNA of tobacco was isolated and was partially digested with *Sau*3AI restriction enzyme and fractionated on 0.8 % agarose gel. The fragments of the size range of 0.5 kb to 3.0 kb were eluted from the gel and adapters were ligated to both the ends of the fragments as described by Reddy et al. (1998), except that the ends were compatible instead of blunt. PCR was carried out using PCR components

and with 20 ng of adapter ligated *N. tabacum* genomic DNA as template. An approximately 0.6kb fragment of 5' upstream of the *N. tabacum TopI* gene was PCR amplified using T7 primer and gene specific primer designed in antisense orientation from the 5' end of the cDNA(PM1,5′-GCTTTGATGTGGTACTACTTCG-3') located 100 bp downstream of ATG (Fig. 1A). The amplified fragment was from a region 482 bp upstream of the translation initiation codon with 100 bp overlap of the coding region. The amplification product was gel purified, its ends polished using T4 DNA polymerase and subsequently digested with *Not* I before cloning into *EcoR* V and *Not* I digested pBluescript plasmid. The insert DNA was completely sequenced and promoter sequence was deposited in Genbank (AY043311). This clone was used as a template for introducing it into various vectors.

Cloning of 5′ Upstream Regulatory Regions of N. tabacum TopI in pGWB433

The 482 bp 5' flanking sequence of *N. tabacum TopI* was PCR amplified using the following primers: Prom For- 5′-CACCTGGTGCGCCGCCGCGGGCGA-3' and Prom Rev- 5′-GATCGTACTCTATGCTATA-3'. The amplified fragment was cloned into TOPO Entry vector followed by recombination into pGWB433. The promoter was placed upstream of promoterless Gus. It was transformed by the freeze-thaw method into *Agrobacterium tumefaciens* (GV3101) by following the protocol of Sambrook et al. (Sambrook et al., 1989). The transformation was confirmed by colony PCR and restriction digestion analysis.

Transcription Start Site Mapping

A 22-mer oligonucleotide was synthesized corresponding to 87 nucleotides downstream of ATG of *N. tabacum TopI* cDNA in the antisense orientation, 5'-GCTTTGATGTGGTACTACTTCG-3'. The oligonucleotide primer was end labelled with [^{32}P] γ-ATP using T4 polynucleotide kinase. Fifty nanograms of radiolabelled primer was annealed to 10 µg of total *N. tabacum* RNA at 55°C for 10 min and the primer extension was carried out for 1 h at 42°C using SuperScript^{TM} (Gibco-BRL). At the end of the extension reaction the products were ethanol-precipitated and resuspended in 20 µl of 1x formamide denaturing dye. The products were heated for 3 min and then cooled on ice prior to running on a 6% polyacrylamide urea gel. The gel was dried and exposed to X-ray film and analysed.

Preparation of Nuclear Extract

Nuclei were isolated from young leaves of greenhouse grown *N. tabacum* plants. Leaves (500 g) were homogenized in STM buffer (50 mM Tris pH 8.0, 5 mM $MgCl_2$, 0.5 M sucrose and 15 mM βME). Homogenate was filtered through two layers of cheesecloth and subjected to centrifugation at 6000 rpm in cold for 5 min. The pellet containing chloroplast and nuclei were lysed with 2.5% Triton X-100 in STM buffer for 30 min. on ice. The lysate was centrifuged again to pellet the nuclei and the intact chloroplast. This treatment was repeated 2-3 times to completely lyse the chloroplast and get pure nuclei. Nuclear extract was prepared following Dignam's protocol (Dignam et al., 1983).

Gel Shift Assays

A 200 bp fragment of *N. tabacum TopI* promoter fragment located 157 bp away from ATG (5 upstream 157 to 357 bp) was PCR amplified and cloned in pGEM-T Easy and sequenced to confirm lack of mutations. It was digested with *Not* I (non-cutter of the fragment) and the drop out fragment was gel purified and taken as a substrate for gel shift assays. Labelling of fragment was carried out at room temperature for 1hr using [^{32}P] α-dCTP along with cold dGTP and klenow followed by addition of 1/10 Na acetate and ethanol precipitation. 10 ng (1 µl) of the labelled fragment was incubated with nuclear extract (1µg) in presence of 3µg of Poly dI-dC and binding buffer (2X- 20 mM HEPES pH 7.5, 50 mM KCl, 1 mM EDTA, 10% glycerol, 5 mM DTT, 0.4 mM $MgCl_2$, 60 mM NaCl) in a reaction volume of 30 µl for 3 min. The reaction was terminated by the addition of 10x DNA loading dye. Competition assays were carried out using several fold molar excess of cold probe, and non-specific probes (ssDNA and MCS of pBluescript SK+). The reactions were fractionated on non-denaturing acrylamide gels (4% acrylamide, 0.5X TBE, 2.5% glycerol), dried and exposed to develop autoradiograms.

Sequence Analysis

Promoter motif searches within the 482 bp fragment were carried out and notable sequence motifs were identified using signal scan search-PLACE (http://www.dna.affrc.go.jp) (Higo et al., 1999) and CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Agroinfiltration and Various Stress Treatments in N. benthamiana Leaves

Agrobacterium mediated plant transient expression using leaf infiltration was performed in order to analyze the expression of *TopI* promoter (Yang et al., 2000). Leaves of 3 week old *N. benthamiana* plants were agro-infiltrated and used for different stress treatment analysis after 3 days of infiltration. Leaf discs were cut with the help of cork borer, kept in Petri plates and treated with sodium chloride (NaCl, 200mM), polyethylene glycol (PEG6000, 20%) respectively at normal growth conditions. For water stress, the leaf discs were soaked in Whatman paper filter paper, wet with water at regular time intervals while for dark treatment, leaf discs were completely covered with aluminium foil and kept at room temperature. Similarly the Petri plates having leaf discs were incubated at 4ºC for cold treatment and the discs treated with water. Each treatment was done for 8hrs followed by GUS-staining of discs.

GUS Histochemical Assay

Histochemical GUS staining was followed as described by Malamy and Benfy (1997). For quantitative GUS analysis the protocol of Jefferson et al. (1987) was used. Stress-treated *N. benthamiana* leaf discs were briefly rinsed in sterile 50 mM potassium phosphate buffer (pH 7.0) and were incubated in GUS staining solution, gently degassed for 5min and incubated 8hrs at 37ºC. Chlorophyll clearing from the tissues was done by 3-4 times rinsing in 75% ethanol at 37ºC, followed by visualization using Zeiss Stemi 305 with Magcam DC5 CMOS SENSOR optical camera.

Results

Cloning, Sequencing and in Silico Analysis of 5' Flanking Region of N. tabacum TopI Promoter Show Presence of Stress Responsive Elements

Size fractionated and T7 adaptor ligated tobacco genomic DNA was prepared as previously described (Reddy et al., 1998) and used as template for isolation of tobacco *TopI* promoter. ~0.6kb fragment of 5' upstream of the tobacco *TopI* gene was PCR amplified using T7 primer and gene specific primer designed in antisense orientation from the 5' end of the cDNA located 100 bp downstream of ATG (Fig.1A). PCR amplified fragment containing *N. tabacum TopI* promoter was cloned into pBluescript plasmid followed by sequencing. Resulting sequence was deposited in Genbank (AY043311). Notable sequence motifs within a region upstream of the translation initiation codon were identified using programme CARE and PLACE (Table I). Two canonical CAAT box sequence and consensus sequences matching the putative regulatory factor binding sites such as four GT1 box, I box, two GATA motifs, MARAS motif were observed which form characteristic feature of most of the *Topoisomerase* promoter (Holmes-Davis and Comai, 1998). Abiotic stress-inducible G-BOX, SORLIP1AT, W-BOX, GT1CONSENSUS and I-BOX elements were also present. Tobacco *TopI* promoter sequence contained elements responsive to dehydration (MYB2CONSENSUSAT), salt stress responsive (GT1GMSCAMY), water stress responsive (MYBCORE), cold response related elements (MYCCONSENSUSAT). For light regulated expression GATABOX, GT1CONSENSUSAT, IBOX, IBOXCORE and IBOXCORENT elements were present in the sequence. SORLTIP1AT revealed as the elements over-represented in light induced promoters while TBOXATGAPB as the elements involved in light

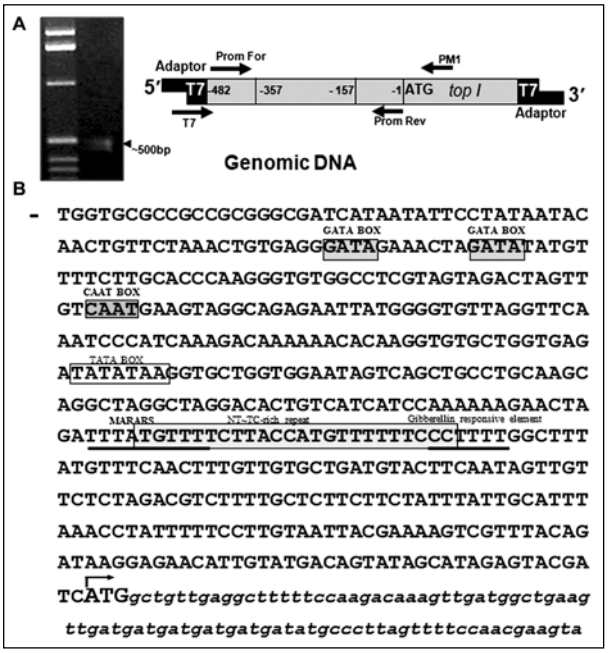

Figure 1. Isolation and in silico analysis of *TopI* promoter. (A) T7 adaptor ligated 1st strand cDNA was used as a template and T7 forward and antisense PM1-*TopI* reverse primer located 100- bp downstream of ATG was used to amplify 482 bp fragment. (B) The promoter fragment was cloned into pBluescript vector and sequenced from both end and completely sequenced and promoter sequence was deposited in Genbank (AY043311). In silico analysis was performed using programme CARE and PLACE and major cis regulatory motifs mainly responsive to dehydration, salt, water, cold and light were found and are marked on sequence.

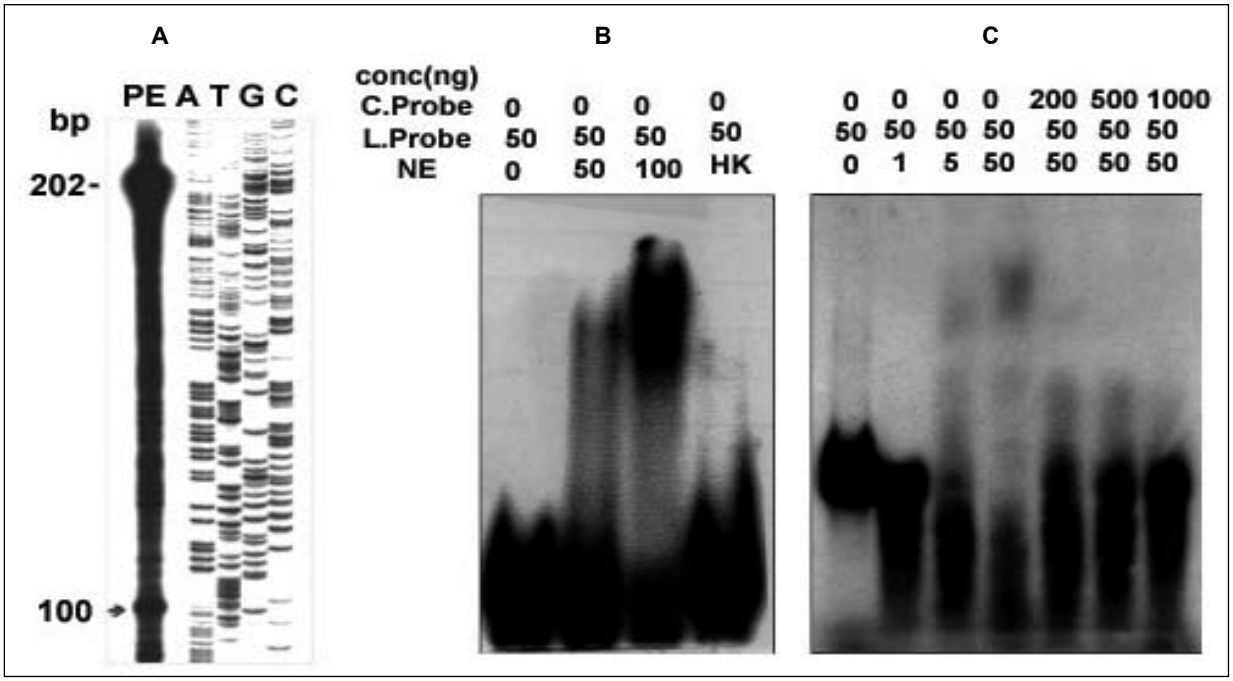

Figure 2. Mapping of transcription start site and DNA binding activity of *TopI* promoter; (A) Primer extension analysis was used to map the transcription start site using a primer-PM1. The two major primer extension products observed were 202 and 100 nucleotides in length. The major transcript was initiated at A 202, the minor transcript at T 100 nucleotides; (B) Gel shift assays were performed using a 200-bp fragment of the *N. tabacum TopI* promoter. Nuclear extract (NE) isolated from *N. tabacum* leaves showed binding with this fragment (lanes 2-3), binding was not detected (lane 4) when heat killed NE (HK, NE boiled for 5 min) was used; (C) Specificity of interaction was tested using a cold probe (C probe, No Labelling of fragment was carried out) to compete radiolabelled probe (L probe, Labelling of fragment was carried out using [^{32}P] α-dCTP). The binding was competed out partially with four-fold molar excess of C probe (lane 5) and completely with ten- to twenty-fold molar excess of the C probe (lanes 6-7).

activated transcription (Fig. 1B). Biotic stress responsive TC-rich repeat and BIHD1OS were also identified as defence related elements.OS~SKN-1 motif for high level endosperm expression and TAAAGSTKST1 for guard cell specific expression was also present (Table 1).

Isolated TopI promoter harbours transcription start site at 202 bp upstream of the translation initiation codon and has DNA binding activity with nuclear proteins

The transcription start site was mapped by primer extension analysis using primer PM1. The two major primer extension products observed were 202 and 100 nucleotides in length. The major transcript, as deduced from the predominant extension product, was being initiated from the A 202 nucleotide corresponding to the transcription start site (arbitrarily defined as position +1), which is 202 bp upstream of the ATG initiation codon. The other minor transcript was initiated at T 100 nucleotides from the transcription start site (Fig. 2A).

In silico analysis of the isolated -482 to -1 revealed several putative cis-regulatory elements (Fig. 1). In order to test if these elements are recognised by specific trans-acting factors in planta we performed electrophoretic mobility shift assay using 200 bp (-157 to -357 bp, Fig. 1B) fragment of *N. tabacum TopI* promoter as probe. This fragment was chosen based on in silico sequence analysis as it contains putative transcription initiation site, AP1 site, MARAS motif, gibberellin responsive element. Nuclear extract prepared from *N. tabacum* leaves showed binding with this fragment, formation of strong low mobility DNA-protein complex was detected (Fig. 2B, lane 2). The complex became more intense at the same position when twice the amount of protein was used (Fig. 2B, lane 3), whereas heat-killed nuclear protein (boiled for 5 min) showed no binding activity (Fig. 2B, lane 4).

To check the specificity of interaction, competition of radiolabelled probe was carried out with cold (unlabelled) probe. The binding was competed out partially with four-fold molar excess of cold fragment and completely with ten- to twenty-fold molar excess of the cold probe (Fig. 2C, lanes 5-7) suggesting that this DNA binding activity was specific to the -157 to -357 bp DNA fragment. Though further characterisation of this binding is required, it does provide preliminary

Table 1. Cis-regulatory elements present in *Top I* promoter identified using programme CARE and PLACE.

Site Name	Position	Database ID	Strand	Sequence	Functions
GCCCORE	7	S000430	(+)	GCCGCC	Ethylene-responsive element
CGCGBOXAT	11	S000501	(-)	VCGCGB	Recognized by AtSR1-6
	11	S000501	(+)		
ROOTMOTIFTAPOX1	25	S00098	(-)	ATATT	Motif found both in promoters of rolD
	26	S00098	(+)	ATATT	
EBOXBNNAPA	40	S000144	(-)	ATATT	E-box of napA storage-protein gene *B. napus*
MYBCORE	40	S000176	(-)	CNGTTR	Responsive to water stress
MYCCONSENSUSAT	40	S000407	(-)	CANNTG	Cold response
	40		(-)		
	226		(-)		
	226		(+)		
EBOXBNNAPA	40	S000144	(+)	CANNTG	
	226		(-)		
MYB2CONSENSUSAT	40	S000409	(+)	YAACKG	Found in the promoters of the dehydration-responsive gene rd22
GTGANTG10	55	S000378	(+)	GTGA	
MYBST1	60	S000180	(+)	GGATA	
GATABOX	61	S000039	(+)	GATA	For light regulated expression
	72		(+)		
	200		(+)		
	440		(+)		
BOXIINTPATPB	62	S000296	(+)	ATAGAA	Important for the activity of this NCII promoter
POLLEN1LELAT52	64	S000245	(+)	AGAAA	For pollen specific activation
	81		(-)		
	290		(-)		
SORLIP1AT	99	S000482	(-)	GCCAC	Over-Represented in Light-Induced Promoters
CACTFTPPCA1	109	S000449	(-)	YACT	key component of Mem1 (mesophyll expression module 1)
	129		(+)		
	255		(+)		
BIHD1OS	120	S000498	(+)	TGTCA	Binding site of OsBIHD1 in disease resistance response homeodomain transcription factor
	258		(+)		
	458		(-)		
WBOXATNPR1	121	S000390	(-)	TTGAC	SA-induced, Regulates NPR1
WRKY71OS	121	S000447	(-)	TGAC	Core of W-box
	224		(-)		
	458		(+)		

Site Name	Position	Database ID	Strand	Sequence	Functions
CAATBOX	123	S00028	(+)	CAAT	CAAT promoter consensus sequence
	351		(+)		
	392		(-)		
	452		(-)		
CANBNNAPA	149	S000148	(-)	CNAACAC	Core of "(CA) element" in storage protein genes in *B. napus*
MYBPLANT	151	S000167	(-)	MACCWAMC	Plant MYB binding site
MYB26PS	151	S000182	(+)	GTTAGGTT	
ACIIIPVPAL2	151	S000194	(+)	GTTAGGTTC	
ARR1AT	161	S000454	(-)	NGATT	"ARR1-binding element"
	280		(+)		
DOFCOREZM	170	S000265	(-)	AAAG	Core site required for binding of DOF proteins in maize
DPBFCOREDCDC3	181	S000292	(+)	ACACNNG	A novel class of bZIP transcription factors
GTGANTG10	196	S000378	(+)	GTGA	
TATABOX4	202	S000111	(+)	TATATAA	TATA box in the 5'upstream region
WBOXHVISO1	223	S000442	(-)	TGACT	
WBOXNTERF3	223	S000457	(-)	TGACY	
WBOXNTCHN48	223	S000508	(-)	CTGACY	"W box"
INTRONLOWER	232	S00086	(-)	TGCAGG	Consensus sequence for plant introns
MARARS	285	S000454	(+)	WTTTATRTTTW	"ARS element"; Motif found in SAR (MAR)
S1FBOXSORPS1L21	295	S000223	(-)	ATGGTA	
PYRIMIDINEBOXHVEPB1	302	S000298	(+)	TTTTTTCC	Required for GA induction
GT1CONSENSUS	303	S000198	(-)	GRWAAW	Related to light regulated genes & SA inducible gene expression
GT1GMSCAM4	303	S000453	(-)	GAAAAA	Pathogen and salt responsive elements
PYRIMIDINEBOXOSRAMY1A	309	S000259	(+)	CCTTTT	Gibberellin-responsive
TAAAGSTKST1	317	S000387	(-)	TAAAG	Involved in Guard cell specific-expression
TBOXATGAPB	329	S000383	(-)	ACTTTG	Involved in light-activated transcription
RAV1AAT	333	S000314	(-)	CAACA	
CURECORECR	345	S000493	(-)	GTAC	GTAC is the core of a CuRE (copper-response element)
CACTFTPPCA1	346	S000449	(+)	YACT	Tetranucleotide (CACT) is a key component of Mem1 (mesophyll expression module 1)
ACGTCBOX	367	S000131	(-)	GACGTC	"C-box"
ACGTATERD1	368	S000415	(-)	ACGT	Required for etiolation-induced expression of erd1 (early responsive to dehydration)
NODCON2GM	378	S000462	(+)	CTCTT	Putative nodulin consensus sequence

Site Name	Position	Database ID	Strand	Sequence	Functions
OSE2ROOTNODULE	378	S000468	(+)	CTCTT	One of the consensus sequence motifs of organ-specific element
BOXIINTPATPB	384	S000296	(-)	ATAGAA	
CARGCW8GAT	386	S000431	(-)	CWWWWWWWWG	A variant of CArG motif (S000404)
POLASIG1	389	S0000080	(-)	AATAAA	"PolyA signal"
L1BOXATPDF1	394	S000386	(-)	TAAATGYA	
IBOX	440	S000124	(+)	GATAAG	Conserved sequence upstream of light-regulated genes
IBOXCORE	440	S000199	(+)	GATAAG	Light-regulated element
IBOXCORENT	440	S000424	(+)	GATAAGR	"I-box core motif" in the CAMs (conserved DNA modular arrays) associated with light-responsive promoter regions

evidence that this promoter interacts with nuclear proteins in planta.

Stress Response Analysis of TopI Promoter in N. benthamiana Leaves

In vivo analysis of plant promoters using *Agrobacterium* mediated transient expression assay is a also widely accepted tool (Yang et al., 2000).We used similar approach to test responsiveness of cis elements of *TopI* promoter. A chimeric construct of *prom-TopI*-GUS was used to measure the expression activity of *TopI* promoter under different abiotic stress conditions. *N. benthamiana* leaves were first infiltrated with *prom-Top*-GUS binary construct for transient expression. Response of *TopI* promoter was studied under water, dark, NaCl (200mM), cold (4ºC), polyethylene glycol (PEG) 20% and mannitol (300mM) stress treatment of 8hrs (Fig. 3A)**.** Under salt stress treatment GUS staining intensity of blue colour was lowest, compared to all the other treatments with mannitol being a little higher. The blue coloration due to effects of PEG and cold stress treatment were similar and higher than mannitol. *N. benthamiana* leaf discs underwater stress developed intermediate blue colour, indicating moderate GUS activity. Interestingly, GUS expression under dark treatment was found highest overall, and higher than in light or water treatment which showed higher GUS activity than the other treatments. Results indicate that the variability in expression of GUS may be due to differential regulation of *TopI* promoter by different cis-regulatory elements (Fig. 3B).

Discussion

Work on topoisomerases in plant systems has been mostly limited to characterization of the purified proteins (Badaracco et al., 1983; Heath-Pagliuso et al., 1990; Makarevitch and Somers, 2005). More recently, studies have been conducted to explore the roles of topoisomerases in response to different abiotic and hormonal stresses (salt, light, cold, ABA and SA). There are relatively few studies on plant topoisomerase I under abiotic stress (Mudgil et al., 2002).

In the current study in order to understand the role and regulation of *TopI* promoter from *N. tabacum,* cis elements were identified using in silico analysis. The promoter region under study revealed the presence of two GATA motifs, a GT-1 consensus site and 3, I-Box consensus sequences in the negative strand. These elements have been shown to confer light responsiveness in a number of genes (Argiiello-Astorga and Herrera-Estrella, 1998; Terzaghi and Cashmore, 1995). In *N. tabacum TopI* promoter MARAS motifs are present that are characteristic features of most *TopI* promoters (Holmes-Davis and Comai, 1998). Stress-inducible (anoxic, elicitor, wound, heat, drought, cold, ABRE, SA), defence-related TC-rich repeat, P-box (gibberellin responsive element) and OS~SKN-1 motif for high-level endosperm expression are also present. Previous in silico analysis of *Tdp1α* and *Tdp1β* promoter sequences in *Arabidopsis* had revealed the cis- response elements for light (Box 4, Box I, G boxes), heat stress (HSE), salicylic acid (TCA-element) as well as elements for low temperature (LTR) and light response (3-AF1 binding site, GATA-motif, GTGGC-motif, I-box) (Sabatini et al., 2017). Isolation and functional characterization of the promoter region of the *top1β* gene from carrot also showed a *myb*-related motif and the Dof element NtBBF-1, which correlates with the inducible expression pattern of this gene (Balestrazzi et al., 2003). Features of the *TopI* promoters from all

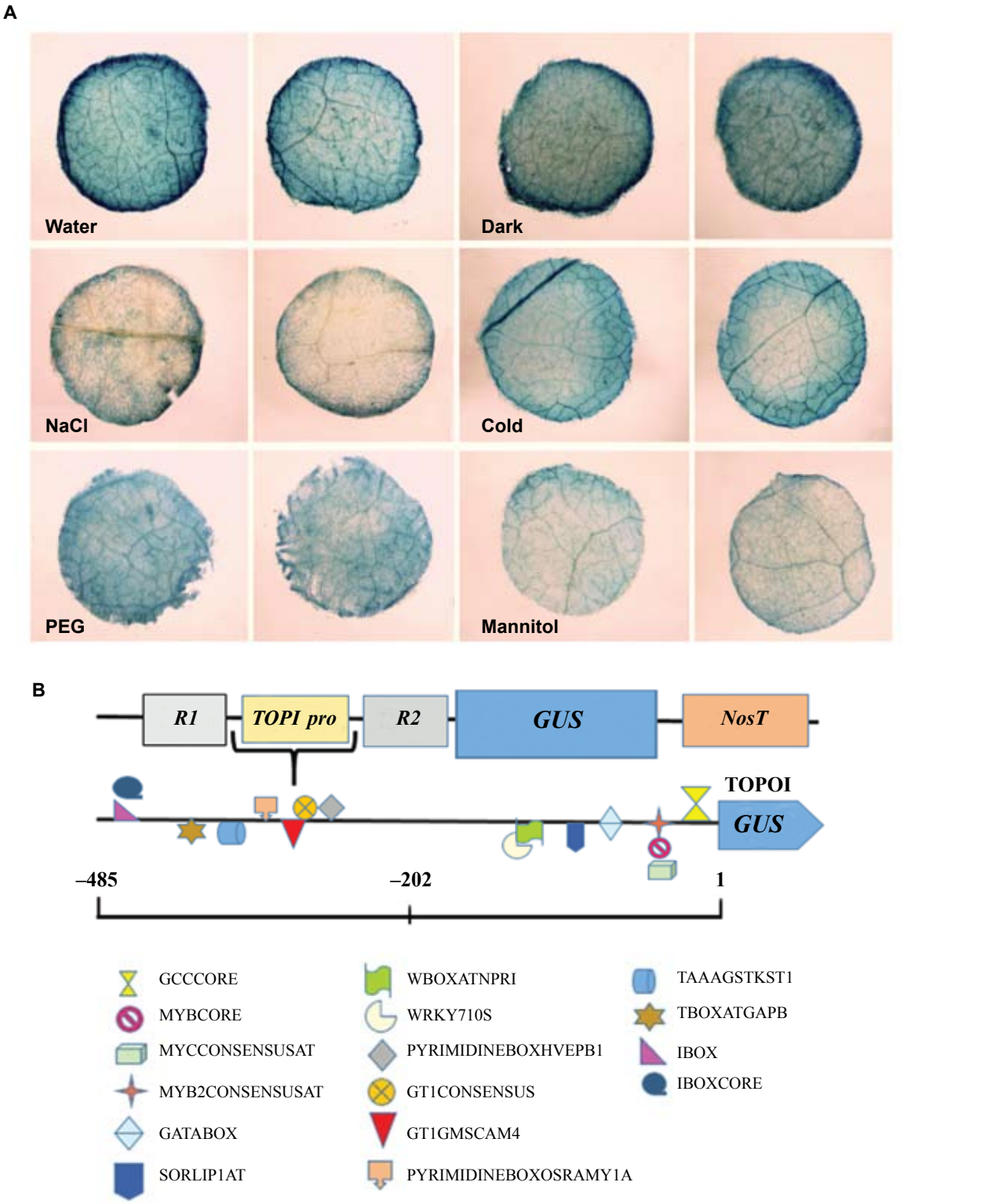

Figure 3. Stress response analysis of *TopI* chimeric promoter-GUS construct. (A) Qualitative histochemical GUS localization analysis was done by in agro-infiltrating *N. benthamiana* leaves with promoter-*TopI*-GUS vector construct in pGWB433. After 48-72 hr post infiltration leaves were cut into equal size discs with a cork borer and various stress treatments were given for 8 hr followed by histochemical GUS staining. Leaf discs were treated with water, dark, NaCl (200 mM), Cold (4ºC), polyethylene glycol (PEG) 20% and mannitol (300 mM). ≥ 4 discs were used per-treatment and technical replicates were done, representative replicate discs for each treatment are shown. The experiment was repeated three time and the same pattern were observed; (B) Schematic representation of promoter-*TopI*-GUS vector constructs. The main cis-regulatory elements are represented with different shapes colours.

these studies indicate a general stress inducible nature of this promoter in plants.

We showed, using primer extension analysis, that the transcript of *N. tabacum TopI* was initiated from A-202 bp from ATG which was also found to have a putative CAAT box in its vicinity, suggesting it to be an authentic start site. A minor transcript start site was observed at T 100 nucleotides from the translation start site. 5′-upstream region of the *top1β* in case of carrot showed major transcription start site by primer extension analysis 164 nt upstream the ATG translation start codon and presence of a canonical TATA-like box at position −35 bp (Balestrazzi et al., 2003).

Our gel retardation assay showed that the *N. tabacum TopI* promoter binds to proteins in the nuclear extract, suggesting a role in regulation of expression, but further experimentation is required to state the specificity and affinity of binding conclusively.

We used an approach similar that of Yang et al., (2000) to test the responsiveness of cis elements of the *TopI* promoter. We used a fusion of the promoter in a vector containing promoterless GUS to show that promoter responsiveness was in accordance with our previous transcript analysis.

Stable lines of the *TopI* promoter-GUS construct we have developed would be valuable as a routinely used tool in molecular biology for further functional characterization of the promoter in vivo. Various studies suggest that there is a significant role of TopI and other proteins involved in DNA/RNA metabolism in stress responses, though it needs further probing. The exact mechanism of the effect of stress on *TopI* is not yet clear. It maybe that, under physiological stress conditions, modulation of *TopI* expression levels is required to maintain desirable chromatin structure to carry out stress responsive gene expression in order to regulate vital cellular processes (Fig. 4).

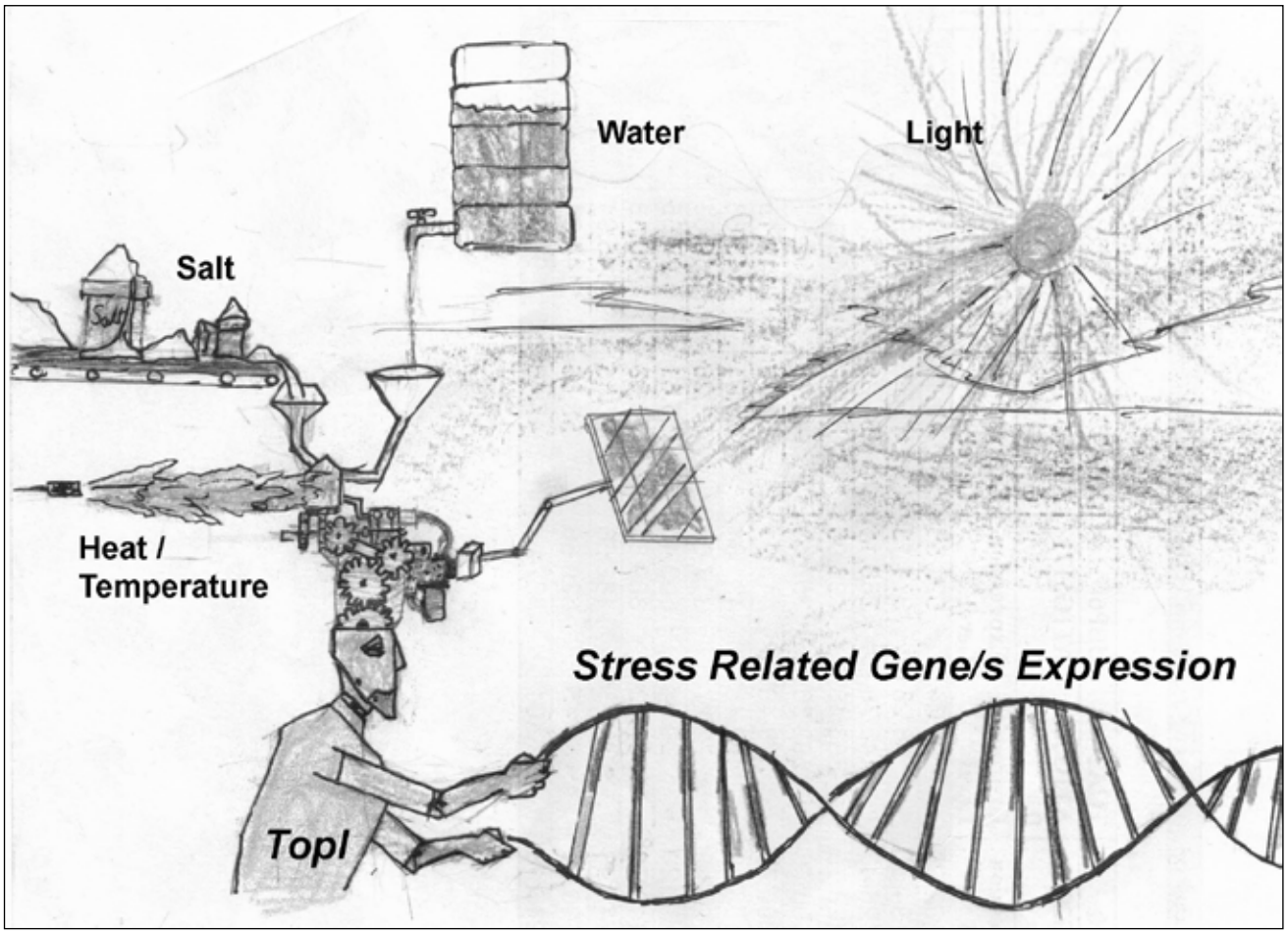

Figure 4: Pictoral representation of a model showing *TopI* function in stress responses. Stress signaling results in integration of signal at the TopI level. TopI perceives these stress-specific signatures and in turn switch on or switch off the expression of various stress responsive genes by regulating the topology of the DNA.

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