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CLONING AND FUNCTIONAL ANALYSES OF *NtPIN1a* PROMOTER UNDER VARIOUS ABIOTIC STRESSES IN *NICOTIANA TABACUM*

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Abstract

The plant-specific auxin efflux proteins PIN-FORMED (PIN) have been well depicted in many plant species for their essential roles in regulating the transport of auxins in several phases of plant growth. Little is known about the various functions of the PIN family genes in the *Nicotiana tabacum* (*N. tabacum*) species during plant growth. To define the expression pattern of the *NtPIN1a* gene under abiotic stresses and hormone treatment, transgenic tobacco with *promoter NtPIN1a::GUS* construct was employed. Comprehensive computational analyses of the *NtPIN1a* promoter confirmed the existence of common core promoter elements including CAAT-box, TATA-box, hormone, and abiotic stress-responsive elements such as ABRE, P-box, MYC, MYB, ARE, and GC-motifs. The transgenic plants with the promoter of *NtPIN1a* displayed a promising expression of β-glucuronidase (GUS) in germinating seeds, root tips, shoot-apex, and developing leaves under optimal conditions. While the differential expression of GUS in moderate salt, drought, low potassium stresses, and externally high auxin level at two different time points, suggested *NtPIN1a* played a key role in growth processes and the plants' response to abiotic stresses. This analysis provides a foundation for more in-depth discoveries of the biological functions of *NtPIN1a* in Nicotiana species and this promoter may be employed in genetic engineering of other crops for enhanced stress tolerance.

Key words: Tobacco; *Nicotiana tabacum*; PIN; Promoter; GUS; Abiotic stresses; Auxin.

Introduction

The differential distribution of phytohormone auxin contributes to plants' growth and development and their adaptation to ever-changing environments (Casanova-Sáez & Voß, 2019). Shoot-originated bulk flow of auxin was considered as a major contributor to changes in auxin concentration. Recent reports, however, advocates substantial contributions via short distance auxin transport under abiotic stresses (Korver *et al*., 2018). IAA levels progressively rose in both leaves and roots of inoculated saplings, and the time-course expression patterns of PodaAUX/IAA altered in tandem with IAA levels (Yao *et al*., 2018). The polar auxin transport (PAT) is facilitated via many known auxin transporter families, however, the polarly localized PIN-FORMED proteins finely corresponds to the direction of auxin movement that highlights *PINs* as key regulators of asymmetric distribution of auxin in plants (Zhou & Luo., 2018). The role of the *PINs* in transport of auxin was first described through the phenotypes of the pin-formed1 (*pin1*) mutants of Arabidopsis. The *pin1* mutants produced PIN like inflorescence due to defective PAT (Okada, 1991; Galweiler, 1998). Successive findings uncovered the PIN proteins as auxin efflux carriers (Zhou & Luo, 2018; Zwiewka *et al*., 2019). To date homologs of the *PIN* genes have been distinguished in several plant species, including monocots and eudicots. The PIN transcript has been analyzed for genome-wide and tissue-specific expression in rice (Wang *et al*., 2009), maize (Carraro *et al*., 2006; Forestan *et al*., 2012), Sorghum (Shen *et al*., 2010; Balzan *et al*., 2014), tomato (Pattison & Catalá, 2012), soybean (Wang *et al*., 2015) and Populus (Carraro *et al*., 2012). Extensive recent research has been carried out on Arabidopsis regarding the epigenetic regulation of the different *PIN* genes (Mateo-Bonmati *et al*., 2019). Analysis of the PIN transcripts in rice unveiled differential responses of the *OsPINs* to various phytohormones (Wang *et al*., 2009). Transcript analysis of the *OsPIN1b* in rice revealed that the gene is highly downregulated under abiotic stresses, and the phenotypic analysis in the *ospin1b* mutant revealed shorter seminal roots than wild type (Sun *et al*., 2018). The overexpressed *OsPIN3t* transgenic lines in rice showed drought tolerance and *OsPIN3t* promoter driven *GUS* activity, enhanced in auxin and polyethylene glycol (PEG) treatment (Zhang *et al*., 2012). The *PIN* genes (*SlPIN1* to *SlPIN10*) identified in tomato showed heterogeneous spatiotemporal expression with tissues and developmental stages specificity. The altered shoot architecture in RNAi-based co-silenced *SlPIN3* and *SlPIN4* suggesting that the genes play a critical role in architecting tomato plants (Pattison & Catalá, 2012). The expression of *PIN* genes in soybean (*Glycine max*) was differentially regulated by abiotic stresses and phytohormones, which triggered auxin redistribution to help soybean plants to acclimatize to the changing environment (Wang *et al*., 2015).

The promoter of a gene plays a crucial role as a major cis-acting factor in the regulation, expression, and transcription. Some important cis-regulating elements in the 5'UTR of *Brassica napus NHX1* gene, which is salt stress-responsive, were reported by Gharelo &

Bondehagh (2017). They are CAAT box, TATA box, TC-rich repeats and WUN motifs, which are involved in plants' response to abiotic stresses such as salinity, drought, mechanical injuries, and TMV-infection, etc. (Li *et al*., 2015), characterized the promoter of violaxanthin de-epoxidase (*CsVDE*) gene of *Cucumis sativus* L. in Arabidopsis, which protects photosynthetic apparatus from photo-damage. The *CsVDE* promoter activity was noted to be positively regulated by IAA and negatively by PEG and sodium chloride. Together with the light-responsive cis-elements (AE-box, Box-I, G-box, GAG-motif, GA-motif, GT-1 motifs, and Sp1) drought-inducible MBS binding site, TC-rich repeats (defense and stress-responsive), HSE (heat stress-related), GARE and P-box in gibberellin responsive-elements were reported on the promoter of *CsVDE* gene (Li *et al*., 2015).

N. tabacum is an important cash crop and is widely used as a good model plant (Cai *et al*., 2016). Recently 20 *NtPIN* genes were identified by genome-wide analysis in *N. tabacum*. Higher expression in shoots and stems and CRISPR/Cas9-based *NtPIN4* mutation indicated that *NtPIN4* was involved in auxin-mediated branching of tobacco plants (Xie *et al*., 2017). Higher expression of *PIN* genes under drought stress played a critical role in lateral root formation of tobacco by mediating downward transport of auxin from shoots to roots (Wang *et al*., 2018). Among the *PIN* genes, *AtPIN1* is the central mediator in developing plant tissues via polar auxin transport. This is why several factors that are assumed to be involved in auxin distribution are often tested in the regulation of *AtPIN1* expression (Omelyanchuk *et al*., 2016). Compared with other family members, lack of *AtPIN1* activity results in most severe phenotypes suggesting a crucial role of this protein in auxin transport (Okada, 1991; Friml *et al*., 2003; Blilou *et al*., 2005). The current study aimed to validate and unveil the expression patterns of *NtPIN1a* in the *N. tabacum* under abiotic stresses through analysis of its promoters' activity.

Materials and Methods

Identification of NtPIN1a and isolation of its promoter in *N. tabacum*: The putative PIN proteins of *N. tabacum* were identified by blasting the protein sequence of the AtPIN1 (AT1G73590.1) gene of Arabidopsis in the tobacco genome database of China (http://218.28.140.17/tools/blast/blast.php?action=run). Twenty *NtPIN* related genes were found and based on the bioinformatics analysis *NtPIN1a* gene (Gene ID: Ntab0448440.1) was selected for the study.

Afterwards, all the protein sequences obtained were further examined by SMART (http://smart.embl-heidelberg.de/) to verify the existence of domains. By searching the tobacco genome dataset with PF03547 of the Pfam Hidden Markov Model (HMM) model, top hits for putative PIN proteins were retained by high score and low E-value (score≥0 and E value≤0.1) (Fig. 2).

To validate the promoter region from Ntab_scaffold_2554 upstream *NtPIN1a* gene, that whether the *NtPIN1a* promoter sequence was present in the *N. tabacum* genome, sequence-specific primers (forward 5'-TAAATCGGTTAATCATCACGC-3', reverse 5'-GGCTAGTTTCTTGAAATACCC-3') were designed based on the DNA sequences of two ancestral diploid species (Fig. 1), The promoter (proNtPIN1a) was then amplified from *N. tabacum* cv. HonghuaDaJinYuan (HD), using ExTaq DNA polymerase enzyme with promoter-specific primers designed at different positions on the sense strands. The PCR was performed at 95°C for 5 min, followed by 30 cycles of 95°C for 30 sec, 56°C for 30 sec, 72°C for 2 min, and with a final cycle at 72°C for 10 min using 100 ng genomic DNA of *N. tabacum*. The PCR product was detected by 1% Agarose gel electrophoresis. After purification by kit, the amplified fragment of the promoter was ligated in the pMD19-T vector.

For the histochemical promoter-GUS constructs, about 3 kb upstream sequences of the selected *PIN* gene, were obtained from the China tobacco database. The product was transformed into *Escherichia coli* DH5α. The positive recombinant clones were verified by PCR and sequencing. To construct the expression vector for *Agrobacterium tumefaciens*, the *NtPIN1a* promoter segment in pMD19-T vector, was re-amplified with infusion primers containing restriction sites (HindIII and BamHI). The amplified fragment was then sub-cloned in pBI121 (treated with the same restriction sites) vector, replacing the *CaMV35S* promoter upstream of the *GUS* reporter, to get the pNtPIN1a::GUS constructs.

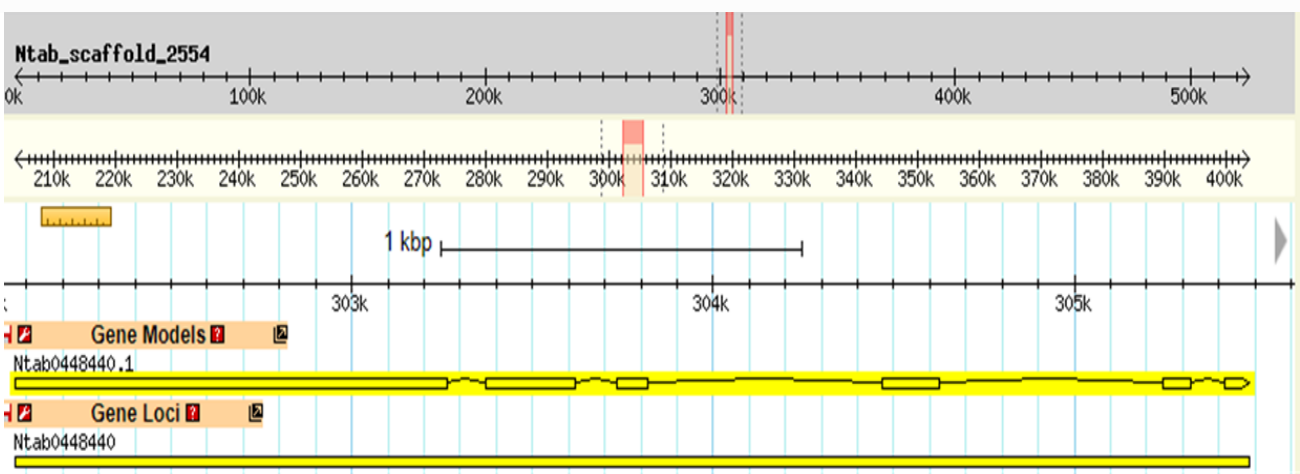

Fig. 1. Genome annotation of the *NtPIN1a* promoter region.

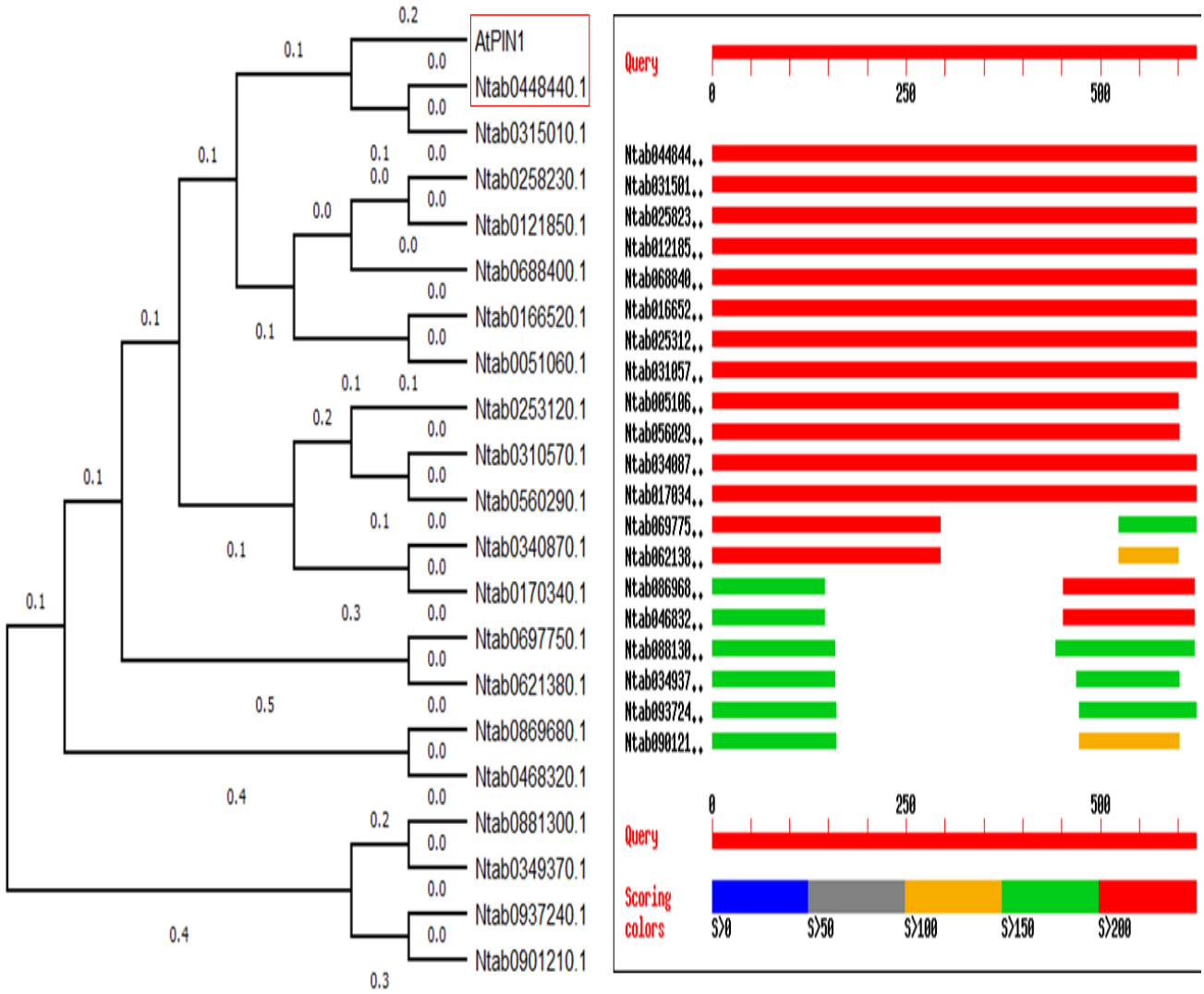

Fig. 2. Evolutionary analysis of *NtPIN1a* genes; phylogenetic tree of the query protein AtPIN1, blast in tobacco database. The red bar represents the NtPINs aligned sequences similar to that of AtPIN1.

Phylogenetic analysis

The 20 NtPINs protein sequences from *N. tabacum* were taken to design the phylogenetic tree by MEGA 8.0, using the maximum likely hood method with 1000 bootstrap replications (Kumar *et al*., 2018) (Fig. 2).

Plant materials and growth conditions: Seeds of *N. tabacum* cv. Honghua Da Jin Yuan, provided by the Tobacco Research Institute, CAAS, were used as source material for promoters cloning and as study materials for tobacco experiment. Agrobacterium mediation leaf disc method was used to generate proNtPIN1a::GUS transgenic line, by transforming pBI101-proNtPIN1a: GUS vector into tobacco (Horsch *et al*., 1985). The T2 generation seeds were screened on kanamycin added Murashige and Skoog (1/2MS) media plates. Two weeks later 10 independent transgenic lines were selected for the experiment, in which two homozygous lines (Z1-8 and Z2-6) were selected as representative. Seeds of the selected T2 lines were sown on vermiculite and kept in a growth chamber with temperatures set at 25/18^{o}C air temperature and day length and night length set at 16/8 h respectively. Two weeks later, identically vigorous seedlings were transferred to one-quarter strength Hoagland solution (HS) and after two days the transgenic tobacco seedlings were exposed to 50 mM NaCl, 100 µM potassium, 2.5% PEG-6000 (m/v) and 10 µM IAA in 1/4HS for simulating moderate salt, low potassium, drought stress, and hormonal treatment respectively. Samples were taken 24 and 48 h after treatments. The control seedlings were also grown in 1/4HS without any abiotic stress factors.

GUS-Histochemical assay: To demonstrate organ-specific patterns of the NtPIN1a promoter in N. tabacum histochemical GUS staining was performed for seedlings with 5-bromo-4-chloro-3-indoyl glucuronide (X-Gluc) as a substrate (Cervera, 2005). Samples were stained at 37^{o}C for 4 h and then was put into 100% ethanol until chlorophyll content was removed. For rehydration to make the tissues soft, samples were put into water for 15 min and then photographed with a microscope (LEICA S8APO).

Real-time quantitative PCR (RT-qPCR): To verify the organ-specific pattern of the NtPIN1a gene, RT-qPCR analysis was performed. The primers used are; forward primer 5'-TTCAAGAAGGTCGCAGGGA-3', reverse primer 5'- CACCAGCAACCATTGAGTAAAA-3'. The expression of the tobacco actin NtL25 gene was used as an internal positive control (Schmidt *et al*., 2010). Primers used for the actin gene are; forward 5'- CAAAAGTT ACATTCCACCG-3', reverse 5'-TTTCTTCGTCCCAT CAGGC-3'. Primer gene-specificity was examined by PCR and agarose gel analysis. The sixteen days old seedlings were treated and the total RNA was extracted by hot phenol method (Dong *et al*., 2015) from the whole root and second pair of leaves of three seedlings after 24 and 48 h. For RT-qPCR, using HiScript® III-RT SuperMix (+gDNA wiper) (Vazyme), cDNA was synthesized from 1 µg total RNA. Using ChamQTM SYBR ® qPCR Master Mix (Vazyme), the PCRs were performed on Light cycler 96 (Roche). The amplification reactions were carried out at a total volume of 20 µL, containing 10 µL 2×ChamQ SYBR qPCR Master Mix, 7.2 µL ddH_2O, 0.8 µL reverse and forward primers (10 µM), and 2 µL cDNA (10 times diluted after synthesis). PCR was executed as follows: 95°C for 1 min, then 40 cycles of 95°C for 10 s and 60°C for 30 min. Three biological repeats were taken for quantification.

Results

Bioinformatics analysis of NtPIN1a gene: To identify NtPIN1a protein in *N. tabacum*, the AtPIN1 (AT1G73590.1) protein sequence was taken from the genome database (arabidopsis.org) and was blasted in China tobacco database (http://218.28.140.17/tools/blast/blast.php?action=run). Twenty putative PIN proteins in *N. tabacum* were identified from the database.

To validate the promoter region from Ntab_scaffold_2554 upstream *NtPIN1a* gene, that whether the *NtPIN1a* promoter sequence was present in the *N. tabacum* genome, primers were designed on the *N. tabacum* genome sequence (Fig. 1) and the fragment was obtained via polymerase chain reaction (PCR). After cloning into pMD19-T cloning Vector (TransGen Biotech Inc., Beijing, China), was sequenced.

Evolutionary analysis of NtPIN1a: In the study *PINs* were searched in the completely sequenced *N. tabacum* genomes using a hidden Markov model profile of the PIN domain (Pfam database: accession no. PF03547). Among 20 *PINs*, identified in the *N. tabacum* genome, 2 *PINs* (Ntab0448440.1 and Ntab0315010.1) were found similar to the *AtPIN1*, suggesting common ancestor of the genes (Fig. 2).

The phylogenetic analysis suggested that, the putative NtPIN1a protein was likely to act as an auxin transporter and based on the sequence similarity was selected for further analysis.

In silico analysis of NtPIN1a promoter: The 1980 bps NtPIN1a promoter cloned was analyzed for cis-elements using online databases PlantCARE (Lescot *et al*., 2002) and Plantpan2 (Chow *et al*., 2015). The predicted cis-elements distributed on NtPIN1a promoter including core promoter elements (TATA-box), enhancer element (CAAT-box), hormones responsive elements (ABRE and P-box), photoreactive elements (AE-box, Box4, G-box GT1-motif, I-box, and TCT-motif), tissue-specific (AAGAA-motif and RY-element), defense and stress-responsive elements (ARE, GC-motif, MYC, MYB, WUN-motif, and TC-rich repeats). In addition, the conserved TF_motif_seq_0254 at the position 2985 is predicted, which describes transcription factor (TF) RAP2.11, regulate high-affinity K^+ uptake transporter AtHAK5 and other components of the low-potassium signal transduction pathway, in response to low potassium. The RAP2.11 was identified by activation tagging of Arabidopsis lines containing a luciferase marker powered by the promoter AtHAK5 that is normally induced only by low potassium (Kim *et al*., 2012). The motif, enriched in the upregulated genes under low potassium, was also contained in the NtPIN1a promoter. This can be hypothesized that the motif in NtPIN1a might also be responsible for K^+ responsive TFs.

Similarly, in the promoters of auxin-regulated genes the TF_motif_seq_0439 at the position 2605 bps (Fig. 3) was reported as auxin response elements (AuxRE), depending on a specific domain of the proteins either activate or repress transcription of the genes (Li *et al*., 2016).

Also, the conserved motif TFmatrixID_0058 codon at position 1070 bps are responsible for the homeobox-leucine zipper protein (HAT5/HB-1), which were identified previously responded to salt stress (Valdés *et al*., 2012), revealed that Arabidopsis HD-Zip I group members; AtHB7 and AtHB12 participate in drought and salinity stress responses through ABA sensing and transduction.

In addition, MYC has been reported as a drought response element (Roy, 2016) and cold-inducible (Ryu *et al*., 2009), while ABRE is salt responsive and can bind to ABA-dependent transcription factors (Park *et al*., 2012). The analysis indicated that the transcription of NtPIN1a might be regulated by various external signals such as light, hormone, and abiotic stresses, which hinted that *NtPIN1a* might contribute in tobacco plants growth and development (Fig. 4) (Table 1).

In short, the in silico Analysis of *NtPIN1a* Promoter was further confirmed by experiments.

Expression analysis of NtPIN1a promoter in response to abiotic stresses and auxin via GUS histochemical assay: To observe the NtPIN1a gene expression in different organs of the N. tabacum species under normal, abiotic stresses and externally applied auxin (IAA), promoter of the gene was cloned and histochemical detection of promotorNtPIN1a::GUS was carried out. The amplicon was cloned in the pMD19-T vector successfully and was validated by the enzyme digestion method and by sequencing. Then was sub-cloned in the expression vector (pBI121), replacing CaMV35S upstream promoter of the reporter gene beta-glucuronidase (GUS).

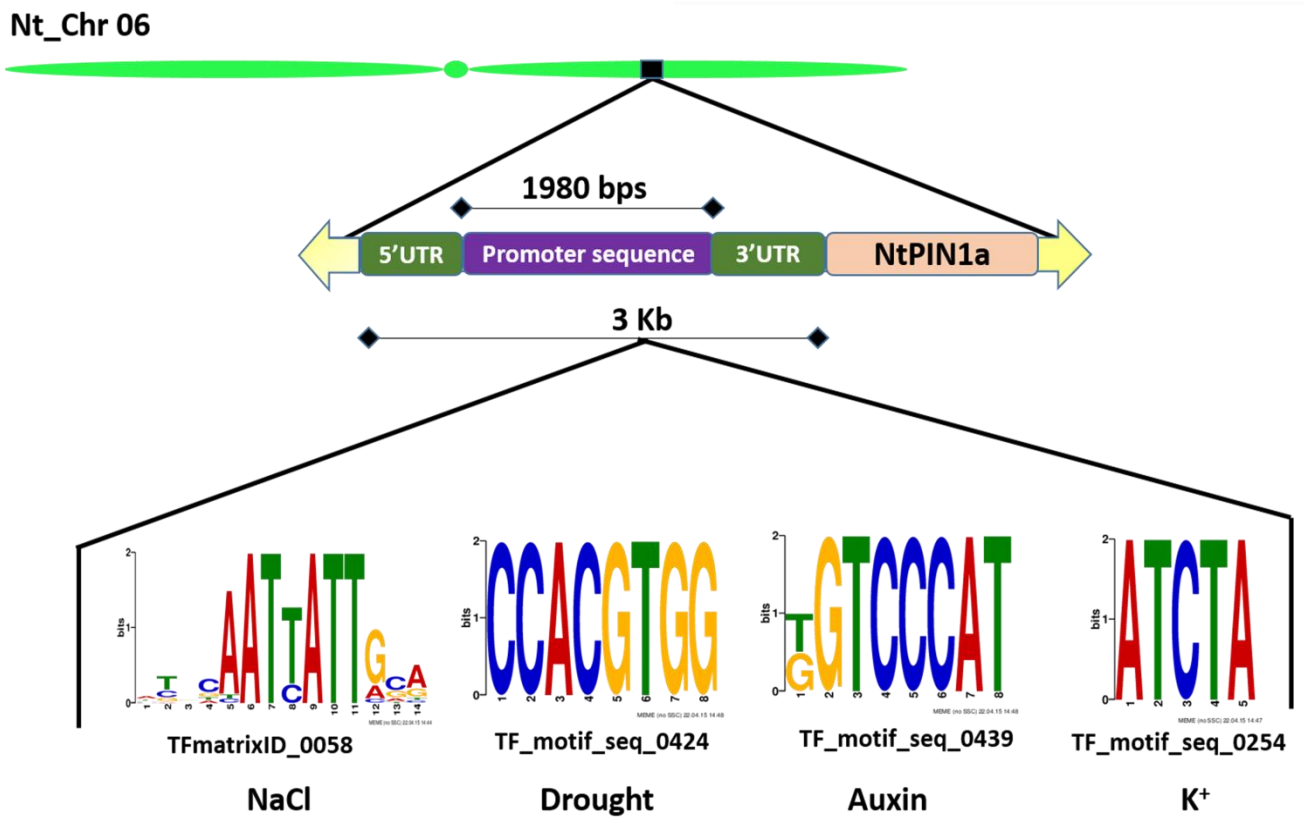

Fig. 3. Conserved cis-elements in the *NtPIN1a* promoter. Motif positions are indicated relative to the start codon. (For further details see Table 1).

Table 1. Descriptions of some key cis-acting elements of the *NtPIN1a* promoter.

Element type	Site name	Repeats	Core sequence	Function
Core element	TATA-box	37	TATA/ATTATA ccTATAAAaa	core promoter element around -30 of transcription start
Enhancer element	CAAT-box	23	CAAAT/CAAT/CCAAT	common *cis*-acting element in promoter and enhancer regions
Hormone response element	ABRE	2	CGTACGTGCA/ACGTG	abscisic acid responsiveness cis-elements
	TF_motif_seq_0439	1	GTCCCAT	Auxin responsive element
	P-box	1	CCTTTTG	gibberellin-responsive element
	AE-box	1	AGAAACAA	part of a module for light response
Light response element	Box 4	3	ATTAAT	part of a conserved DNA module involved in light responsiveness
	G-Box	1	TACGTG	*cis*-acting regulatory element involved in light responsiveness
	GT1-motif	5	GGTTAAT/ GGTTAA	light responsive element
	I-box	1	GTATAAGGCC	light responsive element
	TCT-motif	1	TCTTAC	part of a light-responsive element
Tissue specific	AAGAA-motif	2	GAAAGAA	Seed specific
	RY-element	1	CATGCATG	Seed specific
Defense and stress response element	ARE	2	AAACCA	Essential for the anaerobic induction
	GC-motif	1	CCCCCG	Anoxic specific inducibility
	MYC	4	CATTTG/CATGTG	Stress responsive element
	MYB	5	CAACCA/TAACCA/ CAACAG	Abiotic stress responsive
	TF_motif_seq_0254	1	ATCTA	K^+ stress responsive
	TFmatrixID_0058	1	AATTATT	Salt and drought stress responsive element
	TF_motif_seq_0424	1	CCACGTGG	Drought responsible element
	WUN-motif	2	TAATTACTC/AAATTACTA	Wound stress
	TC-rich repeats	1	GTTTTCTTAC	Defense and stress responsiveness element

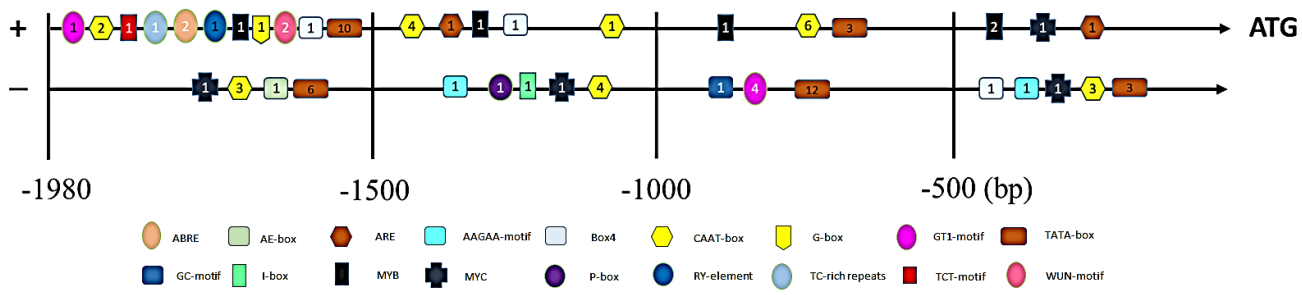

Fig. 4. Schematic distribution of cis-acting elements of the *NtPIN1a* promoter of the *N. tabacum*. Shapes and colors portrayed various cis-acting elements of the promoter. The characters in the graph indicate the numbers of predicted elements. "+" and "-" represent the sense and antisense strand, respectively.

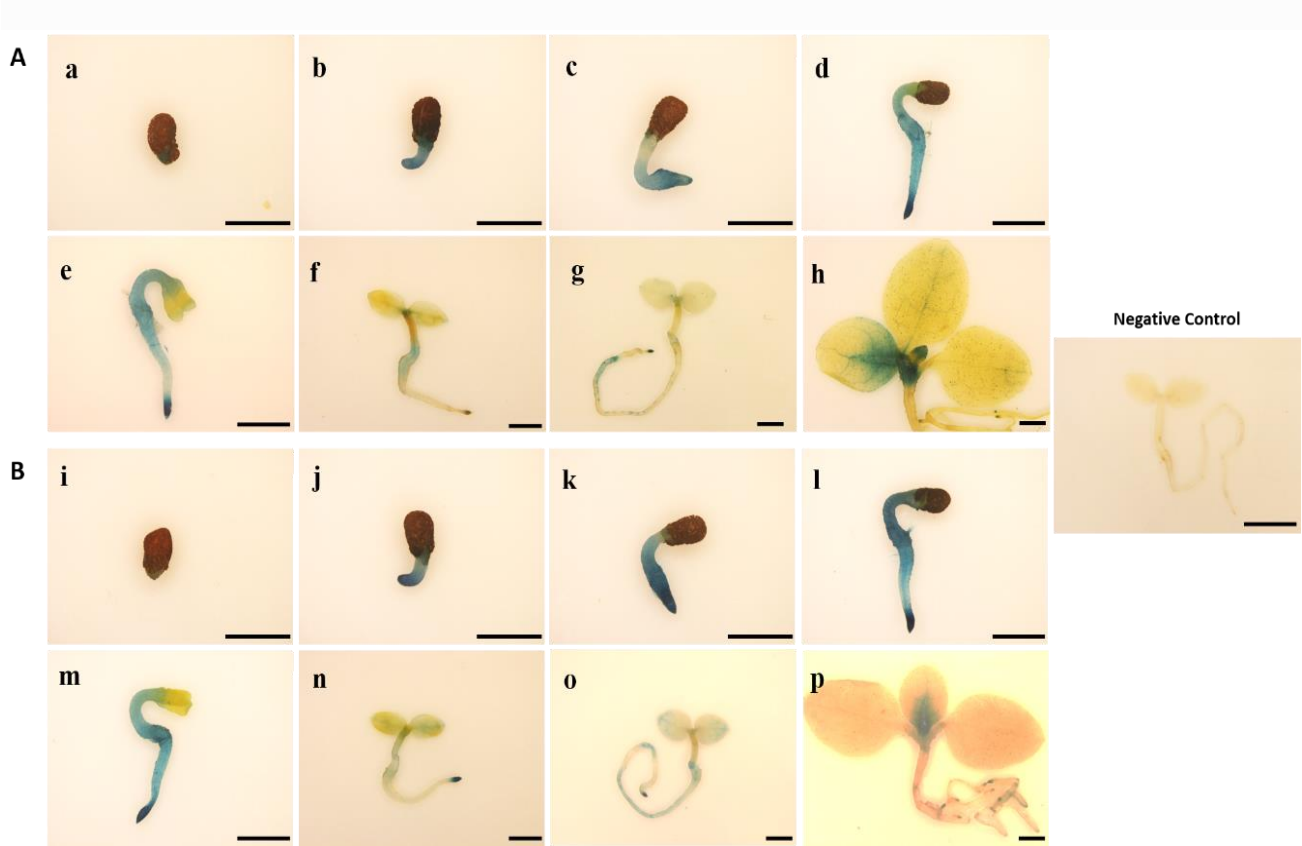

Fig. 5. The *NtPIN1a* promoter drives the *GUS* expression under the normal condition from germination to the seedling stage (n = 6 seedlings). (A) Z1-8 and (B) Z2-6 are two independent lines of the *pNtPIN1a::GUS* construct. The stages include (a,i) micropylar endosperm rupture (b,j) radicle elongation (c,k) and hypocotyl elongation during 3~3.5 days after sowing (DAS) (d,l), cotyledon emergence at 3.5~5 DAS (e,m), cotyledon expansion during 5~6 DAS (f,n) cotyledon maturation during 6~8 DAS (g,o), emergence of the first two leaves at 10 DAS (h,p) and expansion of the first two leaves at 14 DAS. The wild type of tobacco was used as a negative control. Scale bar is 1 mm using ImageJ.

The *NtPIN1a* gene promoter efficiently mediated *GUS* expression in the lines studied under normal conditions. The expression was different at various developmental stages. During germination, the expression of GUS is visibly higher, while the expression was observed at shoot apex and root. As the seedlings grew, the visibility of the GUS expression was decreased while interestingly enhanced at the seedling stage having three and four leaves. However, the expression pattern was more visible in newly developed leaves, in the leaves' vascular system, and root. To compare the lines of *NtPIN1a*, in this study, Z2-6 (Fig. 5B) had more visible expression than Z1-8 (Fig. 5A). The visibly higher expression of GUS with the promoter of *NtPIN1a* as driver showed the importance of the gene in developing tissues via distribution of auxin.

For the abiotic stresses and auxin treatment, samples for the GUS expression analysis were taken after 24 hours (h) (Fig. 6, T1) and 48 h (Fig. 6, T2) of the treatments. The abiotic stresses effectively induced the promoter activities in two lines of *NtPIN1a*. The *GUS* signal was induced in salt-stressed seedlings at both time points, however, the signal was stronger in roots compared to other tissues. As for seedlings under low potassium regime, PEG and IAA, there was no visible difference between control and treatments at a first-time point, and the GUS expressed only in root tips, apical meristem, and newly developing leaves. However, after 48 h of the

treatment with low potassium and IAA, the GUS expression was enhanced in seedlings, especially in lateral roots, stem, and developing leaves. While in PEG stressed seedlings, the promoter activity was elevated visibly in the apical meristem and developing leaves compared with control. In addition, the *GUS* signal became weaker in salt-induced stress after 48 h of the treatment. Overall, the stronger expression pattern of *NtPIN1a*, especially under moderate salt and low potassium stress, showed its importance in the development of tobacco plants. However, due to its large gene family, distinct organ-specific patterns and versatile mode of regulation under internal and external cues make it very challenging to unveil the precise roles of a certain *NtPIN* gene in the tobacco plants development.

Expression pattern of NtPIN1a under abiotic stresses and IAA treatment as validated by RT-qPCR: To accurately define the NtPIN1a expression pattern in different organs under abiotic stresses and exogenous application of IAA, RT-qPCR was performed (Fig. 7). The NtPIN1a gene expression was exceedingly higher in leaf under NaCl treatment for 24 h compared with control. While the external application of auxin (IAA), low potassium stress (K^+), and PEG had no significant effect on NtPIN1a gene expression in leaves comparing that with control. Likewise, for 24 h treated root samples, the expression of the gene was exceedingly higher in NaCl treatment, however, the expression was extremely decreased in PEG treated root sample comparing with control (Fig. 7A). In 48 h treated leaf samples, the gene expression had no significant differences. However, in 48 h treated root samples, the gene expression was highly induced by treatments compared to control (Fig. 7B). The NtPIN1a gene expression results corresponded well with the organ-specific expression pattern analysis of the gene (Fig. 6).

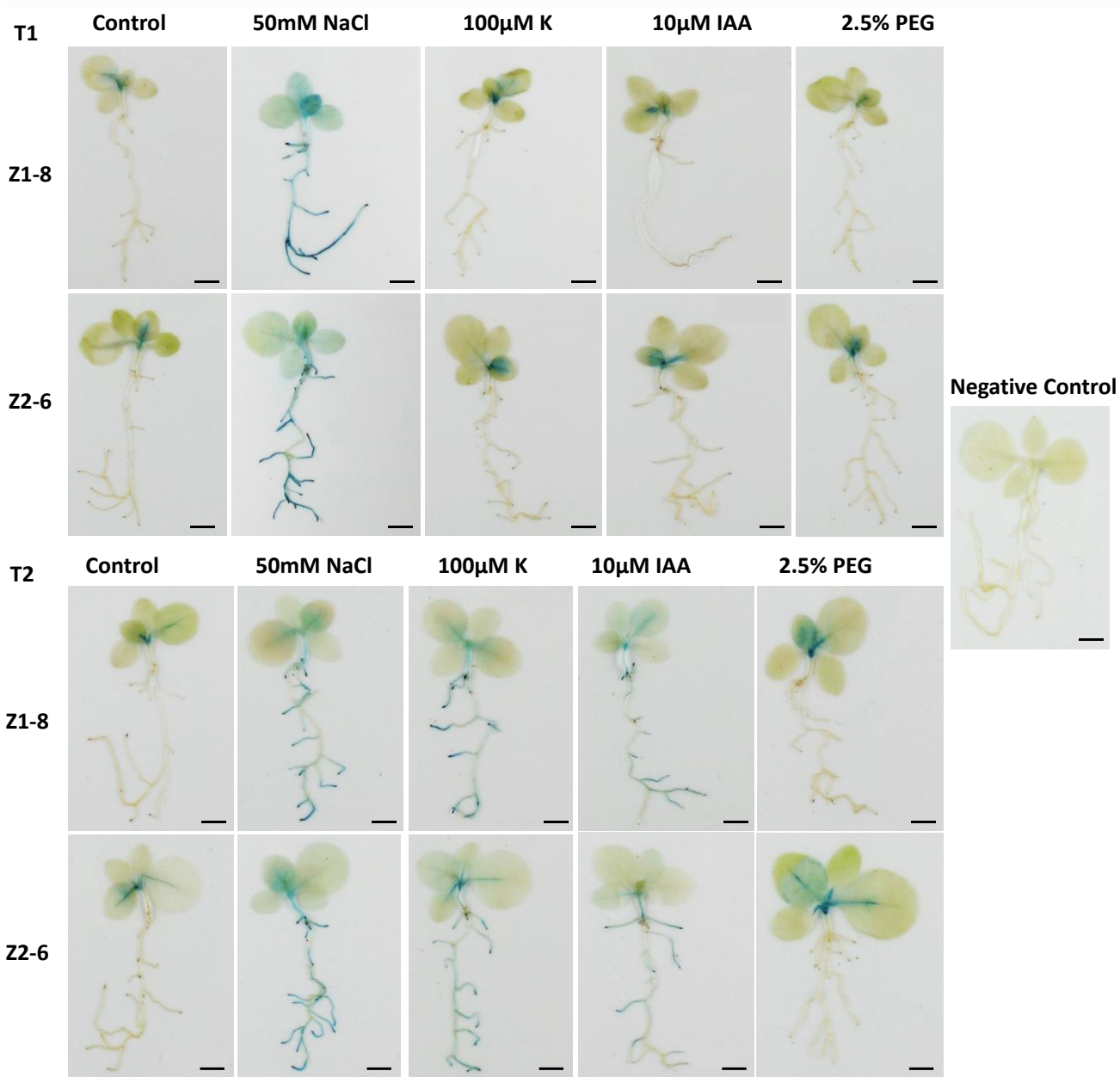

Fig. 6. The *NtPIN1a* promoter drives the *GUS* expression under various stress conditions. (T1) samples were taken after 24 h and (T2) 48 h of stress. Z1-8 and Z2-6 are the two lines of the promoter. The negative control was wild type tobacco. Scale bar is 0.5 cm.

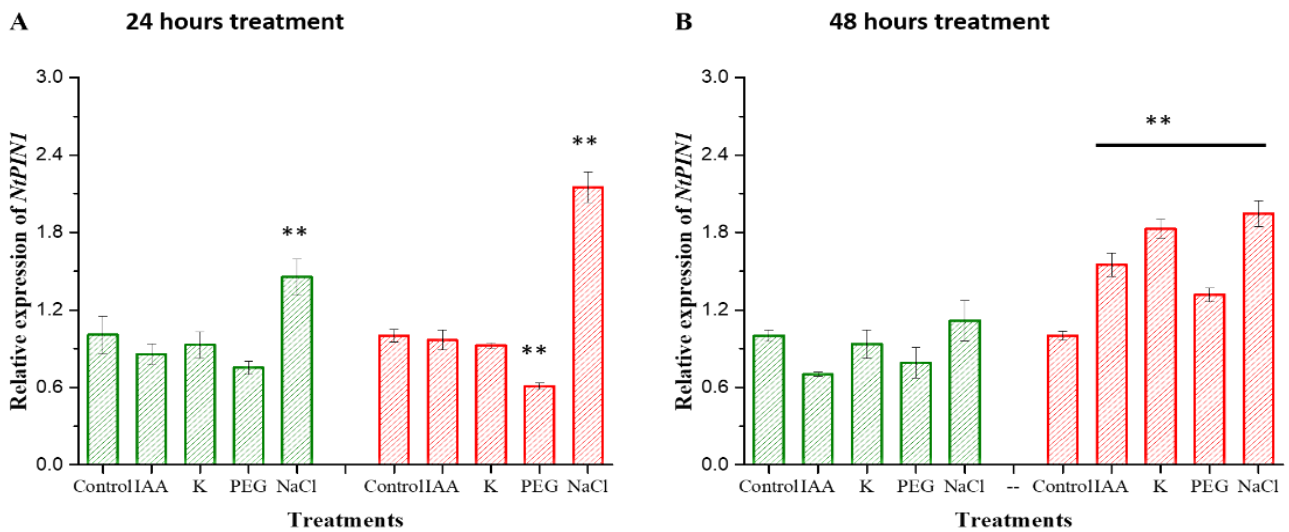

Fig. 7. Expression analysis of the *NtPIN1a* gene in leaves (green color bars) and roots (red color bars) by real-time quantitative PCR (RT-qPCR) analysis. (A) Expression analysis of the gene after 24 h and (B) 48 h treatment. The tested sample was composed of the first pair of leaves and root (n = 6 seedlings). Data were shown as means ± SD (three biological repeats) using the Duncan test, $p<0.01$.

Discussion

Though there are many approaches to enhance tolerance in plants to stresses. The transgenic method has recently acquired significant consideration as an efficient way of boosting tolerance in plants to stresses (Bhatnagar-Mathur *et al*., 2008). It was documented in previous experiments that transgenic plants harboring promoter constructs displayed distinct expression of GUS under various stressful conditions, unveiling its essential function in tolerance to abiotic stresses by enhancing the gene expression (Niu *et al*., 2018). To generate transgenic lines/cultivars via genetic engineering, the availability of a potential gene or transgene source as well as an efficient promoter is a prerequisite for achieving a desirable trait or significant degree of activity in the host plant.

The current study was started to clone the *NtPIN1a* promoter of tobacco and study their organ-specific expression pattern in tobacco under normal and stressful conditions. The up-regulation of the *NtPIN1a* during seed germination and developing seedlings showed the significance of the gene in tobacco growth and development. The promoter, the upstream sequences of the gene, can control the transcription initiation and the RNA polymerase activation to bind precisely to the template (Hernandez-Garcia & Finer, 2014). Our results confirmed that abiotic stresses (low potassium, osmotic, and salt stress) and exogenous application of auxin enhanced the GUS expression, driven by the *NtPIN1a* promoter cloned, compared with control. The transgenic tobacco harboring *NtPIN1a* promoter:GUS, are assumed to enhance the binding of abiotic stress-related TFs and auxin response factors in response to an external stimulus, which boost *NtPIN1a* promoter activity that induces the expression of *GUS* gene (Niu *et al*., 2018).

Our results are well supported by the bioinformatics analysis on the *NtPIN1a* promoter sequence, revealing stress response elements such as CAAT box, TATA box, ABRE, MYB, MYC, P-box, RY-element as described in (Table 1). This indicated that the Ntab_scaffold_2554 upstream *NtPIN1a* gene consisted of various cis-regulatory elements played a major role in the plant response to abiotic stresses, such as auxin, salinity, drought and K^+ (Kim *et al*., 2012; Park *et al*., 2012; Valdés *et al*., 2012). Thus we further presume that TF_motif_seq_0254 (ATCTA) had corresponded to TF RAP2.2 of family AP2; ERF, whereas RAP2.2 was identified through the activation tagging of Arabidopsis lines that contained a luciferase marker driven by the *AtHAK5* promoter that is normally only induced by low potassium (Kim *et al*., 2012). Likewise, the AuxR (TF_motif_seq_0439) with GTCCCAT codon, predicted to be AuxRE (Auxin responsive element) of pea (P.s.) PS-IAA4/5 gene. The relevant genes, IAA1 to IAA14 are homologs of PS-IAA4/5 and PS-IAA6 from pea. Characterizing the IAA expression in seedlings of etiolated pea reveals specificity for auxin inducibility (Abel *et al*., 1995). In particular, ARF proteins interaction and structural studies have provided novel insights into the molecular basis of auxin-regulated transcription. The findings provide the basis for predicting *ARF* genes' contributions to other plant biology (Li *et al*., 2016). In the current study, treatment with auxin might cause the activation of ARFs and their binding with AuxRE in the promoter might induce the pNtPIN1::GUS expression. In addition TF_motif_seq_0424 with sequence CCACGTGG (Table 1) represents ABA-dependent water-stress responsive elements (ABRE) identified in maize (rab28). ABRE is a class of cis-elements, present in the promoters of many genes, which respond to ABA-dependent TFs, and regulate the genes under salinity, drought, dehydration, and low-temperature stress (Guiltinan *et al*., 1990; Narusaka *et al*., 2003). Besides, studies have demonstrated that TC-rich repeats and WUN-motif are present on the promoters of salt and drought stress-responsive genes (Chen *et al*., 2005; Qin *et al*., 2012; Valifard *et al*., 2015). Transgenic plants were tested for stress tolerance and resulted in sustained growth under polyethylene glycol (PEG)-mediated dehydration compared to wild-type controls (Amara *et al*., 2013).

In our study, the stronger expression pattern of *NtPIN1a*, especially under moderate drought, salt, low potassium, and external application of auxin show its importance in the tobacco plant development under abiotic stresses, suggesting that the promoter may be employed in engineering other crops with enhanced tolerance to stresses.

Conclusions

Computational analysis using PlantCARE and Plantpan2 databases, abiotic stresses response cis-elements, defense responsive, hormones response cis-acting elements, light-responsive, seed-specific, and common core promoter elements involved in the development of the plant were confirmed in the *N. tabacum NtPIN1a* promoter cloned. The differential mode of the *NtPIN1a* promoter in *N. tabacum* under abiotic stresses and externally applied auxin revealed several specific functions and roles of the gene at definite environmental conditions thereby regulating the auxin redistribution enabling the tobacco plants to adapt to abiotic stresses in the ever-changing environment. Since 20 *PIN* genes have been identified in *N. tabacum*, these genes might be functionally redundant. Identification of the functions of a specific member gene of the PIN family can be a very big challenge. Further in-depth functional characterization of the *NtPIN1a* gene by creating a single or group of duplication gene mutations may significantly enhance our understanding of their biological roles in plants' response to abiotic stresses and aid our efforts in developing more stress-resistant plants. Therefore, we presumed that there might exist unknown motifs in *NtPIN1a* promoter contributing to stress-specific expression in *N. tabacum*, which forecasting a layout for future studies.

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