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The Transcription Factor *Sl*NAC6 from *Suaeda liaotungensis* Positively Regulates Stress Tolerance in Transgenic *Arabidopsis thaliana*

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Abstract

NAC transcription factors (TFs) constitute a major plant-specific regulatory family that coordinates diverse biological processes, including organ growth, developmental progression, and responses to unfavorable environmental conditions. In this study, we isolated a *NAC* gene from *Suaeda liaotungensis* whose expression was induced by salt treatment and named it *SlNAC6*. The coding region of *SlNAC6* is 1,059 bp in length and encodes a protein comprising 353 amino acids. Subcellular localization analysis based on fluorescent signals revealed that *Sl*NAC6 is distributed in both the nucleus and cytoplasm. Yeast one-hybrid assays further confirmed that *Sl*NAC6 functions as a transcriptional activator, with its activation capacity primarily attributable to the C-terminal region. Expression profiling further showed that *Sl*NAC6 is constitutively transcribed in the roots, stems, and leaves of *S. liaotungensis*, although its transcript level is highest in the leaves. In addition to salt, drought, cold, and abscisic acid treatment markedly enhanced *Sl*NAC6 expression. To characterize the biological function of *SlNAC6*, two independent *Arabidopsis* lines constitutively expressing this gene were established. When exposed to salt or drought stress, the *SlNAC6*-overexpressing lines displayed greater stress resistance and survival rate than wild-type (WT) plants and pBI121-GUS transgenic controls (VT). These phenotypic improvements were accompanied by elevated proline (Pro) levels, higher maximum quantum efficiency of photosystem II photochemistry (Fv/Fm), and increased activities of peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT). By contrast, relative electrical conductivity and malondialdehyde (MDA) accumulation were significantly reduced. Ectopic expression of *SlNAC6* also enhanced the transcription of several stress-associated genes. These observations demonstrate that *SlNAC6* expression improves *Arabidopsis* tolerance to salinity and drought, potentially by promoting reactive oxygen species (ROS) detoxification and limiting membrane lipid peroxidation. Thus, *SlNAC6* may serve as a valuable genetic resource for improving plant resilience to adverse environmental conditions.

Keywords: *Suaeda liaotungensis*; NAC transcription factor; Abiotic stress; Transgenic *Arabidopsis*

1. Introduction

Progressive deterioration of the natural environment has intensified plant exposure to

unfavorable conditions such as salinity, water deficit, and temperature extremes, with substantial consequences for plant growth, development, and productivity (He et al., 2018). Abiotic stress is therefore a major constraint on agriculture because it reduces both worldwide food production and the amount of land suitable for cultivation (Kumari et al., 2022). The Food and Agriculture Organization of the United Nations (FAO) estimated that environmental stresses, including abiotic factors, resulted in economic losses of approximately USD 96 billion from 2005 to 2015, with Asia accounting for nearly half of these losses (USD 48 billion) (Sarić et al., 2020). Clarifying the molecular and physiological mechanisms that govern plant adaptation to salt stress is consequently essential for breeding crops with improved salinity tolerance. Plants have developed multiple regulatory strategies that enable them to survive under adverse environmental conditions. Their reaction to high salt levels involves a multi-gene mechanism of adaptive changes in their morphology, physiology, biochemistry, and molecular level (Mansour and Hassan, 2022). Such adaptation is controlled by interconnected signal pathways involving the recognition of the stress signal (Zhu, 2002; Sánchez-Barrena et al., 2005), signal transduction, and gene expression regulation (Singh and Laxmi, 2015; Pérez-Clemente et al., 2013). There is a growing amount of evidence suggesting that TFs play an important role in regulating these processes by affecting gene transcription downstream from them and inducing biochemical and developmental reactions improving plant stress resistance (Hu and Xiong, 2014).

The TFs of the NAC (NAM, ATAF1/2, and CUC2) family constitute an array of plant-specific regulators involved in various biological functions from growth and development to metabolism and environmental stress responses (Xiong et al., 2025). The NAC proteins have been found in plants ranging from the model species *Arabidopsis thaliana* to the agriculturally significant crops rice (*Oryza sativa*) and wheat (*Triticum aestivum*). In most plant genomes, this family includes more than 100 members (Ooka et al., 2003; Guérin et al., 2019). Accumulating evidence indicates that NAC TFs exert multifaceted regulatory effects during plant responses to both abiotic and biotic challenges. These effects are mediated through several processes, including osmotic homeostasis, ROS detoxification, phytohormone signaling, transcriptional control of stress-associated genes, and coordination with other regulatory proteins (Ma et al., 2026). For instance, the A*rabidopsis* NAC protein AtNAP may repress the expression of *AREB1*, resulting in reduced transcription of downstream stress-related genes, including *RD20* and *RD29B*, and consequently diminished salt tolerance (Seok et al., 2017). In rice, *OsNAC3* is transcriptionally activated by SS and promotes abscisic acid (ABA)-mediated responses and salinity resistance by modulating stress-associated gene expression and maintaining Na^+ balance in shoots (Zhang et al., 2021). Similarly, *TaNAC29* improves salt resistance by enhancing antioxidant capacity, limiting H_2O_2 accumulation and membrane injury, and modulating signaling pathways associated with abiotic stress (Xu et al., 2015). The halophyte *Tamarix hispida* provides another representative example. Wang et al. (2014) found that 21 *ThNAC* genes were upregulated in the roots, stems, and leaves of *T. hispida* after exposure to high salinity, water deficit, heavy metals, and ABA. These expression patterns imply that *ThNAC* genes contribute to environmental stress adaptation and are strongly linked to ABA-dependent signaling networks.

Suaeda liaotungensis is an annual halophyte with exceptional salt tolerance that naturally inhabits coastal saline-alkaline soils, making it an appropriate model for examining plant stress-

adaptation mechanisms. Saline-alkaline conditions constitute a major environmental limitation and can severely impair plant growth and survival. Accordingly, the discovery of resistance-related genes from halophytic plants may offer useful genetic materials for enhancing crop resilience through molecular breeding or genetic engineering. Nevertheless, functional information is currently available for only a limited number of NAC genes from *S. liaotungensis* (Li et al., 2014; Yang et al., 2014; Wang et al., 2021; Wu et al., 2018; Du et al., 2022; Liu et al., 2023). Additional *SlNAC* members therefore need to be characterized to clarify their distinct functions and possible functional redundancy in plant stress resistance.

From the leaves of *S. liaotungensis*, we identified and cloned a previously undescribed NAC TF gene, which was designated *SlNAC6*. Examination of its predicted amino acid sequence indicated that *Sl*NAC6 contains the characteristic structural features of NAC proteins: a conserved DNA-binding module located near the N terminus and a transcriptional regulatory region positioned toward the C terminus. These sequence properties support its classification within the NAC transcription factor family. Subcellular distribution experiments demonstrated that the SlNAC6 protein is localized in both nuclear and cytoplasm, while transcriptional activation assays performed in yeast confirmed that it possesses transactivation capacity. Constitutive expression of *SlNAC6* in *Arabidopsis thaliana* produced transgenic lines that were markedly more resistant to salinity and drought stress than WT plants. To investigate the mechanisms underlying this improved stress performance, quantitative real-time PCR (qRT-PCR) was subsequently used to measure changes in the transcript abundance of genes associated with abiotic stress signaling and defense. Taken together, these results broaden the current understanding of NAC-mediated stress regulation in *S. liaotungensis* and indicate that *SlNAC6* represents a potentially useful genetic resource for breeding crops with greater resilience under unfavorable environmental conditions.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The green leaves and seeds of wild-type *S. liaotungensis* were collected from the saline-alkali land of Yingchengzi, Dalian City, Liaoning Province, China. In preparation for RNA extraction and *SlNAC6* cloning, the collected leaf samples were rapidly frozen in liquid nitrogen and kept at -80°C. The *SlNAC6* expression patterns were analyzed and plants were cultivated using the seeds.

Phenotypic and genetic studies were conducted on *Arabidopsis thaliana*, with a focus on the Columbia ecotype. Plants were cultured in a controlled setting that maintained a constant temperature of 22°C, a light intensity of 250 μmol $m^{-2}s^{-1}$, a relative humidity of 60%, and a photoperiod of 16 hours of light and 8 hours of dark. Solid Murashige and Skoog (MS) medium was used to sow *Arabidopsis* seeds after they had been stratified at 4℃ for 3 days. The seedlings were placed in soil-filled containers and returned to the incubator after one week of growing in a controlled environment.

2.2. Cloning and Sequence Analysis of the *SlNAC6* Gene

Utilizing RNAiso Plus (TaKaRa), the leaves of *S. liaotungensis* were subjected to total RNA extraction. The PrimeScript™ RT Reagent Kit (TaKaRa) was used to synthesize cDNA (complementary DNA). Utilizing first-strand cDNA as the template and primers NAC6-EF and NAC6-ER, which were designed according to the expressed sequence tag of *SlNAC6*, a partial sequence of *SlNAC6* was amplified by polymerase chain reaction (PCR). With the 3′-Full RACE Core Set Ver. 2.0 (TaKaRa) as a starting point, we were able to construct the primers NAC6-GSP0 and NAC6-GSP1 from the 3′-end of the partial *SlNAC6* sequence. Finally, the pEASY-T1 vector (TransGen Biotech, Beijing, China) was used to clone the full-length *SlNAC6* sequence with primers NAC6-FL-F and NAC6-FL-R. A complete list of the primers is provided in Supplementary Table S1. Theoretical calculations for the molecular weight, isoelectric point, and grand average hydropathicity of the *Sl*NAC6 protein were performed using the ExPASy ProtParam tool. To find *Sl*NAC6 homologous sequences, we performed BLAST searches against the NCBI database. We performed multiple sequence alignment using ClustalW in MEGA 5.0 and identified conserved domains of *Sl*NAC6 with GeneDoc. A neighbor-joining phylogenetic tree was constructed using iTOL (https://itol.embl.de).

2.3. Analysis of the *SlNAC6* Expression Profile

Soil was used to germinate *S. liaotungensis* seeds for 7 days before they were moved to a greenhouse where they were grown at 25°C with a 12-hour light/12-hour dark photoperiod and 50%-60% relative humidity. At the 6-8 true leaves growth stage, the plants were subjected to stress treatments. The seedings were placed in Hoagland nutritional solution with 200 mM NaCl, 10% PEG 6000, 0.1 mM ABA and 4°C. At 0, 0.5, 1, 3, 6, 12, and 24 hours post-treatment, we took leaf samples. We collected the roots, stems, and leaves of untreated *S. liaotungensis* plants for tissue-specific expression analysis, and as a control, we used seedlings cultivated in normal Hoagland nutritional solution. The samples were promptly placed in liquid nitrogen and kept at -80°C until RNA extraction.

We followed the procedure outlined by TaKaRa (Dalian, China) to extract total RNA from *S. liaotungensis* samples. PrimeScript® RT Reagent Kit with gDNA Eraser from TaKaRa was used to create first-strand cDNA. SYBR® Premix Ex Taq™ (Perfect Real Time) was used in a qRT-PCR on a Thermal Cycler Dice Real Time System TP800 (TaKaRa). The primers used were *Sl*NAC6-qF and *Sl*NAC6-qR. The primers *Sl*Actin-qF and *Sl*Actin-qR were used to amplify the *Slβ-actin* gene, which served as the reference gene. The $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to determine the relative abundance of *SlNAC6*. Supplementary Table S1 contains a complete inventory of the primers utilized in this study.

2.4. Subcellular Localization Analysis of *Sl*NAC6

Using the pEGAD-GFP vector (BioVector, China), a fusion construct was created to investigate the subcellular localization of *Sl*NAC6 in plant cells. The complete coding sequence of *SlNAC6*, excluding the stop codon, was inserted into the pEGAD-GFP vector using the *Sma*I and *Xba*I restriction sites to generated the 35S:*Sl*NAC6-GFP. The 35S:*Sl*NAC6-GFP and the control 35S:GFP vectors were introduced into onion epidermal cells using a Bio-Rad PDS-1000/He™ biolistic particle delivery system. Using confocal laser-scanning microscopy (LSM 700; Zeiss, Germany), the GFP fluorescence was observed after 16 hours of incubation.

2.5. Analysis of *Sl*NAC6 Transcriptional Activation Activity

The transcriptional activation activity was examined in yeast cells by fusing the N-terminal (N) and C-terminal (C) domains of *SlNAC6*, in addition to the entire coding sequence, into the pGBKT7 vector. The recombinant plasmids pGBKT7-*Sl*NAC6, pGBKT7-*Sl*NAC6-N, and pGBKT7-*Sl*NAC6-C, along with the empty pGBKT7 vector, were introduced separately into yeast strain AH109 (*Saccharomyces cerevisiae*). The modified yeast cells were cultured at 30°C for three days after being plated on SD/-Trp and SD/-Trp-His-Ade medium. The yeast growth and a β-galactosidase filter-lift assay were used to assess the transcriptional activation activity of each protein, following the procedures detailed in the Yeast Protocols Handbook (Clontech, USA).

2.6. Generation of Transgenic *Arabidopsis* Plants Overexpressing *SlNAC6*

To create *Arabidopsis* plants overexpressing *SlNAC6*, the pBI121-GUS plant expression vector driven by the CaMV 35S promoter was used. The open reading frame of *SlNAC6* was inserted into the pBI121-GUS vector. *Arabidopsis thaliana* (Columbia ecotype) was transformed using the floral-dip method from positive *Agrobacterium tumefaciens* colonies. Competent *Agrobacterium tumefacien*s GV3101 cells were transfected with the recombinant pBI121-*SlNAC6* vector and the control vector, pBI121-GUS. The homozygous transgenic *Arabidopsis* lines were screened on medium containing 100 μg/mL kanamycin and confirmed by genome DNA PCR. In this study, qRT-PCR was used to assess the *SlNAC6* expression levels in two T3 homozygous overexpression lines.

2.7. Analysis of Salt and Drought Tolerance in Transgenic *Arabidopsis*

To test for salt and drought tolerance, seeds from pBI121-*Sl*NAC6 transgenic *Arabidopsis* lines L1 and L2, along with those from the empty-vector transgenic line (VT) and wild-type (WT) *Arabidopsis*, were used.

Seeds from WT, VT, L1, L2 plants were germinated on MS medium. The medium was either without supplementation (control) or supplemented with 400 mM D-mannitol or 200 mM NaCl. Thirty seeds were sown for each line. Every 24 hours, under both normal and stress conditions, we counted the number of germinated seeds using radicle emergence as the germination criterion.

Seeds from the WT, VT, L1, L2 were sown on MS solid medium containing 100 mM NaCl or 200 mM D-mannitol. The plates were placed in a growth chamber that was set at 22°C, had a 16-hour light/8-hour dark photoperiod, and 60% relative humidity. After a week, we used ImageJ to quantify the length of the primary roots (Mishra et al., 2021).

After four true leaves had developed, the *Arabidopsis* seedlings were moved to soil and maintained in a controlled environment identical to those used for seed germination. After 7 days, we applied salt stress by watering the plants four times with 200 mM NaCl solution. The treatment for drought was withholding water for 21 days, followed by 5 days of rewatering. Survival rates were then determined.

2.8. Determination of Stress-Related Physiological Parameters

At two weeks of age, WT, VT, L1, and L2 *Arabidopsis* seedlings were either irrigated four times with 200 mM NaCl every seven days, or subjected to drought salt by withholding water for twenty-one days, then watering them again for five days. Fluorescence imaging of chlorophyll was used to quantify the maximal quantum efficiency of photosystem II photochemistry (Fv/Fm) in both untreated and stress-treated plants (Imaging-PAM; Walz GmbH, Germany).

Leaves of each line was collected when leaves were fully expanded after salt or drought treatment. The relative electrical conductivity was measured using a DDS-11A conductivity meter manufactured by Shanghai Pengshun Technology Co., Ltd. in China. The procedures according to Wu et al. (2018) were used to determine the levels of MDA and Pro, as well as the activities of SOD, POD, and CAT.

2.9. Expression Analysis of Abiotic Stress-Responsive Genes

The leaves of both transgenic and WT *Arabidopsis* plants were used to extract total RNA. PrimeScript™ RT Reagent Kit from TaKaRa was used to synthesize first-strand cDNA. Using primers that were specific to each gene and the generated cDNA, qRT-PCR was carried out. For this study, the reference gene was the *Arabidopsis* Actin2 gene (*AtActin2*, NM_112764). The $2^{-\Delta\Delta CT}$ method was used to determine the relative transcript levels of genes that respond to abiotic stress. Supplementary Table S1 contains a complete inventory of the primer sequences utilized in this research.

2.10. Statistical Analysis

All statistical analyses were conducted using SPSS 18.0. Comparisons among experimental groups were performed with Duncan's multiple range test, and differences were regarded as significant at $P < 0.05$. Three biological replicates were used for gene expression assays. Survival, seed germination, and root growth experiments included 30 samples per group, whereas physiological measurements were based on five samples per group.

Results

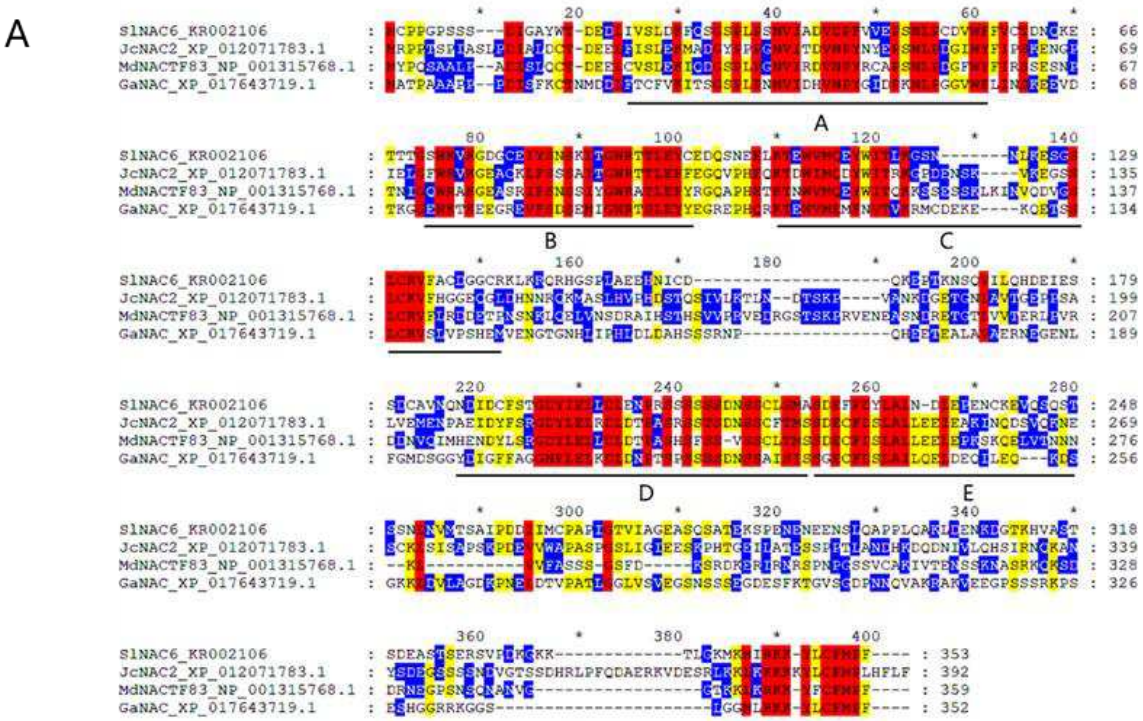
3.1. *SlNAC6* Encodes a Protein Containing a Conserved NAC Domain

A NAC TF gene was cloned from *S. liaotungensis* using PCR and rapid amplification of cDNA ends (RACE), using an expressed sequence tag that was retrieved from the transcriptome database. The gene, which was submitted in GenBank with the accession number KR002106, was designated *SlNAC6*. A polypeptide consisting of 353 amino acids is encoded by its full-length cDNA, which spans 1,301 bp and contains an open reading frame of 1,059 bp. A putative isoelectric point (pI) of 4.46 and a molecular mass of 39.033 kDa were predicted for the *Sl*NAC6 protein. It has been determined that *Sl*NAC6 is hydrophilic, since the grand average of hydropathicity (GRAVY) was -0.631.

We used GeneDoc to analyze the *Sl*NAC6 amino acid sequence and see how it compared to NAC proteins from other plant species. Figure 1A shows that the N-terminal region of *Sl*NAC6 contains a highly conserved NAC domain with five distinct subdomains. The evolutionary

position of *Sl*NAC6 was determined by a phylogenetic analysis of *Sl*NAC6 and other homologous NAC proteins using MEGA 5.0. *SlNAC6* shared the greatest sequence similarity with *Mn*NAC78 from *Morus notabilis* and *Pt*NAC from *Populus trichocarpa*. It was also phylogenetically close to ANAC063 and ANAC064, which belong to the NAC family of *Arabidopsis thaliana*. In contrast, *Sl*NAC6 was not grouped with the previously identified NAC proteins from *S. liaotungensis*. According to the classification proposed by Ooka et al. (2003), these results assigned *Sl*NAC6 to the ANAC063 subfamily within Group II (Figure 1B).

A



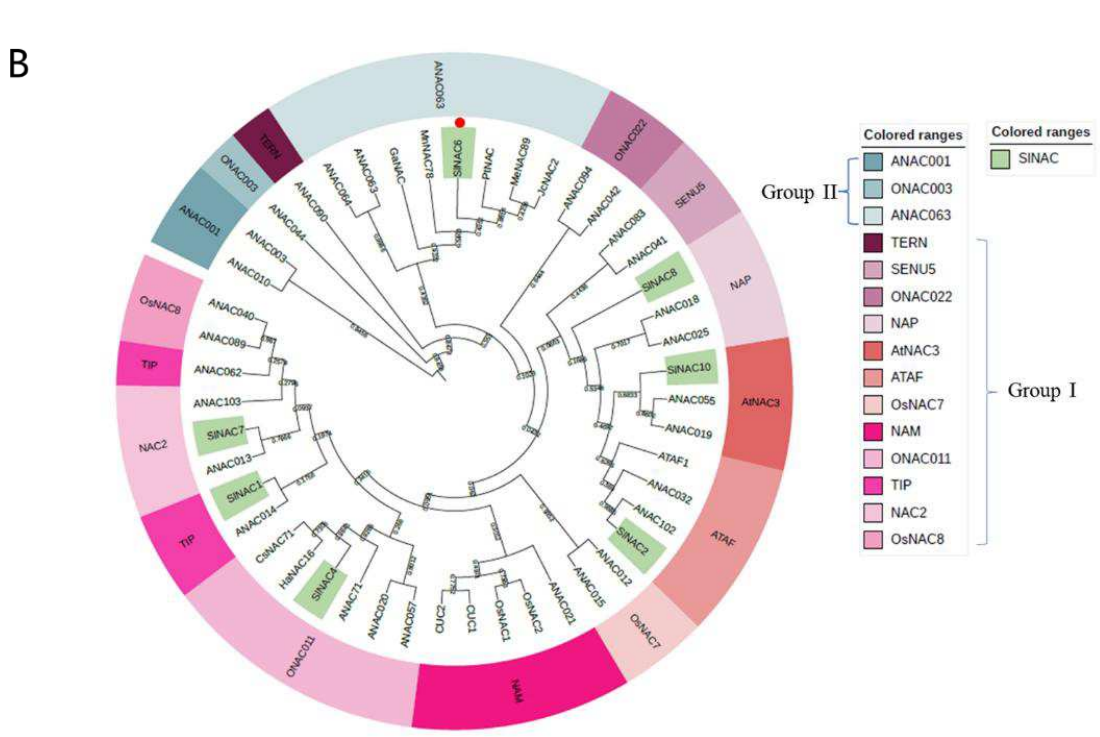


FIGURE 1. Sequence comparison and phylogenetic relationships of *Sl*NAC6 and homologous NAC proteins. (A) Alignment of the *Sl*NAC6 amino acid sequence with NAC proteins from different plant species. Amino acid residues shared by all sequences are highlighted, and the five conserved NAC subdomains are marked with lines and labeled A-E. (B) Neighbor-joining phylogenetic tree showing the relationship between *Sl*NAC6 and other plant NAC proteins. *Sl*NAC6 is marked with a red dot. Sequences were aligned using ClustalW, and phylogenetic reconstruction was carried out in MEGA 5.0 with 1,000 bootstrap replications.

3.2. Abiotic Stresses and ABA Induce *SlNAC6* Expression

The expression pattern of *SlNAC6* was quantitatively evaluated using qRT-PCR in different tissues of *S. liaotungensis* and under different environmental stresses including abscisic acid treatment. *SlNAC6* gene expression was detected in the root, stem, and leaf tissues, and it was highest in leaves and lowest in the root tissues (Figure 2A).

Analysis of *SlNAC6* expression following NaCl treatment showed that the gene responded rapidly to salt stress. Its expression was induced within 0.5 h and reached a maximum at 6 h after treatment (Figure 2B). Under drought stress, the relative expression level of *SlNAC6* initially increased and subsequently decreased, reaching its maximum at 1 h (Figure 2C). Under cold stress, *SlNAC6* expression increased continuously and significantly, peaking at 6 h at approximately threefold higher than that at 0 h (Figure 2D).

To determine whether *SlNAC6* responds to hormonal signals, *S. liaotungensis* seedlings were treated with 0.1 mM ABA, and *SlNAC6* expression was assessed at different time points. *SlNAC6* expression was induced at 1 and 6 h after treatment, with a more than threefold increase at 6 h compared with that at 0 h (Figure 2E). The responsiveness of *SlNAC6* to salt, drought,

cold, and ABA treatments suggests that it is involved in abiotic stress responses and may participate in ABA-mediated signal transduction.

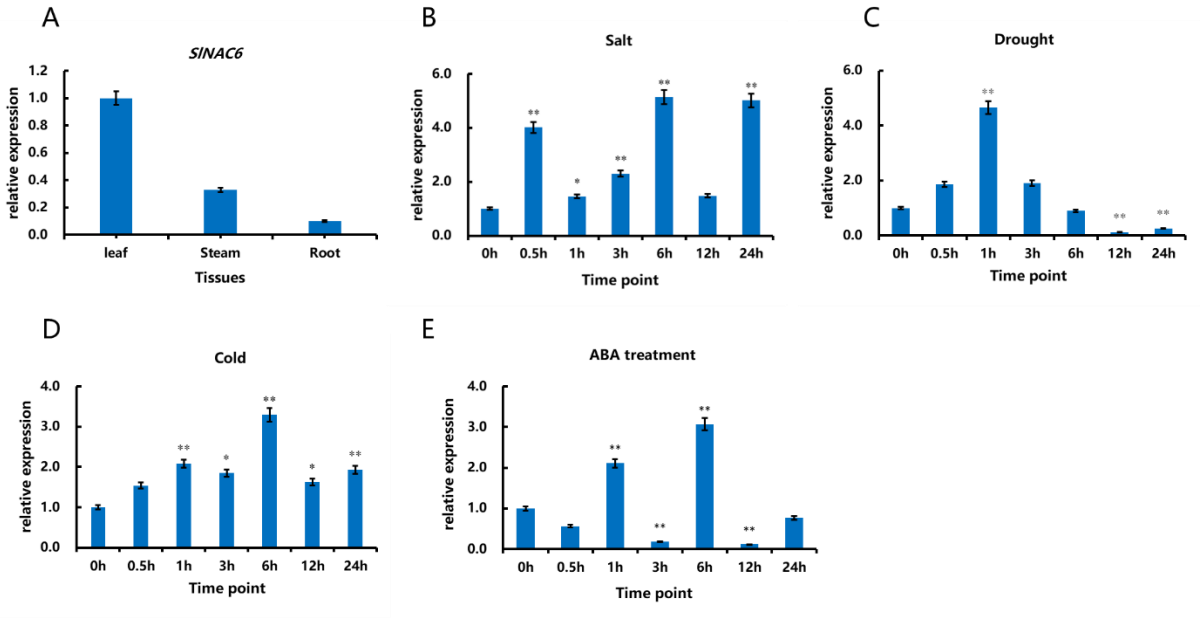


FIGURE 2. Tissue-specific and stress-responsive expression profiles of *SlNAC6* in *S. liaotungensis*. (A) Relative transcript abundance of *SlNAC6* in roots, stems, and leaves under unstressed conditions. (B-E) Temporal changes in *SlNAC6* expression in seedling leaves following exposure to salt stress (200 mM NaCl) (B), drought stress (DS; 200 mM D-mannitol) (C), cold stress (4 °C) (D), or abscisic acid treatment (0.1 mM ABA) (E). Transcript levels were normalized to the corresponding value at 0 h.

3.3. *Sl*NAC6 Is Localized in Both the Nucleus and Cytoplasm

To study the subcellular localization of *Sl*NAC6, a GFP fusion vector was generated. The coding sequence of *Sl*NAC6 (without the stop codon) was fused in-frame with GFP under the control of the CaMV 35S promoter, generating the 35S:*Sl*NAC6-GFP construct. This plasmid, together with the empty 35S vector control, was transiently introduced into onion epidermal cells. Confocal microscopy revealed the presence of the GFP expressed by the control construct throughout the nucleus and cytoplasm (Figure 3). The GFP fusion with *Sl*NAC6 was localized in a similar way; the fusion protein produced the fluorescence signal both in the nucleus and in the cytoplasm. Colocalization with DAPI stained nuclei confirmed the nuclear localization of *Sl*NAC6. Therefore, *Sl*NAC6 is localized in the nucleus and cytoplasm.

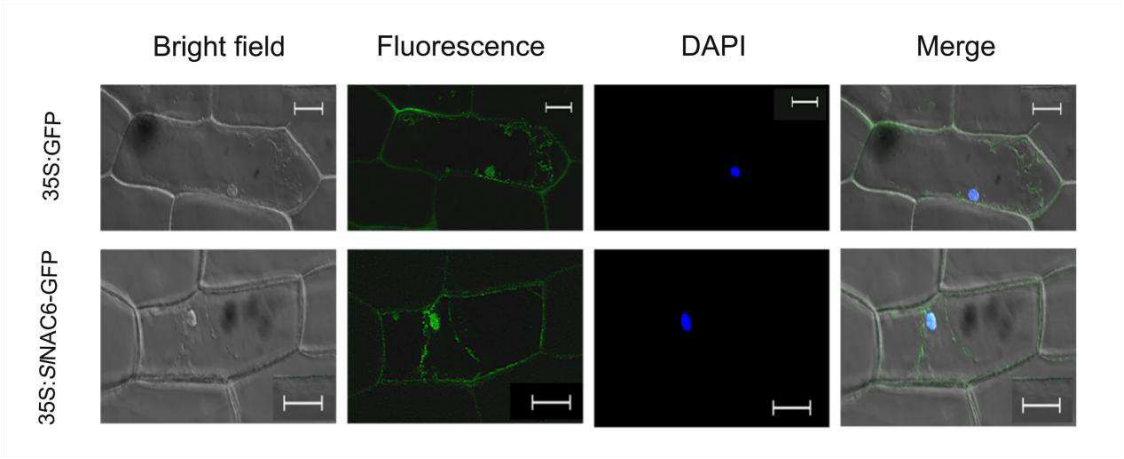


FIGURE 3. Intracellular localization of *Sl*NAC6. Onion epidermal cells were transiently transformed with either the 35S control plasmid or the 35S fusion construct and subsequently examined by confocal microscopy. Bright field, transmitted-light image; GFP, green fluorescence signal; DAPI, fluorescence from nuclear staining; Merge, overlay of the bright field, GFP, and DAPI channels.

3.4. *Sl*NAC6 Possesses a C-Terminal Transcriptional Activation Domain

Figure 4A shows the results of cloning DNA fragments encoding the full *Sl*NAC6 protein, its N-terminal region, or its C-terminal region into the pGBKT7 vector. These constructs were used to determine the protein's transcriptional activation function. The AH109 yeast strain was transformed with the recombinant plasmids and the empty pGBKT7 control. Figure 4B shows that the transformants with each design were successfully transformed when grown on SD/-Trp medium. Yeast expressing either full-length *Sl*NAC6 or its C-terminal region were the only ones that showed growth on the more restrictive SD/-Trp/-His/-Ade medium. These two transformants also showed detectable β-galactosidase activity. In contrast, the highly selective medium did not support the survival of yeast harboring either the N-terminal region or the empty vector (Figure 4B). Overall, these findings suggest that *Sl*NAC6 can activate transcription and pinpoint the functional activation domain to its C-terminal region.

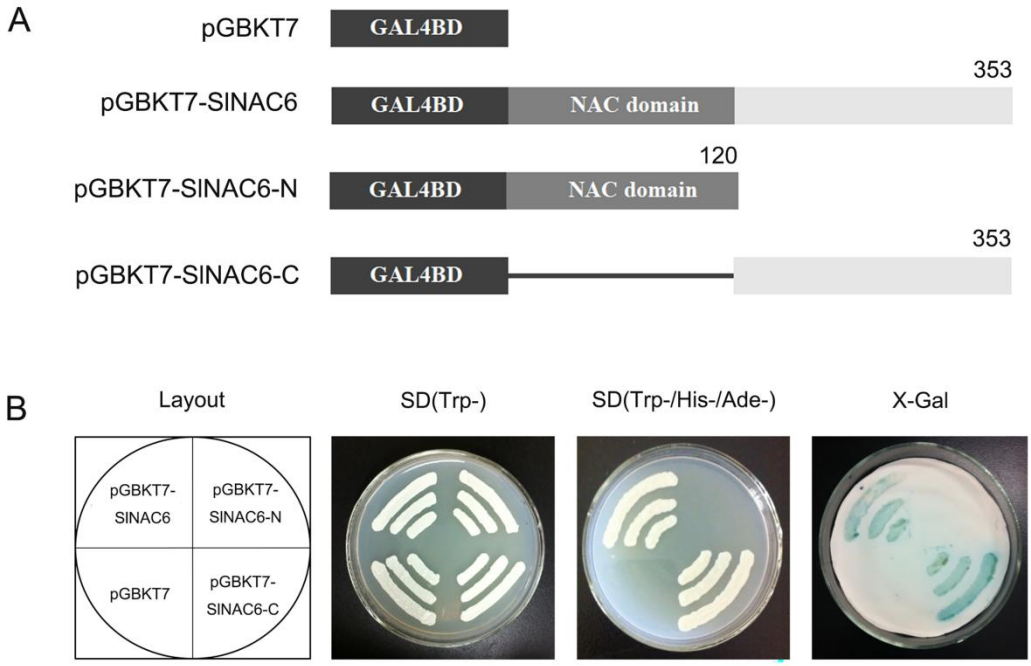


FIGURE 4. Yeast-based assessment of *Sl*NAC6 transcriptional activation activity. (A) Schematic representation of full-length and truncated *Sl*NAC6 fragments fused to the GAL4 DNA-binding domain of pGBKT7. Amino acid coordinates are indicated. (B) Growth phenotypes of yeast transformants expressing the indicated constructs on selective media. The empty pGBKT7 plasmid served as the negative control, and *β*-galactosidase activity was detected using X-Gal.

3.5. Overexpression of *SlNAC6* Enhances Salt and Drought Tolerance in Transgenic *Arabidopsis*

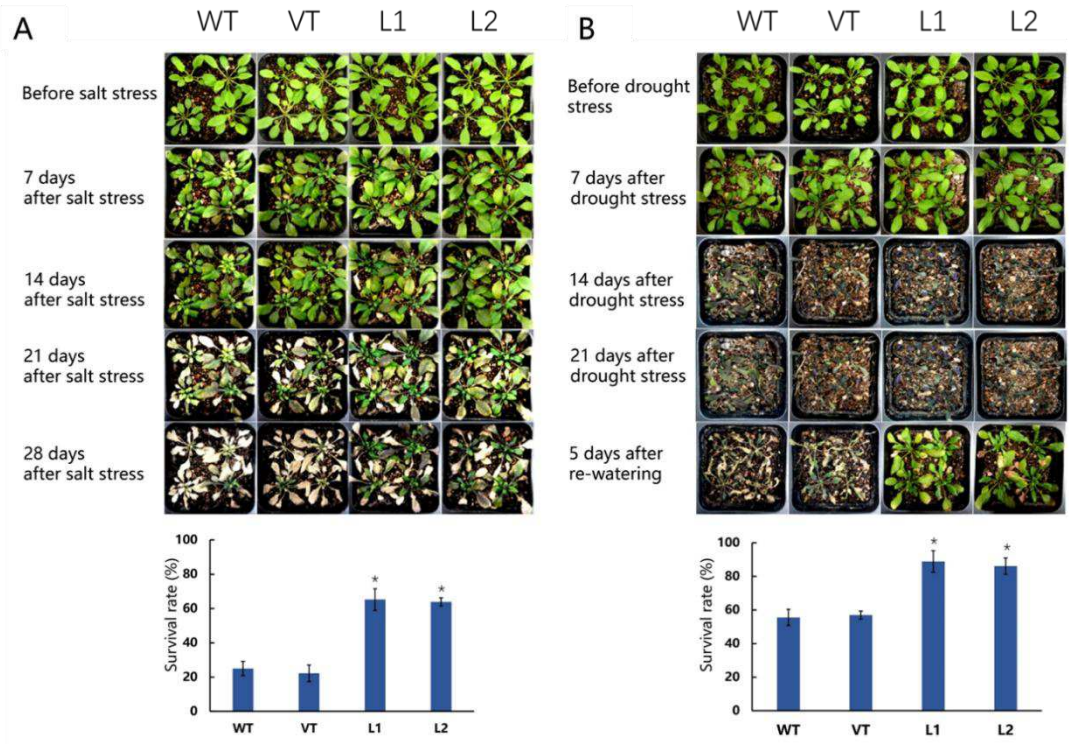
Previous research has shown that several NAC genes derived from *S. liaotungensis* can confer increased salinity and drought resistance when expressed in *Arabidopsis*. We therefore examined whether *SlNAC6* exerts a comparable protective effect. The pBI121-*Sl*NAC6 overexpression construct and the corresponding empty pBI121 plasmid were separately introduced into *Arabidopsis*. After kanamycin selection and PCR identification, homozygous plants with pBI121-*Sl*NAC6 and the control empty vector were generated. Two overexpression lines with relatively high expression of *Sl*NAC6, referred to as L1 and L2, were chosen for further investigation. A plant with an empty vector from pBI121, named VT, and WT plants were used as controls.

All lines were seen to suffer leaf yellowing and wilting after exposure to 200 mM NaCl and drought, respectively (Figure 5A, B), but the L1 and L2 lines showed significantly better viability than the controls. At the end of 28 days, 65.2% of L1 plants and 63.8% of L2 plants survived, compared with 25.0% and 22.2% of WT and VT lines, respectively (Figure 5A). The same trend was shown when the plants were subjected to drought stress. Following 5 days of rehydration, most L1 and L2 plants recovered and resumed growth, producing survival rates of 88.9% and 86.1%, respectively. In comparison, nearly all WT and VT seedlings continued to exhibit severe wilting (Figure 5B).

Seed germination was then monitored for seven successive days on MS medium under control, saline, or osmotic-stress conditions. In the absence of stress, germination proceeded similarly among WT, VT, L1, and L2 seeds, and no statistically significant differences were detected. By day 4, the respective germination percentages were 96.7%, 97.8%, 97.8%, and 96.7% (Figure 5C). NaCl exposure delayed germination in all lines; however, the two *SlNAC6*-overexpressing lines consistently outperformed the controls from day 4 onward. On the seventh day, germination reached 82.2% in L1 and 80.0% in L2, compared with 50.0% in WT and 47.8% in VT (Figure 5D). Under mannitol-induced drought salt, L1 and L2 also germinated more rapidly during the initial period than WT and VT. The largest differences occurred on day 4 but became progressively smaller at later time points. At day 7, germination percentages were 97.8%, 97.8%, 98.9%, and 98.9% for WT, VT, L1, and L2, respectively, and the differences were no longer significant (Figure 5E).

The effects of SlNAC6 expression on root development were additionally evaluated after 15 days of cultivation. Under standard growth conditions, primary root elongation did not differ markedly among the four lines (Figure 5F). In contrast, when exposed to 100 mM NaCl or 200 mM mannitol, L1 and L2 seedlings had significantly longer primary roots than the WT and VT controls.

As survival and root elongation are commonly used indicators of plant performance under environmental stress, the combined results demonstrate that constitutive expression of *SlNAC6* increases *Arabidopsis* tolerance to both salinity and drought.



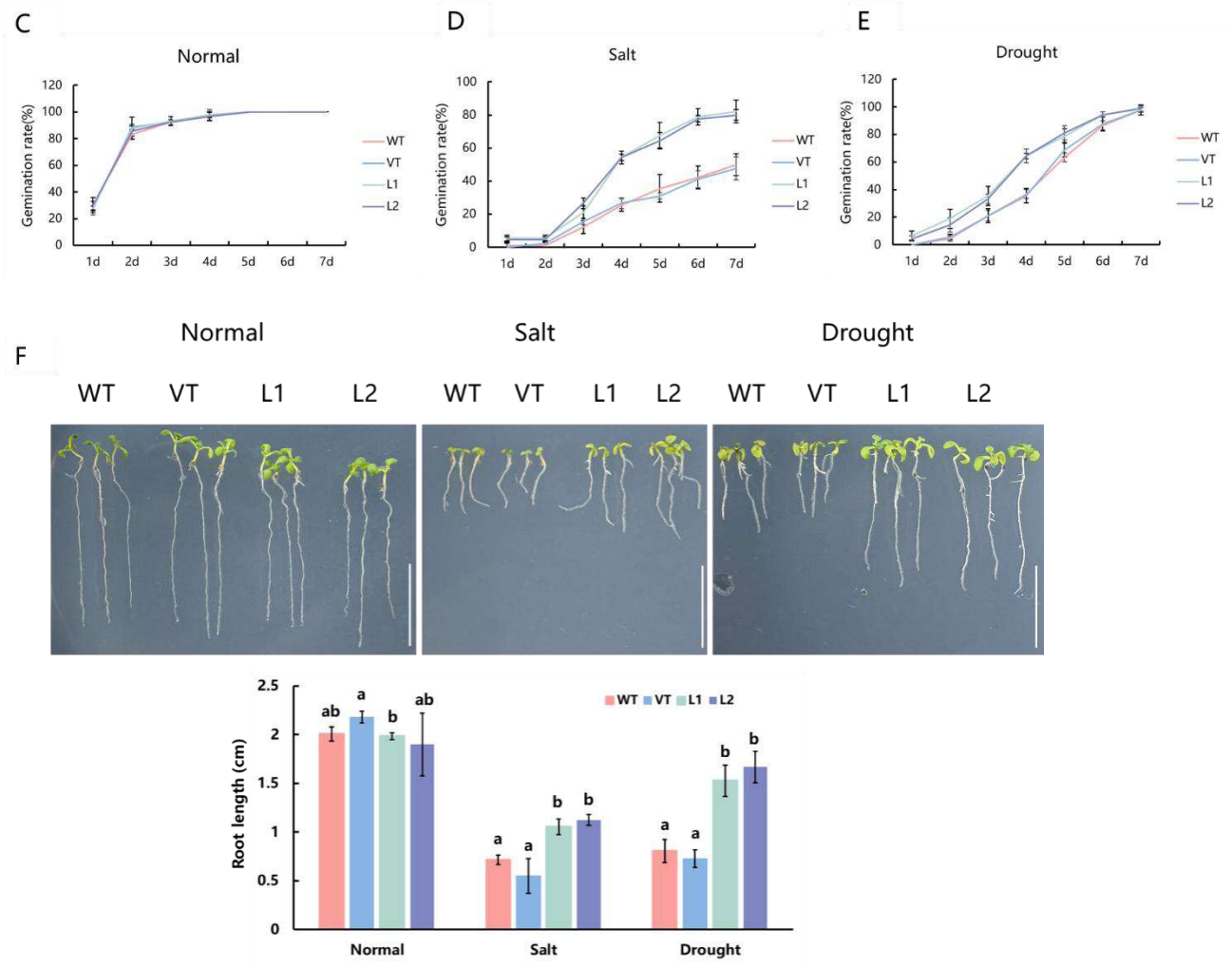


FIGURE 5. Enhanced salinity and drought resistance conferred by *SlNAC6* overexpression in *Arabidopsis*. (A, B) Representative growth phenotypes and survival percentages of WT, pBI121 empty-vector (VT), and pBI121-*SlNAC6* overexpression (L1 and L2) plants following exposure to salinity (A) or water-deficit conditions (B). (C-E) Seed germination dynamics of WT, VT, L1, and L2 on standard medium (C), medium supplemented with 100 mM NaCl to impose salt stress(D), or medium containing 200 mM mannitol to simulate drought stress (E). (F) Primary root growth of the four lines under 100 mM NaCl or 200 mM mannitol treatment.

3.6. Physiological and Biochemical Responses of *SlNAC6*-Overexpressing *Arabidopsis* Plants Under Stress Conditions

A measure of the photochemical efficiency and functional integrity of PSII is the maximal quantum yield of photosystem II photochemistry, denoted as Fv/Fm. Fv/Fm is commonly used to indicate photosynthetic injury caused by adverse conditions because it typically shows a significant decrease when exposed to environmental stress. The blue-purple fluorescence was mostly present in *Arabidopsis* leaves under control conditions, and the Fv/Fm values ranged from 0.77 to 0.81, indicating that PSII was unharmed. After 21 days of salt treatment, Fv/Fm values were maintained at 0.518 and 0.569 in L1 and L2, respectively, but declined to 0.319 in WT and 0.328 in VT. At the same time, most WT and VT leaves emitted green or reddish-

brown fluorescence (Figure 6A), indicating substantial impairment of PSII. Continuous drought led to a marked reduction in Fv/Fm for all four lines. After five days of recovery in water, the value of Fv/Fm in L1 and L2 increased back to 0.584 and 0.566, respectively. In contrast, the values of Fv/Fm for WT and VT decreased towards zero, and their fluorescence images appeared black (Figure 6B), showing death of the plants. This indicates that continuous expression of *SlNAC6* protects plants against PSII damage caused by stress.

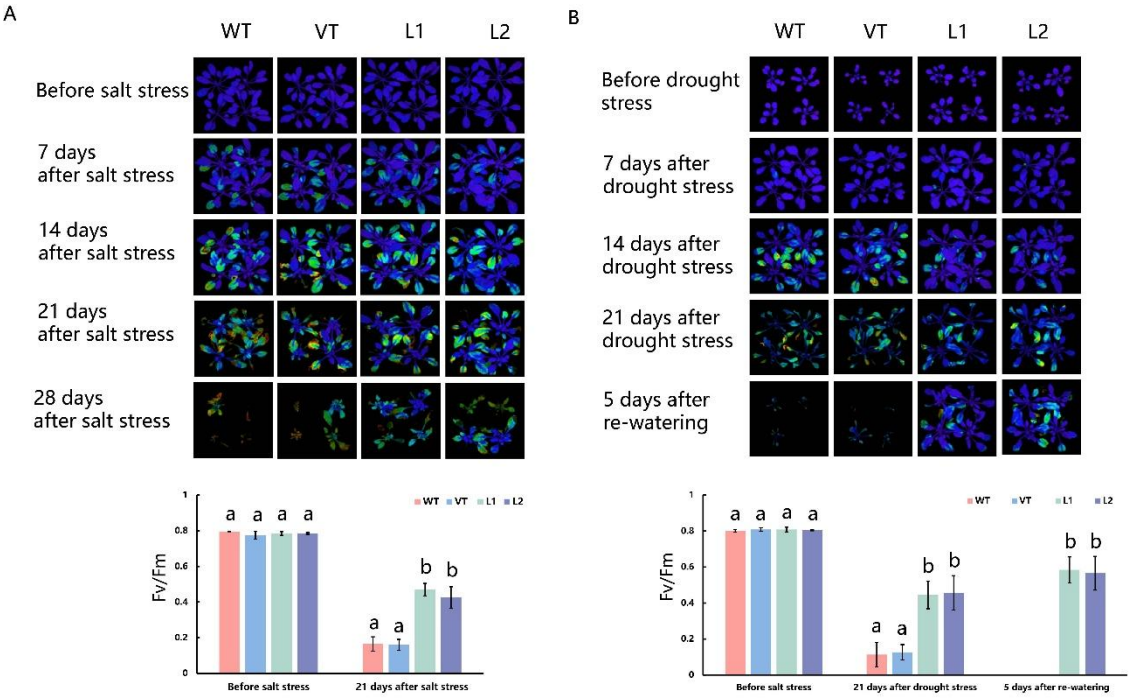


FIGURE 6. Chlorophyll fluorescence characteristics and maximum quantum yield of photosystem II photochemistry (Fv/Fm) in *Arabidopsis* lines subjected to abiotic stress. Chlorophyll fluorescence images and Fv/Fm values of WT, pBI121 empty-vector (VT), and pBI121-*SlNAC6* overexpression (L1 and L2) plants under salt (A) and drought (B) treatments.

Antioxidant enzymes constitute an important defense system that removes excessive ROS and limits oxidative injury to cellular membranes. Under unstressed conditions, CAT, SOD, and POD activities were comparable among WT, VT, L1, and L2 plants. Following exposure to either NaCl or drought, however, all three enzyme activities were significantly greater in the SlNAC6-overexpressing lines than in the two controls lines (Figure 7A-C).

Membrane lipid peroxidation as well as MDA production is usually caused by abiotic stress which disturbs redox homeostasis in cells and increases ROS generation. Membrane degradation causes loss of its selective properties and higher permeability for electrolytes. Since stress can damage membranes, relative conductivity and MDA levels can be used to identify this damage. In the control condition, none of the four *Arabidopsis* lines showed any significant variations in these two characteristics. Figure 7D and 7E shows that relative conductivity and MDA content were much lower in the overexpression lines compared to the WT and VT lines after salt or DS treatment, suggesting that the overexpressed lines had less membrane degradation.

Proline acts as a compatible osmolyte and contributes to membrane protection during exposure to adverse environmental conditions. Salinity and water deficit induced substantially greater proline accumulation in L1 and L2 plants than in the WT and VT controls (Figure 7F). Taken together, these physiological responses suggest that the superior stress resistance of the *SlNAC6*-overexpressing plants is associated with strengthened antioxidant defenses and enhanced osmotic adjustment. Accordingly, ectopic expression of SlNAC6 alleviates oxidative injury, suppresses membrane lipid peroxidation, and preserves cellular membrane stability under salt and DS.

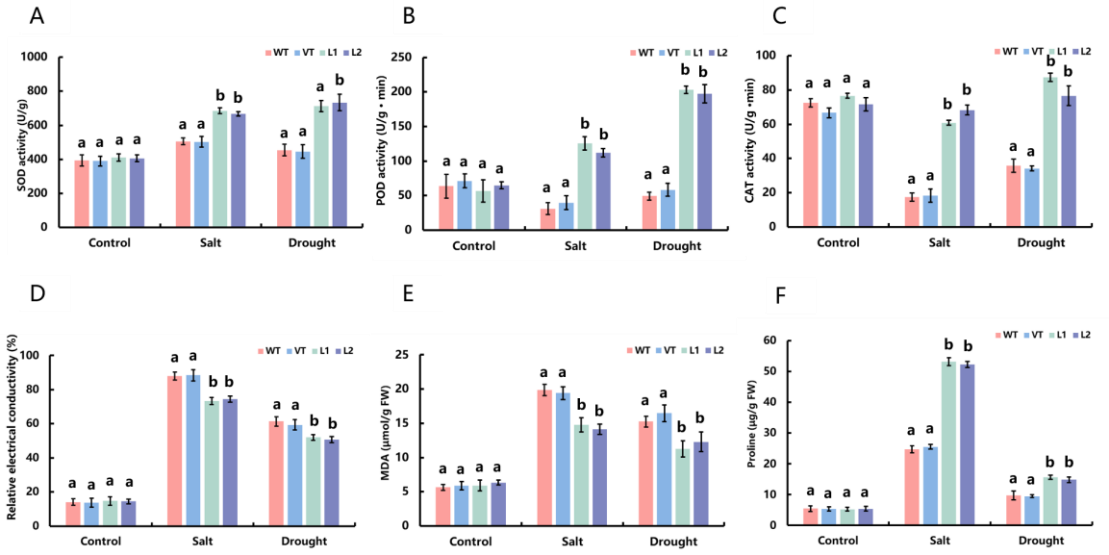


FIGURE 7. Physiological and biochemical responses of *Arabidopsis* genotypes under control and stress conditions. Measurements were obtained from WT, pBI121 empty-vector (VT), and pBI121-SlNAC6 overexpression (L1 and L2) plants. (A) SOD activity; (B) POD activity; (C) CAT activity; (D) relative electrical conductivity; (E) MDA concentration; and (F) proline accumulation.

3.7. *SlNAC6* Upregulates the Expression of Several Stress-Responsive Genes

To elucidate the molecular mechanisms by which *SlNAC6* overexpression enhances stress tolerance in transgenic *Arabidopsis*, qRT-PCR was used to determine the relative expression levels of 12 stress-responsive genes: *RD29A*, *RD29B*, *RD20*, *SGR2*, *ERD10*, *COR47*, *PORA*, *IAA6*, *VSP1*, *AZF1*, *GSTF6* and *MYB*. Compared with WT plants, transgenic *Arabidopsis* plants exhibited significantly increased expression of *RD29A*, *RD29B*, *RD20*, *SGR2*, *ERD10*, *COR47*, *PORA*, *IAA6*, *VSP1*, *AZF1* and *GSTF6,* with increases of 1.37-, 2.83-, 1.17-, 1.19-, 1.33-, 1.79-, 4.21-, 2.55-, 2.77-, 1.64-, and 9.55-fold, respectively. By contrast, *MYB6* expression did not change significantly (Figure 8). These findings suggest that the upregulated genes may be downstream targets of *Sl*NAC6.

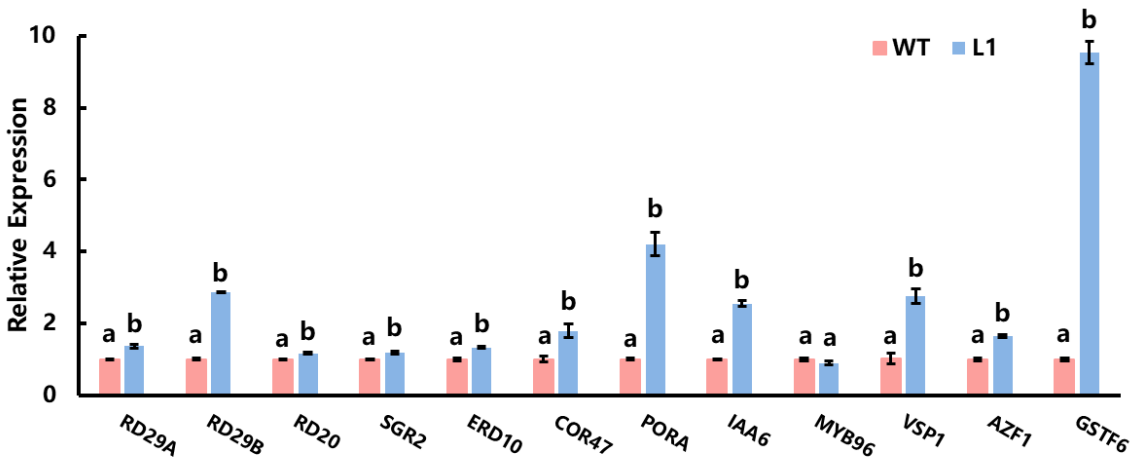


FIGURE 8. Relative expression levels of stress-responsive genes in the *SlNAC6*-overexpressing *Arabidopsis* line L1 under normal growth conditions, as determined by qRT-PCR using gene-specific primers.

4. Discussion

4.1. *Sl*NAC6 Is a Stress-Responsive NAC TF

The role of NAC TFs in plant development, growth, and response to stress has been well-documented in the literature. Sequence analysis in this study confirmed that *Sl*NAC6 has the NAC domain, a feature of this protein family that comprises five conserved subdomains labeled A-E. Base on similarities in their amino acid sequences, Ooka et al. classified the NAC proteins found in rice and *Arabidopsis thaliana* into two groups and multiple s subgroups. To determine the evolutionary position of *Sl*NAC6, a phylogenetic analysis was conducted using *Sl*NAC6, previously identified NAC proteins from *S. liaotungensis*, and homologous proteins from other plant species. *Sl*NAC6 did not cluster with other NAC proteins from *S. liaotungensis,* indicating that it is distantly related to these family members despite sharing a similar capacity to enhance plant salt tolerance. *Sl*NAC6 was most closely related to ANAC063 and ANAC064, two NAC family members from *Arabidopsis*. On this basis, *Sl*NAC6 was classified into the ANAC063 subfamily of Group II (Ooka et al., 2003).

A defining property of many TFs is the presence of a nuclear localization signal that facilitates their transport into the nucleus, where they modulate the transcription of target genes. However, most NAC proteins exhibit nuclear localization, some members have been found to be localized in the plasma membrane, cytoplasm, or endoplasmic reticulum (Wang et al., 2016; Mathew et al., 2016). The current study reveals that *Sl*NAC6 is localized in both nucleus and cytoplasm, a pattern similar to that previously observed for *OsNAC23* (Li et al., 2022). Such variation in cellular distribution may be associated with differences in the biological activities and regulatory modes of NAC proteins during plant development and environmental adaptation (Liu et al., 2023). Structurally, NAC TFs generally possess a conserved N-terminal DNA-binding region and a divergent C-terminal regulatory region capable of either activating or repressing stress-associated genes and signaling pathways

(Yamaguchi et al., 2010; Puranik et al., 2012). Both the complete *Sl*NAC6 protein and its C-terminal fragment activated transcription in yeast, demonstrating that the functional activation region resides within the C-terminal portion.

An increasing number of evidence shows involvement of NAC proteins in adaptive response of plants to water deficit, cold and salinity. For instance, according to Nakashima et al. (2007), the transcript level of *OsNAC6* was barely detectable in rice without any stress treatment, but the transcription was greatly induced by drought, salt, cold and ABA treatments. Additionally, overexpression of *OsNAC6* increased the transcription of a number of stress - responsive genes. To assess whether *SlNAC6* may perform a comparable function in *S. liaotungensis*, its transcriptional response to several environmental stimuli was examined. The expression of *SlNAC6* increased substantially after exposure to salinity, drought, cold, and ABA, showing a response profile similar to that of *OsNAC6* in rice (Nakashima et al., 2007).

Taken together, its conserved structural features, dual nuclear-cytoplasmic distribution, C-terminal transcriptional activation capacity, and inducibility by environmental stimuli support the classification of *SlNAC6* as a stress-responsive NAC TF potentially involved in adaptation to multiple abiotic stresses.

4.2. *Sl*NAC6 Enhances Stress Tolerance in Transgenic *Arabidopsis*

Previous studies by our group have shown that *Sl*NAC proteins from distinct phylogenetic subfamilies can modulate stress-related downstream genes, modify physiological and biochemical responses under unfavorable conditions, and improve the stress resistance of transgenic plants (Li et al., 2014; Yang et al., 2014; Wu et al., 2018; Wang et al., 2021; Du et al., 2022; Liu et al., 2023). Transgenic *Arabidopsis* thaliana plants overexpressing *SlNAC6* were used to study the role of *SlNAC6* in plant adaptation to stress. When exposed to salt and water stress, the transgenic plants outperformed the WT and empty-vector controls in terms of stress phenotypes and survivability. Root development and seed germination were both improved in the overexpression lines when subjected to salt and drought stress. These phenotypic variations suggest that salt and drought tolerance in *Arabidopsis* is enhanced by ectopic expression of *SlNAC6*.

Analysis of chlorophyll fluorescence represents an essential method to determine photosynthetic performance in plants. Changes in chlorophyll fluorescence reflect the sensitivity to salinization-alkalinization stress and provide information on the light energy absorption, transportation, distribution, dissipation, and transformation in the photosynthetic machinery (Strasser et al., 2002). The maximum quantum yield of photosystem II photochemistry, or Fv/Fm, is a measure of photosynthetic efficiency of a plant that indicates how well it can convert light energy that it absorbs into chemical energy. In order to gauge how well plants have adapted to and are able to withstand various stresses, Fv/Fm is used (Hou et al., 2018). Analysis of chlorophyll fluorescence showed that overexpression of *SlNAC6* led to higher Fv/Fm comparison with WT and VT lines in the presence of salinity and drought. It can be stated that *Sl*NAC6 overexpression leads to the preservation of photosynthetic activity in the stressful environment.

Salt stress causes disruption in cellular redox homeostasis because it results in excess production of ROS that interferes with regular physiological functions and may even cause death due to severe oxidative injury (Gill and Tuteja, 2010). Plants activate their antioxidant defense system through non-enzymatic and enzymatic mechanisms in order to create an equilibrium between ROS production and scavenging (Hasanuzzaman et al., 2020). Excess ROS generated in unfavorable conditions will also trigger lipid peroxidation of membrane leading to increased MDA contents and dysfunction in selective membrane permeability. Hence, levels of MDA and relative electrolyte leakage are used as markers for cellular membrane damage and stress tolerance. To prevent ROS-induced oxidative damage to plant cells, several antioxidant enzymes work together, including SOD, CAT, and POD. In lines of *Arabidopsis* overexpressing *SlNAC6*, the activity of all three enzymes was significantly higher under salt and drought stress compared to control lines. These changes were accompanied by lower MDA levels and reduced relative electrolyte leakage. Thus, *SlNAC6* expression appears to strengthen antioxidant defenses and preserve membrane integrity in transgenic *Arabidopsis* subjected to abiotic stress.

Proline functions as an important compatible osmolyte and contributes to stabilization of the photosynthetic machinery and enhancement of antioxidant defenses in plants (Martinez-Garcia and Rodriguez-Concepcion, 2023). Following salt or drought treatment, free proline accumulated to higher levels in the *SlNAC6*-overexpressing plants than in the WT and VT controls. A comparable response was reported by Liu et al. (2025), who observed markedly increased proline accumulation in sunflower plants overexpressing HaNAC146 relative to WT plants under unfavorable conditions. Because improved osmotic adjustment is generally associated with stronger stress resistance, the increased accumulation of free proline may represent another mechanism through which *SlNAC6* promotes abiotic stress tolerance in *Arabidopsis*.

4.3. *SlNAC6* Regulates the Expression of Several Stress-Responsive Genes

NAC TFs primarily recognize CAGG-containing *cis*-acting elements or NAC recognition sequences (NACRs), thereby directly or indirectly activating or repressing the transcription of downstream stress-responsive genes (Mao et al., 2018). Gene cloning, functional characterization, and the analysis of underlying regulatory mechanisms of NAC TFs have been characterized in recent years. Numerous studies have shown that NAC TFs improve plant stress tolerance through controlling a cascade of stress-responsive genes. As a TF, *Sl*NAC6 may control how genes respond to environmental stressors.

In this study, 12 stress-responsive genes were selected for analysis of their relative expression levels in transgenic plants. The results showed that R*D29A, RD29B, RD20, ERD10, SGR2, COR47, PORA, IAA6, VSP1, AZF1, and GSTF6* were significantly upregulated in the transgenic lines, whereas *MYB96* expression did not change significantly. The genes upregulated in the transgenic lines participate in a broad range of biological functions, including ROS detoxification (Krajewski et al., 2013; Blée et al., 2014), chlorophyll biosynthesis (Sperling et al., 1998), seed germination (Kato et al., 2002), nutrient storage (Qing et al., 2011), growth and development (Jung et al., 2015; Sakamoto et al., 2004), osmotic adjustment (Guo et al., 1992), and

responses to multiple environmental stresses (Zhao et al., 2023; Kaur et al., 2025; Hanin et al., 2011). These expression changes suggest that *SlNAC6* positively regulates a set of stress-associated genes. Although these genes may be controlled directly or indirectly by *SlNAC6*, additional experiments are needed to identify its downstream targets and clarify the signaling networks through which it regulates abiotic stress responses.

5. Conclusions

According to the current research, *SlNAC6* is a NAC TF that responds to abiotic stress. The physiological responses and growth of *Arabidopsis* plants in response to drought and salinity were significantly altered by constitutive expression of *SlNAC6* (Figure 9). In general, *SlNAC6* is a positive regulator of stress resistance and could be a valuable genetic resource for making crops more resistant to stress.

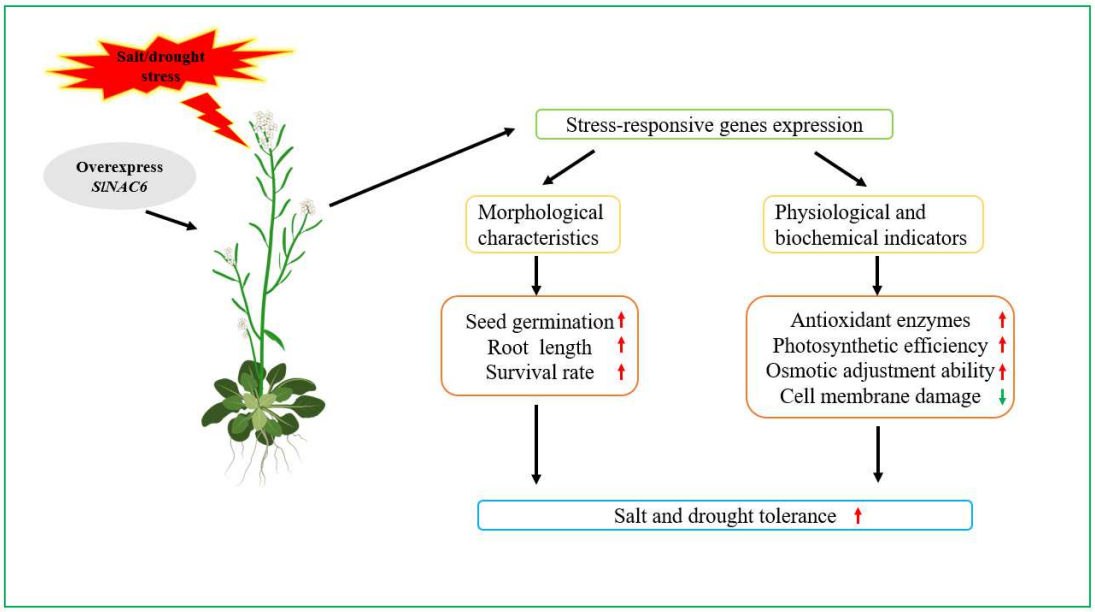


FIGURE 9. Hypothetical regulatory framework for *SlNAC6*-mediated plant growth and abiotic stress resistance in transgenic *Arabidopsis*. Overexpression of SlNAC6 activates stress-associated downstream genes and promotes seed germination, primary root elongation, plant survival, antioxidant enzyme activity, photosynthetic performance, and osmoprotectant accumulation. These coordinated responses reduce cellular membrane injury and enhance plant growth and resistance under adverse environmental conditions.

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Competing Interest

The authors declare no conflict of interest.

Author Contributions

All authors contributed to the study conception and design. QL conceived and designed the experiments; JC, HS, and TL performed the experiments; CL and HW analyzed the data; JS provided some suggestions; JC drafted the manuscript; QL revised the manuscript. All authors have read and approved the content of the article.

Data availability

All data supporting the findings of this study are included in the article and supplementary material. Further questioning can point to the corresponding author.

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