Overexpression of ThSOS from Tamarix hispida improves salt tolerance

Zhongyuan Liu
Northeast Forestry University
Qingjun Xie
Northeast Forestry University
Feifei Tang
Northeast Forestry University
Jing Wu
Northeast Forestry University
Wenfang Dong
Northeast Forestry University
caiqiu gao (gaocaiqiu@nefu.edu.cn)
Northeast Forestry University https://orcid.org/0000-0001-9953-9775

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Abstract

Highlights ● The functional characterization of ThSOS genes were investigated by bioinformatics analysis and molecular characterization. ● ThSOS genes can respond to abiotic stresses (salt and drought) and hormone treatment (ABA). ● ThSOS3 gene overexpression increased ROS-scavenging capability and decreasing lipid peroxidation in cell membrane. ● ThSOS3 could effectively enhance the tolerance of transgenic T. hispida and Arabidopsis to salt stress.

Background

High salinity is the main adverse environmental factor affecting plant growth and development because of osmotic and ionic stresses [1]. Plants have evolved several mechanisms to cope with the harsh environment and adjust their growth under high salt stress [2]. Ca^{2+} is a ubiquitous second messenger involved in the signaling of various environmental and developmental stimuli [3]. In response to these stimuli, intracellular Ca^{2+} concentrations undergo rapid and significant changes, which are detected and decoded by Ca^{2+} sensors, including calmodulins (CaMs), calmodulin-like proteins (CMLs), calcineurin B-like proteins (CBLs) and calcium dependent protein kinases (CPKs) [4]. Ca^{2+} sensors or Ca^{2+}-binding proteins enabled the plants to survive stress conditions by sensing transient Ca^{2+} changes and altering the protein phosphorylation and gene expression of related proteins or genes [5].

SOS3 gene acts as a calcium ion receptor in plants and is involved in calcium signal-mediated related stress responses. SOS3 is a calcineurin-like protein which belongs to the *CBL* family gene, and named *CBL4*. It was reported primarily in Arabidopsis which consist of EF-hand domains in the C terminus and a myristylation site in the N-terminal portion [6]. Myristylation of *SOS3* play an important role in plasma membrane and enhancing salt tolerance [7]. *SOS3* can interacts physically with *SOS2* (a serine/threonine protein kinase) by the 21 amino acid residue FISL motif. In the presence of Ca^{2+}, *SOS3* activates the substrate phosphorylation activity of *SOS2* [8]. An *SOS2-SOS3* interaction in the SOS pathway has also been demonstrated by using *sos3*/ *sos2* double-mutant analysis in Arabidopsis [9]. The *sos2* and *sos3* mutations led to reductions in plasma membrane Na^+/H^+ exchange activity in Arabidopsis. But, transport in these mutants could be restored by adding activated SOS2 protein [10]. The SOS3 phosphorylates *SOS1* and activates *SOS1* activation through binding with SOS2 kinase under salt stress [11]. *SOS1* encode a protein of the CPA1 (cation/proton antiporter 1) family, and play a key role at Na^+/H^+ antiporter to compartmentalize or exclude Na^+ and mediate the K^+/Na^+ balance [12]. Besides the SOS pathway, *SOS3* is an important component in the CBL-CIPK signaling network, and also participate in other signaling pathways to enhance plants salt stress tolerance [13].

In previous reports, *SOS3s* and *CIPK* homologs were induced under abiotic stress in different tissues of various plant species. For example, Arabidopsis *SOS2* (*AtCIPK24*) and *SOS3* (*AtCBL4*) specifically mediate salt stress signaling transduction in roots [10, 14]. *OsCIPK24* and *OsSOS3* act coordinately to activate *OsSOS1* in yeast cells and can be exchanged with their Arabidopsis counterpart to form heterologous protein kinase modules that activate both *OsSOS1* and *AtSOS1* and suppress the salt sensitivity of *sos2* and *sos3* mutants of Arabidopsis [15].

As reported that the *SOS3* gene was involved in plant salt stress response. Such as, *SOS3* has been shown to have an individual role in salt stress responses in Arabidopsis [16]. Specifically, the *SOS3* (*CBL10*) overexpression could increases calcium-mediated signaling capacity in *Eutrema* and confers enhanced salt tolerance in salt-sensitive Arabidopsis [17]. Overexpression of the *SOS3* gene in tobacco increased salt stress by excluding Na^+ from the cytosol and maintaining high K^+ levels to re-establish ion homeostasis [18]. In *Populus trichocarpa*, *PtSOS1*, *PtSOS2* and *PtSOS3* were identified and acted coordinately to activate *PtSOS1*, thus conferring salt tolerance on *P. trichocarpa* [19].

Tamarix hispida is a species of woody halophyte with excellent stress resistance. It can form natural forests in soils with 1% salt content. It is an ideal material for studying salt tolerance mechanisms and for cloning salt tolerance genes. A search of *T. hispida* transcriptome libraries yielded 5 *ThSOS* genes. In this study, 5 *SOS* genes from *T. hispida* (*ThSOS1-ThSOS5*) were cloned and characterized, and their expression levels under different abiotic stress conditions analyzed by means of qRT-PCR. To further study *ThSOS3* function, appropriate vectors for *ThSOS3* overexpression and RNAi-silencing were generated and

transgenic transformed into *T. hispida*. The results showed that *ThSOS3* could significantly improve salt tolerance in transgenic *T. hispida*.

Results

Gene identification and sequence analysis of *ThSOS* genes

In total, 5 candidate *SOS* genes (*ThSOS1*– *ThSOS5*) were selected and identified. The proteins of 5 ThSOS ranged from 213 to 1165 amino acids (aa) in length (Table 2). Large variations were found in theoretical pI values (ranging from 4.76 to 6.42) and MW (from 22.42-128.83 kDa) among the 5 *ThSOS* genes. The prediction results showed that 5 *ThSOS* genes were localized in the plasma membrane, cytoplasm, extracellular space or chloroplasts, respectively (Table 2).

To determine the subclass of *ThSOS* genes, a phylogenetic analysis was performed using the sequences of ThSOS and SOS proteins from other species (Fig. 1A; Tab.S1). The results revealed that *ThSOS1*, *2*, *3* genes were closely related to the *SOS1*, *2*, *3* subfamilies of Arabidopsis. *ThSOS3* belonged to the *SOS3* (*CBL4*) subfamily, being clustered into the same clade with *PtrSOS3*, *MnSOS3* and *AtSOS3.* Multiple sequence alignment analysis showed that *ThSOS3* was closely related to *PtrSOS3* (XP-002318422.1) from *P. trichocarpa*, *MnSOS3* (XP-010100753.1) from *Morus notabilis* and *AtSOS3-1* (AT5G24270) from *Arabidopsis thaliana* (Fig. 1B).

Characterization of cis-elements in *ThSOS* gene promoters

The upstream sequence ~2 kb promoter regions of each of the 3 *ThSOS* genes were cloned and used to identify the cis-elements using the PlantCARE database. Numerous stress related consensus cis-acting elements were detected, including abscisic acid responsiveness (ABRE), antioxidant response element (ARE) and TC-rich repeats. Moreover, *ThSOS1* included one abiotic stress related element, MYB binding site (MBS); nine hormone stress related elements, such as salicylic acid responsiveness element (TCA-element), MeJA-responsiveness element (CGTCA-motif or TGACG-motif), gibberellin-responsiveness element (TATC-box), and auxin-responsiveness element (TGA-element); and one development related endosperm expression element (GCN4-motif). *ThSOS3* contained MBS and LTR abiotic stress response elements, consisting of 5 hormone stress related elements, auxin-responsive elements (TGA-element or AuxRR-core) and salicylic acid responsiveness elements (TCA-element). Similarly, *ThSOS4* contained many abiotic stress and hormone stress related elements, specifically as shown in Fig. 2. This result indicated that *ThSOS* might be involved in stress responses (abiotic stresses and hormone treatment) as well as plant development.

Expression of *ThSOS* genes under abiotic stresses and ABA treatment

To analyze the relative abundance of *ThSOS* genes, the expression profiles of 5 *ThSOS* genes under different stresses (NaCl, PEG_{6000}), or hormone treatment (ABA), were measured using qRT-PCR.

Abiotic stresses

In roots under NaCl stress, most *ThSOS* gene expressions were upregulated. Notably, *ThSOS4* and *ThSOS5* exhibited upregulated expression at all study stress points. The highest expression levels of *ThSOS4* and *ThSOS5* were induced 8.02-fold and 4.86-fold, respectively. The other three *ThSOS* genes, *ThSOS1*, *ThSOS2* and *ThSOS3,* were downregulated at the initial stress time point and upregulated t later stages. The lowest expression levels of these three *ThSOS* genes in roots all occurred at 6 h: the expression levels of *ThSOS1*, *ThSOS2* and *ThSOS3* were 3.55%, 6.11% and 0.20%, respectively. These results indicate that these 3 genes can respond rapidly to salt stress in *T. hispida* roots. In leaves, *ThSOS* gene expression was mainly downregulated during the stress period. *ThSOS2* and *ThSOS3* reached their lowest expression levels, 3.78% and 1.56%, respectively, in the control at 6 h. The relative abundance of *ThSOS1*, *ThSOS4* and *ThSOS5* were similar to those of *ThSOS2* and *ThSOS3* but achieved their lowest expression levels at 24 h (Fig. 3A).

Under PEG_{6000} stress, most of the *ThSOS* genes were significantly upregulated, and all *ThSOS* genes achieved their highest expression levels at 72 h. Interestingly, the expression levels of *ThSOS1*, *ThSOS2* and *ThSOS3* in the roots were significantly downregulated under PEG_{6000} stress at 6 h (5.39%, 16.67% and 0.16% of the control, respectively). In leaves, the expression

levels of *ThSOS1*, *ThSOS2* and *ThSOS3* genes were mainly downregulated throughout the period of stress, and *ThSOS1* and *ThSOS2* achieved their lowest levels of expression at 24 h. However, *ThSOS3* reached its lowest expression level during the early stages of stress (6 h). In contrast to the gene expression patterns of these three *ThSOS* genes, the relative expression of *ThSOS5* was significantly upregulated at almost all stress points (besides 24 h) and reached its highest expression at 72 h. The expression of *ThSOS4* didn't changed significantly under salt stress (Fig. 3B).

ABA treatment

The relative abundance of *ThSOS1*, *ThSOS4* and *ThSOS5* were significantly upregulated in roots. In addition, the gene with the highest percentage of induction was *ThSOS1*, with a peak expression level at 6 h that was 161.28-fold that of the control. Except at 12 h, when expression was only 24.3% of the control, the relative expression of *ThSOS2* was mainly upregulated. However, the expression of *ThSOS3* was clearly downregulated at 6 h (0.4% of the control) and showed no significant changes at any other stress points. In the leaves, no significant changes were found in the expression of any *ThSOS* genes except for *ThSOS2* from 12-72 h. All *ThSOS* genes (*ThSOS1, ThSOS2, ThSOS3, ThSOS4, ThSOS5)* under ABA stress reached their lowest levels of expression at 6 h (0.55%, 0.83%, 0.07%, 1.31% and 3.17% of the control, respectively) (Fig. 3C).

Transient expression of *ThSOS3* in *T. hispida*

To ascertain whether the *ThSOS3* gene in *T. hispida* was transiently overexpressed and suppressed, the transcription levels of *ThSOS3* in the control (empty pROK2), OE (35S::*SOS3*) and SE (pFGC::*SOS3*) plants were examined by using qRT-PCR. Compared to Con plants, *ThSOS3* expression was significantly increased in OE plants and significantly decreased in SE plants under salt stress condition (Fig. 4), indicating that the gain- or loss-of-function of *ThSOS3* in *T. hispida* plants was successfully generated.

ThSOS3 confers salt stress tolerance to transgenic plants

To preliminarily explore the function of the *ThSOS3* gene, diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining and related physiological indexes of three types of plants with different transformed *T. hispida* were studied. DAB and NBT staining showed that levels of H_2O_2 and O^{2-} accumulation in transgenic (OE, SE) and control plants. Under salt stress, the staining strength in OE plants were lower than in Con; however, SE plants were higher than in Con (Fig. 5A-B). In addition, H_2O_2 and MDA contents were measured in different transgenic *T. hispida*. The results failed to demonstrate a difference in H_2O_2 and MDA contents among three kinds transient transgenic plants under normal conditions. However, under salt stress, SE plants showed the highest H_2O_2 and MDA contents, followed by the Con plants, the OE plants had the lowest H_2O_2 and MDA contents. The levels of H_2O_2 and MDA in SE plants were 1.27 and 1.53 times those of Con plants, respectively. However, the H_2O_2 and MDA contents in OE plants were the lowest, with values of only 82.02% and 85.2% of the contents in the Con plants, respectively, at 24 h (Fig. 5D-E).

To further explore the transient expression of *ThSOS3* in *T. hispida*, *ThSOS3* was overexpressed in Arabidopsis. Two independent T_3 homozygous transgenic lines (OE1 and OE2) overexpressing *ThSOS3* were selected and studied. Under salt treatment, the H_2O_2 and MDA levels in both OE lines were lower than those of WT plants, although they had similar H_2O_2 and MDA contents under normal conditions (Fig. S1A, B, D). In addition, OE1 and OE2 had prominently higher root growth and fresh weights than the WT (Fig. S2A-C). Analysis of the growth phenotypes of the Arabidopsis plants grown in soil showed that OE lines grew much better than the WT under salt treatment (Fig. S2D).

ThSOS3 increases improves ROS scavenging capability

Antioxidant enzymes (SOD and POD) are the two most important ROS scavenging enzymes, influencing cellular ROS levels. Thus, we further studied peroxidase (POD) and superoxide dismutase (SOD) activities. Without stress conditions, there were no significantly altered in SOD and POD activities among Con, OE and SE plants. However, SOD and POD activities were raised gradually with the salt treatment time extended. At 24 h, the antioxidant enzymes (SOD and POD) activities in OE plants were 1.35 and 1.24 times those of Con plants, while in SE plants were only 80% and 83.73% those of Con plants, respectively (Fig. 5F-G).

Similarly, in Arabidopsis the activities of SOD and POD exhibited no obvious differences among all the studied lines without stress conditions. Under salt stress, two OE lines showed the highest antioxidant enzymes (SOD and POD) activities compared with WT plants (Fig. S1E-F), which is consistent with the results obtained from *T. hispida*; therefore, overexpression of *ThSOS3* significantly increased SOD and POD activity under salt stress.

Cell death and electrolyte leakage analysis

Evans blue staining was used to establish a cell membrane damage by the intensity of the stain under salt stress. Evans blue staining indicated that in OE plants presented light blue points of less area in comparison with Con plants under salt stress; while in SE plants were the opposite (Fig. 5C). We then measured electrolyte leakage. There was no significantly altered in three types of plants with different transformed *T. hispida* under normal conditions. The relative electrolyte leakage rates of SE plants were the highest at 24h which was 1.14 times that of Con plants, meanwhile those of OE were 0.822 folds those of Con plants under salt stress (Fig. 5H). Meanwhile, we further detected changes in the content of corresponding physiological indicators in Arabidopsis. The results showed that Arabidopsis and *T. hispida* were consistent (Fig. S1C, G).

Discussion

The *SOS* gene plays a significant role in plants. *SOS* gene function has been studied in *Arabidopsis thaliana* [30-32], *Nicotiana tabacum* [33], *Oryza sativa* [1], *Gossypium raimondii* [34], *Lycopersicon esculentum* [35], *Zea mays* [36], *P. trichocarpa* [19, 37] and other plants, especially Arabidopsis [38]. However, there are few studies on the salt tolerance function of *ThSOS* in *T. hispida*.

In a recent study, it was found that plants under stress initiated a series of cellular signaling pathways to direct the expression of downstream resistance genes, such as the MAPK cascade pathway and calcium signaling pathways. Furthermore, the calmodulin-mediated downstream pathway and inositol synthesis pathway have been found to be important pathways for plant stress responses [39]. The SOS pathway is a saline-alkali stress response pathway regulated by the calcium signaling pathway. Increases in intracellular calcium concentration activate SOS3 and opens the downstream SOS pathway [40]. The SOS signal transduction pathway was first reported by Zhu Laboratory, and five groups of Arabidopsis salt-sensitive *SOS* mutants were screened to identify five SOS signaling pathway genes that strongly responded to the induction of salt stress signals [12].

In ours study, 5 monomorphic and intact *ThSOS* genes were selected. An unrooted phylogenetic tree and multiple sequence alignment analysis showed that *ThSOS3* shared 85.92%, 84% and 70% identity with *PtrSOS3*, *MnSOS3* and *AtSOS3-1*, respectively. It has been reported that *AtSOS3* enhances salt tolerance in Arabidopsis [41]. Moreover, in *P. trichocarpa, PtrSOS3* is involved in salt stress regulation to improve salt tolerance [19]. Therefore, we hypothesized that ThSOS3 may be similar to the proteins AtSOS3-1 or PtrSOS3 and may play an important role in salt stress.

The regulatory elements of the promoter sequences are essential for the temporal, spatial, or cell type-specific control of gene expression [42]. Previous studies have shown that many abiotic stress and hormone stress related elements have been found on the promoter of *ThSOS* genes. As shown in Fig. 2, abscisic acid responsiveness (ABRE), antioxidant response element (ARE) and TC-rich repeats were found in the promoters of 3 *ThSOS* genes. The low-temperature-responsive element (LTR) was found in promoter of *ThSOS3* gene. MYB-binding sites (MBS) was abundant in *ThSOS1*, *3* gene promoters. *ThSOS3* also contained 5 hormone stress related element, auxin-responsive element (TGA-element or AuxRR-core) and salicylic acid responsiveness element (TCA-element). Moreover, previous studies showed that *OSBZ8* mediates salt and dehydration stress tolerance by binding to ABRE motif [43]. *AtMYB44* inhibits oxidative damage and hypersensitivity to abiotic stresses by binding to MYB-binding sites to activate expression of downstream related genes [44]. These results suggest that the *ThSOS* genes may also be involved in the abiotic stress response with some transcription factors binding to these cis-acting elements.

The relative abundance of most of the *ThSOS* genes in *T. hispida* were significantly changed under NaCl, PEG_{6000} and ABA stresses. Notably, the expression of *ThSOS3* was significantly downregulated at 6 h under salt stress. SOS3 is a calcium-

regulated upstream regulatory protein of the SOS pathway and plays an important role in plant salt stress response pathways [30]. Yang [30] reported that *SOS3* could effectively increase the salt tolerance of transgenic plants. Combining the results of cis-elements in *ThSOS* genes promoters and the phylogenetic analysis, *ThSOS3* was shown to contain abundant abiotic stress and hormone stress-related elements and exhibits a closely related to *AtSOS3* and *PtrSOS3*. Therefore, we predict that *ThSOS3* might also play a role in responses to salt stress.

Plants produce high levels of reactive oxygen species (ROS) in adverse environments. ROS play a role, acting as signaling molecules to control several physiological processes [27]. Baxter [45] indicated that the H_2O_2 and O^{2-} signaling network responded to abiotic stimuli. In the present study, DAB and NBT staining showed that levels of H_2O_2 and O^{2-} accumulation in transgenic (OE, SE) and control plants. Under salt stress, the staining strength in OE plants were lower than in Con; however, SE plants were higher than in Con. Moreover, H_2O_2 and MDA were measured. The results showed overexpression of *ThSOS3* resulted in the lowest H_2O_2 and MDA accumulation. Conversely, RNAi-silencing revealed the opposite physiological changes among three types of plants with different transformed *T. hispida*. Evans blue staining was performed to study cell death in *T. hispida* plants under salt stress. Evans blue staining indicated that in OE plants presented light blue points of less area in comparison with Con plants under salt stress; while in SE plants were the opposite (Fig. 5C). We then measured electrolyte leakage. There was no significantly altered in three types of plants with different transformed *T. hispida* under normal conditions. The relative electrolyte leakage rates of SE plants were the highest at 24h, which was 1.14 times that of Con plants, meanwhile those of OE were 0.822 folds those of Con plants under salt stress. The electrolyte leakage assay further confirmed these Evans blue staining results.

Antioxidant enzymes (SOD and POD) are the two most important ROS scavenging enzymes, influencing cellular ROS levels. Increasing SOD and POD activity can reduce the accumulation of ROS and enhance the scavenging capacity of ROS. [27]. Thus, we further studied peroxidase (POD) and superoxide dismutase (SOD) activities. Without stress conditions, there were no significantly altered in SOD and POD activities among Con, OE and SE plants. However, SOD and POD activities were raised gradually with the salt treatment time extended. At 24 h, the antioxidant enzymes (SOD and POD) activities in OE plants were 1.35 and 1.24 times those of Con plants, while in SE plants were only 80% and 83.73% those of Con plants, respectively. These results indicated that the overexpression of *ThSOS3* reduced ROS accumulation and enhanced ROS scavenging by improving the activities of SOD and POD.

In summary, these physiological indicators results suggest that *ThSOS3* confers salt stress tolerance by increasing the activities of antioxidant enzymes (SOD, POD), reducing ROS accumulation, enhancing ROS scavenging and reducing membrane injury. However, the molecular mechanism of *ThSOS3* conferring salt stress tolerance in *T. hispida* is unclear. In future studies, the salt stress regulatory mechanism of *ThSOS3*, especially the upstream regulatory gene, will be further investigated.

Conclusions

The SOS gene plays an important role in responses to salt stress. However, there are few studies evaluating the role of ThSOS in salt tolerance in T. hispida. In this study, 5 ThSOS genes were cloned and identified in T. hispida. Their expression patterns in response to different abiotic stresses (NaCl and PEG6000) and hormone (ABA) stress were analyzed using qRT-PCR. The expression levels of most of the ThSOS genes were significantly altered under NaCl, PEG6000 and ABA treatments in at least one organ. Notably, the expression of ThSOS3 was significantly downregulated at 6 h under salt stress. Further, the role of ThSOS3 in salt tolerance was studied. The results showed that overexpression of ThSOS3 confers salt stress tolerance to plants by reducing ROS accumulation and membrane damage via enhancing the activities of antioxidant enzymes and improving ROS-scavenging capability. These results suggest that ThSOS3 might play an important physiological role in salt tolerance in transgenic T. hispida plants. This study provides a foundation for further understanding the salt tolerance mechanisms involving ThSOSs in T. hispida. However, the molecular mechanism of ThSOS3 conferring salt stress tolerance in T. hispida is unclear. Future studies should emphasize the mechanisms of ThSOS3 under salt stress.

Methods

Plant growth and stress treatments

T. hispida seedlings (the Turpan Desert Botanical Garden (Xinjiang,China)) were planted in pots containing a mixture of perlite/vermiculite/soil (1:1:4 v/v) in a greenhouse (70–75% relative humidity; 14 h light/10 h darkness photocycle, approximately 600 mmol m^{-2} s^{-1}; 24°C). Uniformly developed 2-month-old *T. hispida* seedlings watered with a solution of 0.4 M NaCl, 20% (w/v) PEG_{6000}, 0.4 M NaCl solution or 100 μM ABA, and the tissues were harvested at 6 h, 12 h, 24 h, 48 h and 72 h post watering. Seedlings watered with fresh water were harvested at the corresponding time points as controls.

Arabidopsis thaliana Columbia (WT) plants (State Key Laboratory of Tree Genetics and Breeding) were used in this study. Arabidopsis seeds after vernalization were sterilized for 3-5 min with 2.5 % (v/v) sodium hypochlorite and washed three times with distilled water. The seeds were soaked on 1/2 MS solid media plates containing 0.6% agar. One-week-old seedlings were transferred from the plates to pots filling with vermiculite/soil/ perlite (1:3:1) and grown in a greenhouse with the conditions of 16 h light/8 h dark photocycle, 70-75% relative humidity, 500 mmol m^{-2} s^{-1} light intensity, and a stable temperature of 22°C.

Cloning and sequence analysis of *ThSOSs*

The transcriptome libraries database was searched for *ThSOS* genes, which were further verification by the NCBI database (https://www.ncbi.nlm.nih.gov/). A phylogenetic tree was analyzed based on SOS proteins of *T. hispida* and SOS homologues from other species using the neighbor-joining method in MEGA 5.0 and a bootstrap method with 1000 replications [20]. Multiple sequence alignments were performed by using Clustal X with gap extension penalties and a gap open of 10 and 0.1, respectively [21]. The theoretical pI and molecular weight (MW) of the ThSOS proteins were studied with the ExPASy compute pI/Mw tool (http://www.expasy.org/tools/protparam.html). The predicting subcellular localization of the *ThSOSs* was performed by using CELLO v.2.5 (http://cello.life.nctu.edu.tw/).

RNA extraction and qRT-PCR analyses

Total RNA was extracted from *T. hispida* plants with the Plant RNeasy Extraction Kit (BioTeKe, China), and first strand cDNA was synthesized from 1 μg purified RNA using a PrimeScriptTM RT Reagent Kit (TaKaRa, China). qRT-PCR was analyzed in a $qTOWER^3$ G (Analytik Jene AG, Germany) with Actin (FJ618517) and β-tubulin (FJ618519) genes as internal controls. A 20 μL qRT-PCR reaction was described [22]. The relative abundance levels were determined by the $2^{-\Delta\Delta Ct}$ method [23]. Three biological replicates for each sample were performed (primers for qRT-PCR are shown in Table1).

Transient expression of the *ThSOS3* gene in *T. hispida*

A 642 bp cDNA of *ThSOS3* was amplified, fusing into prokII vector by the CaMV 35S promoter and named 35S::*ThSOS3*. A 200 bp truncated inverted-repeat cDNA of *ThSOS3* was cloned into the pFGC5941, at flanking the CHSA intron and generate pFGC5941::*ThSOS3* for silencing the expression of *ThSOS3* (primers for vector construction are listed in Table1).

The transient transformation of *T. hispida* plants was performed according to Zhang [24]. Three groups of transgenic plants were generated by transient transformation with 35S::*ThSOS3* of overexpress *ThSOS3* gene (OE), with pFGC::*ThSOS3* for silencing *ThSOS3* (SE) expression, or the empty pROK2 plasmid as control (Con). After growth for 12 h, 24 h and 36 h under normal conditions or salt treatment, the relative abundance of *ThSOS3* in these transformed *T. hispida* plants was studied using qRT-PCR. Three biological replicates for each sample were performed and contained at least 20 transformed seedlings.

Stress tolerance analysis

Stably transformed Arabidopsis plants were generated by the floral dip method [25]. Two T_3 generation homozygous transgenic lines (OE1 and OE2) of *ThSOS3* were selected to further evaluate stress tolerance. The Arabidopsis seeds after vernalization seeds were sown on 1/2 MS medium and grown for 5 d prior to being transferred to 1/2 MS medium or 1/2 MS medium plus 120 mM NaCl for 10-14 d. Root length and fresh weight were measured, and the seedlings photographed. For assessing salt tolerance in soil, seeds of two T_3 generation homozygous transgenic lines (OE1 and OE2) of *ThSOS3* and WT were sown on 1/2

MS solid medium for 5-7 d and were then transferred to soil. After 3 weeks of growth, the seedlings were watered with a solution of 150 mM NaCl for 5 d and then imaged. The treatments were independently repeated at least three times.

Biochemical staining

Hydrogen peroxide (H_2O_2) and superoxide (O^{2-}) staining were performed by infiltration with 3-3-diaminobenzidine (DAB) or nitro blue tetrazolium (NBT) following the procedures described by Zhang [26]. Evans blue staining was performed to investigate the cell death, as described by Liu [27].

Physiological indexes measurement of transformed plants

The transient transformation of *T. hispida* plants were grown on 1/2 MS solid medium plus 150 mM NaCl for 12-36 h. Four-week-old Arabidopsis seedlings were subjected to 150 mM NaCl for 5 d. After treatment, the seedlings were collected and subjected to physiological index analysis. Superoxide dismutase (SOD) and peroxidase (POD) activities and H_2O_2 content were measured using corresponding reagent kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. MDA content was determined according to Dhindsa [28]. Electrolyte leakage was described by Amor [29]. All of these procedures were performed in triplicate.

Statistical analyses

Statistical analyses were carried out using Excel software. Data were compared using Student's t, and differences were considered to significant if $P < 0.05$. * Indicates significant differences.

Abbreviations

SOS, salt overly sensitive; ABRE, abscisic acid responsive element; MBS, MYB binding site; TCA-element, salicylic acid responsiveness element; CGTCA-motif or TGACG-motif, MeJA-responsiveness element; ARE, antioxidant response element; qRT-PCR, quantitative real time PCR; WT, wild-type of Arabidopsis; OE, overexpression plant of *ThSOS3*; SE, *ThSOS3* RNAi.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Relevant data analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

CG designed research. ZL and FT conducted experiments and data analysis. ZL and QX wrote the manuscript. JW and WD conducted data analysis. CG revised the manuscript. All authors read and approved the manuscript.

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Tables

Table 1 Primers sequences used in this study.

Constructs	Forward and reverse primers (5′–3′)	
Genes	**Primers used in real-time RT-PCR analysis**	
ThSOS1	CTGATGCTGATCTGGATCCTAT	ATGCTAGACTGAAGAAATCGGT
ThSOS2	AGTAGAGGCCTTGTACGAGCT	ACCCAGTATGCCTCAGATCAT
ThSOS3	TGACGTTGATCCGATCAATTC	CCATAACAGGATCACATGCATAT
ThSOS4	CAATTTGCCTTATTCAGGAAT	CCACTATGCTCCCAACGATCT
ThSOS5	ATAGCCCACCATGGACGGCTT	ACCCTTGTGACTGAGAACCT
Actin (FJ618517)	AAACAATGGCTGATGCTG	ACAATACCGTGCTCAATAGG
β-tubulin (FJ618519)	GGAAGCCATAGAAAGACC	CAACAAATGTGGGATGCT
	Primers used in constructing plant plasmids	
pROKII-*ThSOS3*	GCTCTAGAATGGGCTGCTTCCATT CAAAG	GGTACCCCGTACTTCTGAATCT TCAACTT
	ThSOS3-Sense-F: CATGCCATGGATGGGCTGCTTCC ATTCAAAG	*ThSOS3*-Sense-R: GCTCTAGATTATACTTCTGAAT CTTCAACTTCCG
pFGC5941-*ThSOS3*		
	ThSOS3-Anti-F: GCTCTAGAATGGGCTGCTTCCATT CAAAG	*ThSOS3*-Anti-R: CATGCCATGGTTATACTTCTGAA TCTTCAACTTCCG

Table 2. Features of *ThSOS* genes in *T. hispida*.

Name	locus	ORF (bp)	Introns	Protein length	Theoretical pI	Aliphatic index	Molecular Weight (kD)	Localization predictions
ThSOS1	Unigene22889	3498	11	1165	6.42	103.24	128.83	Plasma Membrane
ThSOS2	Unigene13265	1371	2	456	6.29	92.08	51.44	Cytoplasmic
ThSOS3	Unigene1212	642	6	213	4.76	96.53	22.42	Cytoplasmic
ThSOS4	Unigene24293	927	11	308	6.22	105.1	33.58	Extracellular or Chloroplast
ThSOS5	Unigene1744	675	0	224	5.00	88.39	25.57	Cytoplasmic

Supplementary Materials

Fig. S1 Histochemical staining and related physiological change analyses of *ThSOS3*-transformed Arabidopsis. (A) NBT and (B) DAB staining. (C) Evans blue staining. Leaves from *ThSOS3*-transformed and WT Arabidopsis plants treated with 150 mM NaCl for 5 d were used for histochemical staining, respectively. (D–F) Analysis of MDA content, SOD and POD activities and electrolyte leakage in transgenic and WT Arabidopsis plants. Four-week-old Arabidopsis seedlings subjected to 150 mM NaCl for 5 d were used to detect the MDA (D) content, SOD (E) and POD (F) activities and electrolyte leakage (G).

Fig. S2 Salt stress tolerance associated with *ThSOS3*. (A) Growth comparison among OE and WT plants. Arabidopsis plants grown on 1/2 MS medium (control), 1/2 MS medium supplied with 120 mM NaCl for growth analysis. (B) Analysis of root length and (C) fresh weight. At least 30 seedlings in each treatment were analyzed. (D) Comparison of growth phenotypes between OE and WT Arabidopsis lines grown in soil. The plants were treated with 150 mM NaCl for 5 d for analysis. Plants grown under normal conditions were used as controls.

Figures

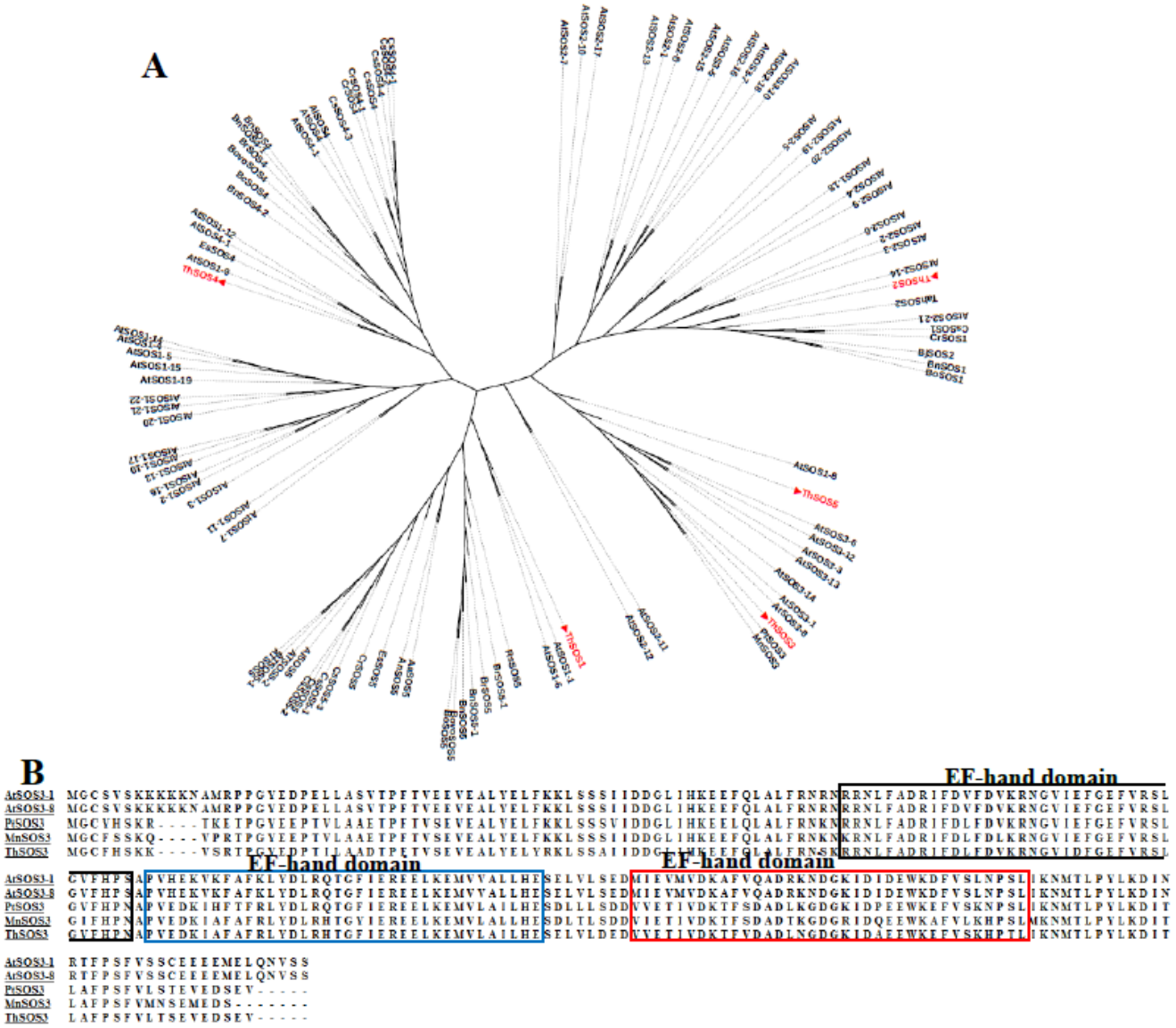
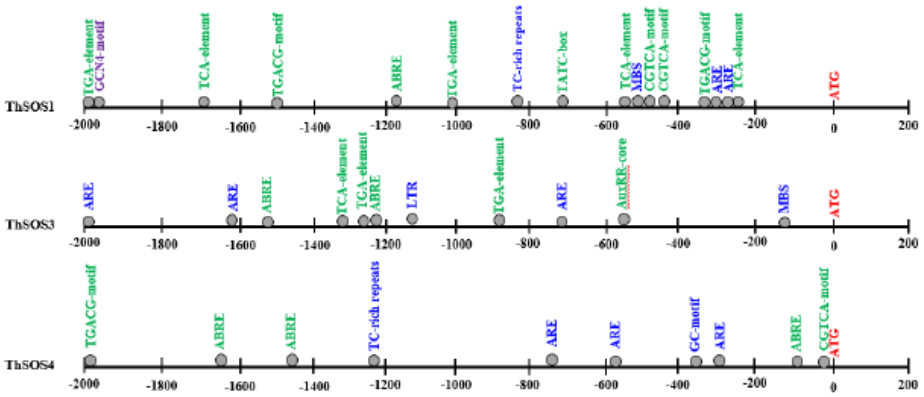


Figure 1

Phylogenetic and sequence analysis of ThSOS proteins. (A) Phylogenetic analysis of ThSOSs and other SOS proteins from different plant species. (B) Alignment of ThSOS3 protein sequence with other plant SOS3 proteins. The sequences of SOS proteins were downloaded from the GenBank database and their GenBank accession numbers are listed in Table S1.

A



B

Class	Element	Description
Hormone	ABRE	cis-acting element involved in the abscisic acid responsiveness
	AuxRR-core	cis-acting regulatory element involved in auxin responsiveness
	CGTCA-motif	cis-acting regulatory element involved in the MeJA-responsiveness
	TATC-box	cis-acting element involved in gibberellin-responsiveness
	TCA-element	cis-acting element involved in salicylic acid responsiveness
	TGACG-motif	cis-acting regulatory element involved in the MeJA-responsiveness
	TGA-element	auxin-responsive element
Stress	ARE	cis-acting regulatory element essential for the anaerobic induction
	MBS	MYB binding site involved in drought-inducibility
	LTR	cis-acting element involved in low-temperature responsiveness
	GC-motif	enhancer-like element involved in anoxic specific inducibility
	TC-rich repeats	cis-acting element involved in defense and stress responsiveness
Development	GCN4-motif	cis-regulatory element involved in endosperm expression

Figure 2

(A) The locations of cis-elements in the promoters of ThSOS genes. (B) The description of cis-elements.

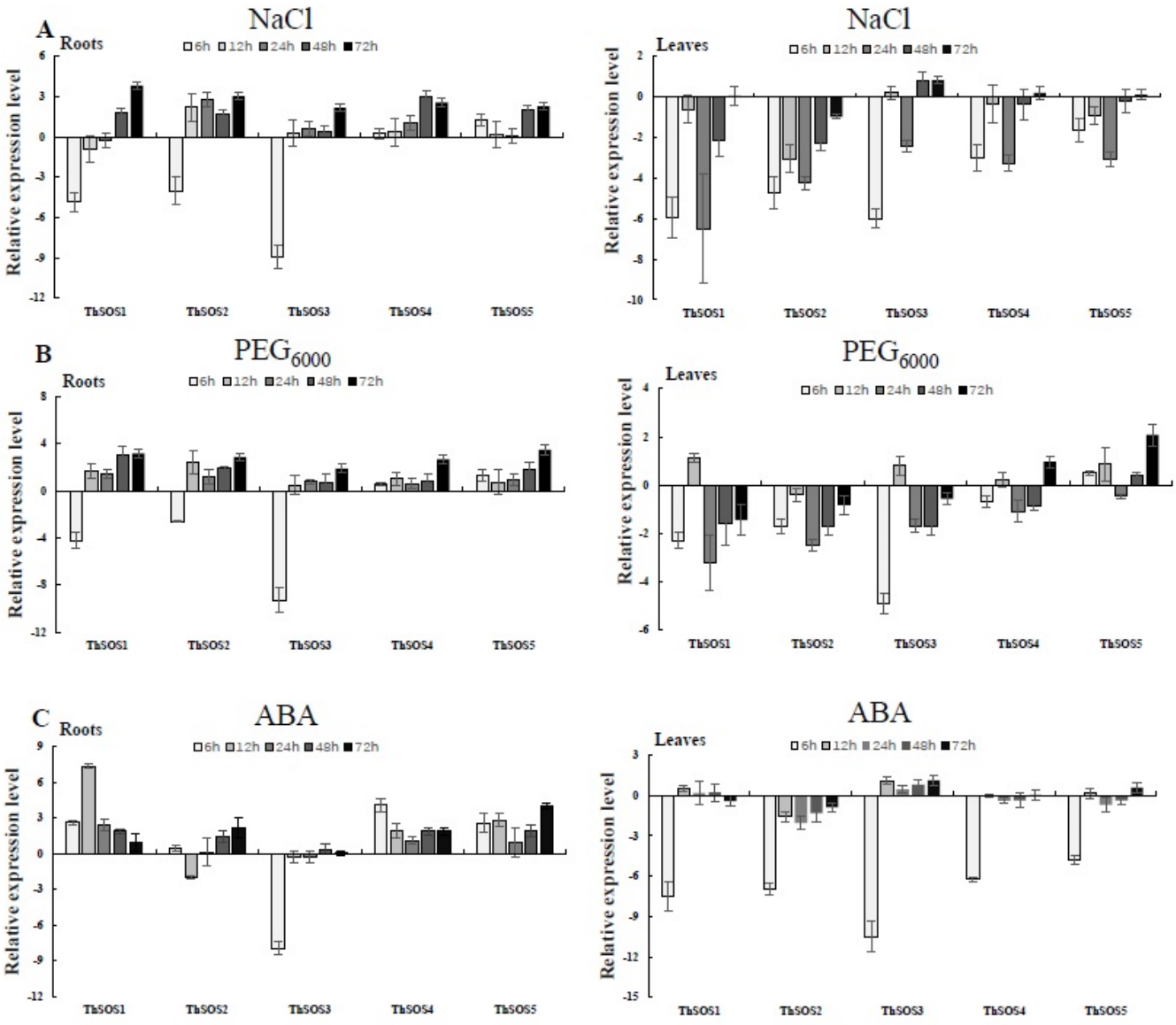


Figure 3

Expression analysis of the 5 ThSOS genes responding to abiotic stresses (NaCl, PEG6000) and hormone treatment (ABA) in roots and leaves. (A) 0.4 M NaCl. (B) 20% (w/v) PEG6000. (C) 100 μM ABA. All relative transcription levels were log2-transformed. The error bars were obtained from multiple replicates of qRT-PCR.

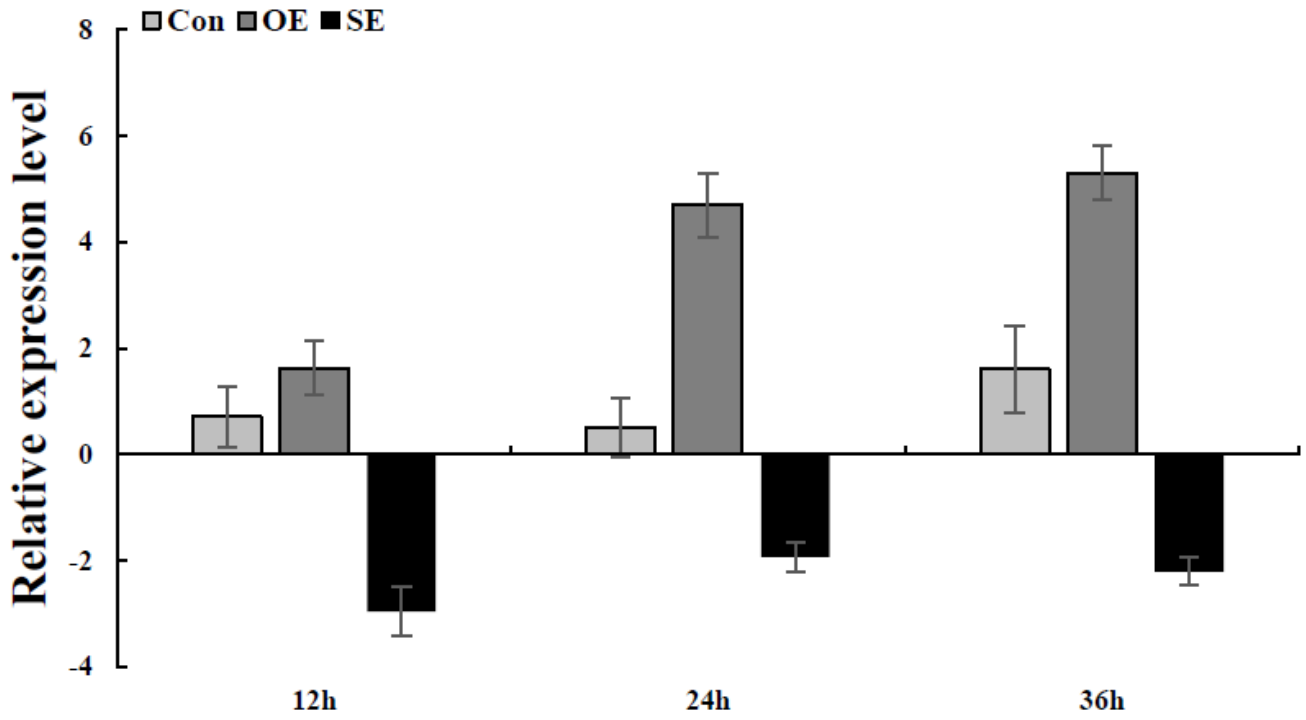


Figure 4

ThSOS3 transcript levels in T. hispida plants with transient overexpression or knockdown of ThSOS3. The expression data were log2- transformed. Two-month-old T. hispida plants were transiently transformed with empty pROKII, 35S::SOS3 or pFGC::SOS3. After transformation for 36 h, T. hispida plants were treated with 120 mM NaCl for 12, 24, or 36 h, and the expression of ThSOS3 was determined. OE: ThSOS3 overexpression; SE: ThSOS3 RNAi; Con: pROKII vector control.

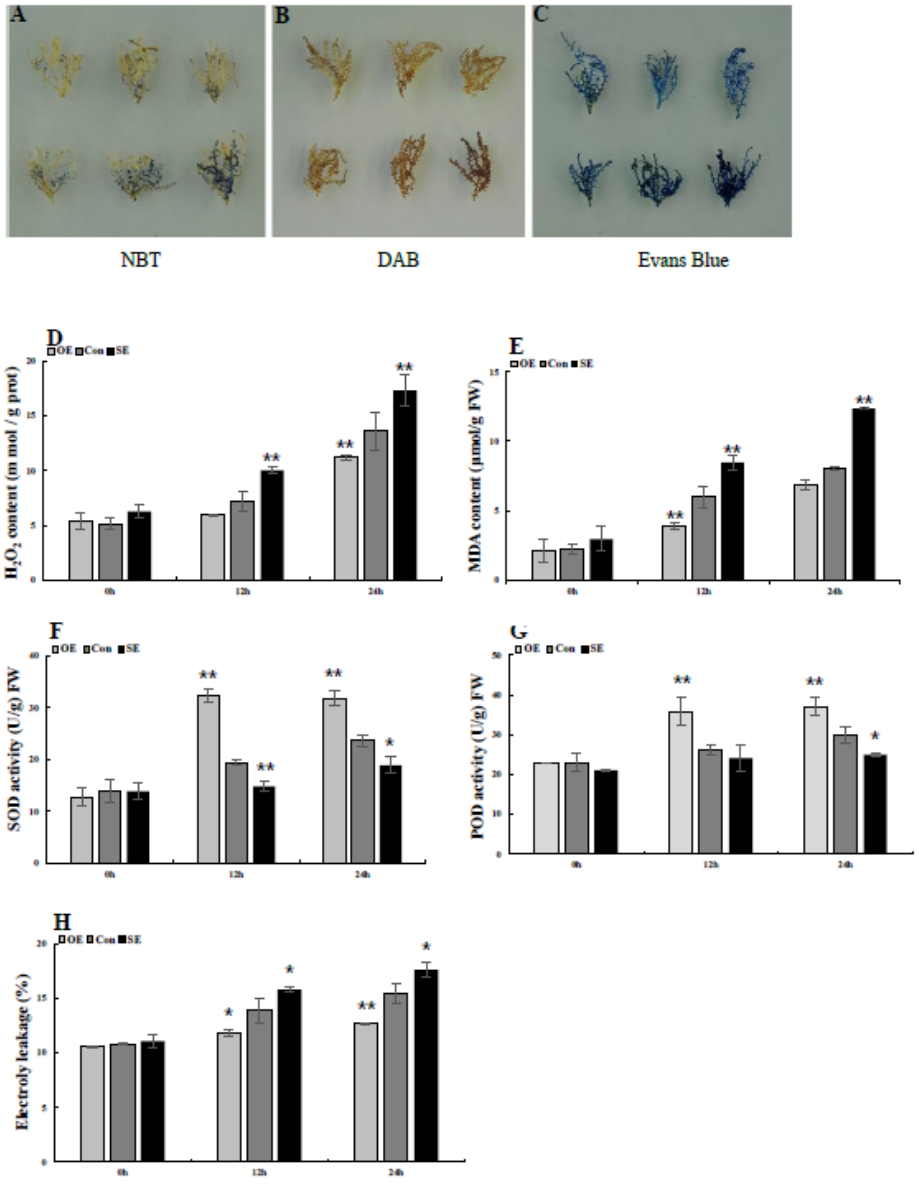


Figure 5

Histochemical staining and related physiological change analyses of transformed T. hispida. (A) NBT and (B) DAB staining to detect O2- and H2O2, respectively. (C) Evans blue staining for analyzing cell death. Young branches from transformed T. hispida plants treated with 150 mM NaCl for 2 h were used for DAB, NBT staining and Evans blue staining. (D–H) Analysis of H2O2 and MDA contents, SOD and POD activities and electrolyte leakage in three different transgenic T. hispida plants. Transformed T. hispida plantlets grown on 1/2 MS solid medium supplemented with 150 mM NaCl for 24 h were used to measure the H2O2 (D) and MDA (E) contents, SOD (F) and POD (G) activities and electrolyte leakage (H).

Supplementary Files

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- Fig.S1.pdf
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- TableS1.pdf