The ThSOS3 Gene Improves the Salt Tolerance of Transgenic Tamarix hispida and Arabidopsis thaliana

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The salt overly sensitive (SOS) signal transduction pathway is one of the most highly studied salt tolerance pathways in plants. However, the molecular mechanism of the salt stress response in Tamarix hispida has remained largely unclear. In this study, five SOS genes (ThSOS1–ThSOS5) from T. hispida were cloned and characterized. The expression levels of most ThSOS genes significantly changed after NaCl, PEG6000, and abscisic acid (ABA) treatment in at least one organ. Notably, the expression of ThSOS3 was significantly downregulated after 6 h under salt stress. To further analyze ThSOS3 function, ThSOS3 overexpression and RNAi-mediated silencing were performed using a transient transformation system. Compared with controls, ThSOS3-overexpressing transgenic T. hispida plants exhibited greater reactive oxygen species (ROS)-scavenging capability and antioxidant enzyme activity, lower malondialdehyde (MDA) and H$_2$O$_2$ levels, and lower electrolyte leakage rates under salt stress. Similar results were obtained for physiological parameters in transgenic Arabidopsis, including H$_2$O$_2$ and MDA accumulation, superoxide dismutase (SOD) and peroxidase (POD) activity, and electrolyte leakage. In addition, transgenic Arabidopsis plants overexpressing ThSOS3 displayed increased root growth and fresh weight gain under salt stress. Together, these data suggest that overexpression of ThSOS3 confers salt stress tolerance on plants by enhancing antioxidant enzyme activity, improving ROS-scavenging capability, and decreasing the MDA content and lipid peroxidation of cell membranes. 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 elucidation of salt tolerance mechanisms involving ThSOSs in T. hispida.

Keywords: ROS-scavenging capability, salt stress, Tamarix hispida, Arabidopsis thaliana, ThSOS

INTRODUCTION

High salinity is a major adverse environmental factor affecting plant growth and development due to osmotic and ionic stress (El Mahi et al., 2019; Yang et al., 2019). Plants have evolved several mechanisms to respond to harsh environments and adjust their growth under high-salt conditions (Munns and Tester, 2008). Ca$^{2+}$ is a ubiquitous secondary messenger that is involved in the signaling of various environmental and developmental stimuli (Hilleary et al., 2018; Srinivasan and Roberto, 2018). Rapid and significant changes in intracellular Ca$^{2+}$ concentrations can occur in
response to such stimuli, and these changes are sensed and decoded by Ca\(^{2+}\) sensors, including calmodulins, calmodulin-like proteins, calcineurin B-like proteins (CBLS), and Ca\(^{2+}\)-dependent protein kinases (Kudla et al., 2010). Ca\(^{2+}\) sensors or Ca\(^{2+}\)-binding proteins can sense transient Ca\(^{2+}\) changes and alter the protein phosphorylation and gene expression of related proteins or genes, thus enabling plants to survive stress conditions (Luan, 2009).

The salt overly sensitive (SOS) 3 gene acts as a Ca\(^{2+}\) receptor in plants and is involved in Ca\(^{2+}\) signal-mediated stress responses. This gene, which encodes a calcineurin-like protein, belongs to the CBL gene family and is also known as CBL4. The SOS3 gene was originally identified in the model plant Arabidopsis (Kamei et al., 2010). The SOS3-encoded protein contains EF-hand domains in its C-terminus and a myristylation site in its N-terminal region. Myristylation is important for recruitment of SOS3 to the plasma membrane and for salt tolerance in plants (Quintero et al., 2002). SOS3 physically interacts with SOS2, a serine/threonine protein kinase. In the presence of Ca\(^{2+}\), SOS3 activates the substrate phosphorylation activity of SOS2. The binding of SOS3 to SOS2 is mediated by the 21-amino acid (aa) FISL motif in SOS2 and activates SOS1, a Na\(^{+}/H^+\) antiporter, leading to Na\(^{+}\) efflux from the cytosol (Quintero et al., 2002; Nutan et al., 2018). Moreover, an SOS2–SOS3 interaction in the SOS pathway has also been demonstrated using sos3/sos2 double-mutant Arabidopsis (Guo et al., 2001). In addition to interacting with SOS3 at the plasma membrane, SOS2 has also been reported to interact with and thereby regulate the activity of several tonoplast-localized transporters, such as the Ca\(^{2+}/H^+\) antiporter, vacuolar V-ATPase, and the Na\(^{+}/H^+\) exchanger (Cheng et al., 2004; Batelli et al., 2007; Huertas et al., 2012).

SOS3s and CIPKs from different plant species have been found to function in different tissues in response to abiotic stress. For example, SOS2 and SOS3 specifically mediate salt stress signal transduction in Arabidopsis roots (Qiu et al., 2002; Zhang et al., 2017). OsSOS2 and OsSOS3 can coordinate to activate OsSOS1 in yeast cells and can be exchanged with their Arabidopsis counterparts to form heterologous protein kinase modules that activate both OsSOS1 and AtSOS1 and suppress the salt sensitivity of sos2 and sos3 Arabidopsis mutants (Martinez-Atienza et al., 2007).

The SOS3 gene has also been reported to be involved in plant salt stress responses. For example, in Arabidopsis, SOS3 has been shown to play a unique role in these responses (Kamei et al., 2010). Notably, duplication of SOS3 increases the Ca\(^{2+}\)-mediated signaling capacity in Eutrema and confers increased salt tolerance on salt-sensitive Arabidopsis (Monihan et al., 2019). Overexpression of the SOS3 gene in tobacco increases salt stress by causing exclusion of Na\(^{+}\) from the cytosol and retention of high K\(^{+}\) levels in the cytosol to re-establish ion homeostasis (Li et al., 2013). In Populus trichocarpa, PtSOS1, PtSOS2, and PtSOS3 have been identified to cooperate in the activation of PtSOS1, thus conferring salt tolerance on P. trichocarpa (Tang et al., 2010).

*Tamarix hispida* is a woody halophyte species with excellent salt stress resistance. It can form natural forests in soil with a 1% salt content and is thus an excellent material for research on salt tolerance mechanisms and for cloning of salt tolerance genes.
cis-acting elements of 3 ThSOS genes were analyzed with the Plant Cis-Acting Regulatory DNA Elements (PLACE) database.

RNA Extraction and qRT-PCR Analyses
Total RNA was extracted from T. hispida plants with a Plant RNeasy Extraction Kit (BioTeke, China), and first-strand cDNA was synthesized from 1 µg of purified RNA using a PrimeScript™ RT Reagent Kit (TaKaRa, Beijing, China). qRT-PCR was performed on a qTOWER™ G (Analytik Jena AG, Germany) with the Actin (FJ618517) and β-tubulin (FJ618519) genes as internal controls. Each 20 µL reaction mixture contained 10 µL of SYBR-Green Real-time PCR Master Mix (Toyobo, Shanghai, China), specific primers (0.5 µM each), and 2 µL of cDNA template. Amplification was performed using the following cycling parameters: 94°C for 30 s; 45 cycles of 94°C for 12 s, 58°C for 30 s, and 72°C for 45 s; and then 82°C for 1 s for plate reading (Liu et al., 2018). The relative abundance was determined by the 2−ΔΔCt method (Livak and Schmittgen, 2001). Three replications were included for each sample (the primers used for qRT-PCR are shown in Table 1).

Transient Expression of the Thsos3 Gene in T. hispida
A 642 bp cDNA sequence of ThSOS3 was amplified, cloned into the prokII vector with the CaMV 35S promoter, and named 35S::ThSOS3. A 200 bp truncated inverted-repeat cDNA sequence of ThSOS3 was cloned into pFGC5941 flanking the CHSA intron to generate pFGC5941::ThSOS3 and used to silence the expression of ThSOS3 (the primers for vector construction are listed in Table 1).

T. hispida plants were transiently transformed according to the methods of Zhang et al. (2018). Three groups of transgenic T. hispida plants were generated by transient transformation: 35S::ThSOS3 plants were generated to overexpress ThSOS3 (OE), pFGC5941::ThSOS3 plants were generated to silence the expression of ThSOS3 (SE), and empty pROKII vector-transformed plants were generated as controls (Con). After growth for 12, 24, and 36 h under normal conditions or salt stress, the relative abundance of ThSOS3 in these transformed T. hispida plants was studied using qRT-PCR. Three biological replicates were included that contained at least 60 transformed seedlings.

Stress Tolerance Analysis
Stably transformed Arabidopsis plants were generated by the floral dip method (Clough and Bent, 1998). Two T3 generation homozygous ThSOS3 transgenic lines (OE1 and OE2) of ThSOS3 and the Con (WT) line were sown on 1/2 MS solid medium for 5–7 days and then transferred to soil. After 3 weeks of growth, the seedlings were irrigated with a 150 mM NaCl solution for 5 days and then imaged. The treatments were independently repeated at least three times. Each sample was analyzed and contained at least 15 transformed seedlings.

Biochemical Staining
Hydrogen peroxide (H2O2) and superoxide (O2−) staining were performed by infiltration with 3-3-diaminobenzidine (DAB) or nitro blue tetrazolium (NBT) following the procedures described by Zhang et al. (2011). Evans blue staining was performed to investigate cell death, as described by Liu et al. (2018).

Physiological Index Measurement of Transformed Plants
Transformed T. hispida plantlets were grown on 1/2 MS solid medium supplemented with 150 mM NaCl for 12–36 h. Four-week-old Arabidopsis seedlings were subjected to 150 mM NaCl for 5 days. After treatment, the seedlings were collected and subjected to physiological index analysis. Superoxide dismutase (SOD) and peroxidase (POD) activity and H2O2 content were measured using corresponding reagent kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer’s instructions. The MDA content was determined according to the methods of Dhindsa et al. (1981). Electrolyte leakage was analyzed as described by Ben-Amor et al. (1999). Each sample contained at least nine harvested seedlings, and all of the experiments were repeated at least three times.

Statistical Analyses
Statistical analyses were carried out using Excel software. The data were compared using Student’s t-test, and differences were considered significant if P < 0.05. *Represents a significant difference (P < 0.05), and **represents a very significant difference (P < 0.01).

RESULTS
Gene Identification and Sequence Analysis of ThSOS Genes
In total, five candidate SOS genes were selected and identified. The 5 ThSOS proteins ranged from 213 to 1,165 aa in length (Table 2). Large variations were found in the theoretical pI values (ranging from 4.76 to 6.42) and the MW values (ranging from 22.42 to 128.83 kDa) of the proteins encoded by the five ThSOS genes. The prediction results showed that the five ThSOS genes were localized in the plasma membrane, cytoplasm, extracellular space, or chloroplasts (Table 2). To determine the subclasses of the ThSOS genes, phylogenetic analysis was performed using the sequences of the ThSOS proteins and SOS proteins from other species (Figure 1A and Supplementary Table S1). The results revealed that ThSOS1, ThSOS2, and ThSOS3 genes were closely related to

http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
the SOS1, SOS2, and SOS3 subfamilies in Arabidopsis. ThSOS3 belonged to the CBL4 subfamily and clustered into the same clade as 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 *A. thaliana* (Figure 1B).

**Characterization of cis-Elements in ThSOS Gene Promoters**
Numerous stress-related consensus cis-acting elements were detected, including an ABA-responsive element (ABRE), an antioxidant response element (ARE), and TC-rich repeats. Moreover, ThSOS3 included 1 abiotic stress-related element, the MYB-binding site (MBS); nine hormone stress-related elements, such as the salicylic acid-responsive element (TCA element), methyl jasmonate (MeJA)-responsive element (CGTCA motif or TGACG motif), gibberellin-responsive element (TATC-box), and auxin-responsive element (TGA element) and 1 development-related endosperm expression element (GCN4 motif). ThSOS3 contained an MBS and low temperature response (LTR) response elements consisting of five hormone stress-related elements, auxin-responsive elements (TGA elements or AuxRR core elements), and salicylic acid-responsive elements (TCA elements). Similarly, ThSOS4 contained many abiotic and hormone stress-related elements, as shown in Supplementary Figure S1.

**Expression of ThSOS Genes Under Abiotic Stress and ABA Treatment**
To analyze the relative abundance of ThSOS genes, the expression profiles of the 5 ThSOS genes were measured under different stresses (NaCl or PEG6000 exposure) or hormone treatment (ABA) using qRT-PCR.

In roots under NaCl stress, the expression of most ThSOS genes was upregulated. Notably, ThSOS4 and ThSOS5 exhibited upregulated expression at all stress time points. The highest expression levels of ThSOS4 and ThSOS5 were 8.02- and 4.86-fold higher than control levels, respectively. The other three ThSOS genes, ThSOS1, ThSOS2, and ThSOS3, were downregulated at the initial stress time point and upregulated at later stages. The lowest expression levels of the three ThSOS genes in the roots all occurred at 6 h. The expression levels of ThSOS1, ThSOS2, and ThSOS3 at this time point were 3.55, 6.11, and 0.20% of control levels, 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% of baseline levels, respectively) in the control plants at 6 h. The relative abundance of ThSOS1, ThSOS4, and ThSOS5 was similar to that of ThSOS2 and ThSOS3, but the lowest expression levels were achieved at 24 h (Figure 2A).

Under PEG6000 stress, most of the ThSOS genes were significantly upregulated, and all ThSOS genes achieved their highest expression levels at 72 h. Interestingly, the expression

**TABLE 1 | Primers sequences used in this study.**

| Constructs | Forward and reverse primers (5′–3′) |
|-----------|-----------------------------------|
| ThSOS1    | CTAGCAGTCTGATCTGGATCTAT ATGCTAGACTGAAGAAATCGGT | ATGCTAGACTGAAGAAATCGGT |
| ThSOS2    | AGTAGAGGCTTGTACGAGGCT | ACGCAGTGATCGCTACGATCAT |
| ThSOS3    | TGAAGTGGTGATCGCAATTC | CCATACAGGATGACCATCATGATAT |
| ThSOS4    | GAAATTTGCTATTAGAGAATGACAT | CCACTATGCTCCAAAGGTATCT |
| ThSOS5    | ATAGCCACCATGGGACGCTTTT | ACCCTTGTGACTGAGAACCT |

**TABLE 2 | Features of ThSOS genes in *T. hispida*.**

| Name      | Locus   | ORF (bp) | Introns | Protein length | Theoretical pl | Aliphatic index | Molecular weight (kD) | Localization predictions |
|-----------|---------|----------|---------|---------------|----------------|-----------------|----------------------|-------------------------|
| ThSOS1    | Unigene222899 | 3,498    | 11      | 1,165         | 6.42           | 103.24          | 128.83               | Plasma membrane          |
| ThSOS2    | Unigene13265 | 1,371    | 2       | 456           | 6.29           | 92.08           | 51.44                | Cytoplasmic             |
| ThSOS3    | Unigene1212 | 642      | 6       | 213           | 4.76           | 96.53           | 22.42                | Cytoplasmic             |
| ThSOS4    | Unigene2429 | 927      | 11      | 308           | 6.22           | 105.1           | 33.58                | Extracellular or Chloroplast |
| ThSOS5    | Unigene1744 | 675      | 0       | 224           | 5.00           | 88.39           | 25.57                | Cytoplasmic             |
levels of *ThSOS1*, *ThSOS2*, and *ThSOS3* in the roots were significantly downregulated after 6 h of PEG6000 stress (5.39, 16.67, and 0.16% of the control levels, respectively). In leaves, the expression levels of the *ThSOS1*, *ThSOS2*, and *ThSOS3* genes were mainly downregulated throughout the stress period, and *ThSOS1* and *ThSOS2* achieved their lowest expression levels at 24 h. However, *ThSOS3* reached its lowest expression level during the early stage 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 (in addition to 24 h) and peaked at 72 h. The expression of *ThSOS4* did not change significantly under PEG6000 stress (Figure 2B).

Under ABA stress, the relative abundance of *ThSOS1*, *ThSOS4*, and *ThSOS5* was significantly upregulated in the roots. The most strongly upregulated gene was *ThSOS1*; its expression peaked at levels 161.28-fold higher than control levels at 12 h. The relative expression of *ThSOS2* was mainly upregulated except at 12 h, when its expression was only 24.3% of the control level. However, *ThSOS3* expression was clearly downregulated at 6 h (0.4% of the control level) and showed no significant changes at any other stress time points. In leaves, no significant changes were found in the expression of any of the *ThSOS* genes except at 6 h. All the *ThSOS* genes (*ThSOS1*, *ThSOS2*, *ThSOS3*, *ThSOS4*, and *ThSOS5*) reached their lowest expression levels under ABA stress at 6 h (0.55, 0.83, 0.07, 1.31, and 3.17% of the control levels, respectively) (Figure 2C).

### Transient Expression of *ThSOS3* in *T. hispida*

To ascertain whether the *ThSOS3* gene was successfully transiently overexpressed and suppressed in *T. hispida*, the *ThSOS3* transcript levels in Con, OE, and SE plants were examined by qRT-PCR. Compared with that in Con plants, the *ThSOS3* expression in OE plants was significantly increased under salt stress conditions, while that in SE plants was significantly decreased (Figure 3), indicating that the gain and loss of function of *ThSOS3* in *T. hispida* plants were successfully achieved.

### *ThSOS3* Confers Salt Stress Tolerance on Transgenic Plants

To preliminarily explore the function of the *ThSOS3* gene, DAB staining and NBT staining were performed, and the related physiological indexes of three differently transformed...
FIGURE 2 | Expression analysis of the 5 ThSOS genes in the roots and leaves in response to abiotic stresses (NaCl and PEG6000 exposure) and hormone (ABA) treatment. (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.

*T. hispida* plants were studied. The reactive oxygen species (ROS) accumulation levels in OE, SE, and Con plants before and after abiotic stress were determined by DAB and NBT staining. Under salt stress, the staining intensity in OE plants was lower than that in Con plants, while that in SE plants was higher than that in Con plants (Figures 4A,B). Additionally, H$_2$O$_2$ and MDA levels were measured in different transgenic *T. hispida*. The results failed to demonstrate differences in H$_2$O$_2$ and MDA levels among the three transiently transgenic plants under normal conditions. However, under salt stress, SE plants showed the highest H$_2$O$_2$ and MDA levels, followed by Con plants; the OE plants had the lowest H$_2$O$_2$ and MDA levels. The levels of H$_2$O$_2$ and MDA in SE plants were 1.27 and 1.53 times those in Con plants, respectively. However, the H$_2$O$_2$ and MDA levels in OE plants were the lowest at only 82.02 and 85.2% of those in Con plants, respectively, at 24 h (Figures 4D,E).

ThSOS3 Improves ROS-Scavenging Capability

The antioxidant enzymes SOD and POD are the two most important ROS-scavenging enzymes influencing cellular ROS levels. Thus, we further studied POD and SOD activity. Under normal growth conditions, there were no obvious differences in activity levels between Con and transgenic *T. hispida*. However,
SOD and POD activity levels were significantly increased in OE plants under salt stress. At 24 h, the activity levels of SOD and POD in OE plants were 1.35 and 1.24 times those in Con plants, while the activity levels in SE plants were only 80 and 83.73% of those in Con plants, respectively (Figures 4F,G).

Similarly, in Arabidopsis, SOD and POD activity did not obviously differ among the studied lines in the absence of stress. Under salt stress, the two OE lines showed higher antioxidant enzyme (SOD and POD) activity than the WT line (Figures 5E,F), consistent with the results obtained in T. hispida.

Cell Death and Electrolyte Leakage Analysis

Evans blue staining was used to assess cell membrane damage on the basis of the intensity of the staining under salt stress. Compared with the Con plants, the OE plants presented light-blue puncta with smaller areas under salt stress, while SE plants presented larger staining areas (Figure 4C). We then measured electrolyte leakage. Electrolyte leakage did not significantly differ among the three differently transformed T. hispida plants under normal conditions. Under salt stress, the relative electrolyte leakage rates of SE plants were the highest at 24 h at 1.14 times those of Con plants, while the rates of the OE plants were 0.82 times those of the Con plants (Figure 4H). Moreover, we detected changes in the levels of corresponding physiological indicators in Arabidopsis, which were consistent with the changes in T. hispida (Figures 5C,G).

DISCUSSION

The SOS gene plays an important role in plants, and its function has been studied in A. thaliana (Qiu et al., 2004; Yang et al., 2009; Wang et al., 2019), Nicotiana tabacum (Yue et al., 2012), Oryza sativa (El Mahi et al., 2019), Gossypium raimondii (Che et al., 2019), Lycopersicon esculentum (Olias et al., 2009), Zea mays (Zörb et al., 2005), and P. trichocarpa (Tang et al., 2010; Zhou et al., 2014). However, few studies have investigated the salt tolerance function of ThSOS in T. hispida.

In our study, five monomorphic and intact ThSOS genes were selected. An unrooted phylogenetic tree and multiple sequence alignment analysis showed that ThSOS shared 85.92, 84, and 70% identity with PtrSOS3, MnSOS3, and AtSOS3-1, respectively. It has been reported that AtSOS3 and PtrSOS3 enhance salt tolerance in Arabidopsis and P. trichocarpa (Quan et al., 2007; Tang et al., 2010). The relative abundance of most ThSOS genes in T. hispida was significantly changed under NaCl, PEG6000, and ABA stresses. Notably, ThSOS3 expression was significantly downregulated under salt stress at 6 h. SOS3 is a Ca2+-regulated upstream regulatory protein of the SOS pathway and plays important roles in plant salt stress response pathways (Yang et al., 2009). Kim et al. (2013) confirmed that AtSOS3 expression is strongly induced by NaCl treatment. In addition, overexpression of LeSOS3-1 enhances salt stress tolerance in tobacco by regulating stress-associated physiological changes, such as by enhancing ROS-scavenging capability and maintaining K+/Na+ homeostasis.

Regulatory elements in promoter sequences are essential for the temporal, spatial, and cell type-specific control of gene expression (Jiang et al., 2014). Previous studies have shown that many abiotic and hormone stress-related elements are present in ThSOS gene promoters. As shown in Supplementary Figure S1, ABREs, AREs, and TC-rich repeats were found in the promoters of three ThSOS genes. The LTR element and MBS element were found in the promoter of the ThSOS3 gene. ThSOS3 also contained 4 hormone stress-related elements (a TGA element, a TCA element, an ABRE, and an AuxRR-core element). This result indicates that ThSOS might be involved in stress responses (to abiotic stress and hormone treatment) as well as in plant
FIGURE 4 | Analysis of ROS-scavenging capability and cell death in *T. hispida* plants with overexpression or RNAi-mediated knockdown of ThSOS3. (A) NBT and (B) DAB staining were performed to detect O$_2^-$ and H$_2$O$_2$, respectively. (C) Evans blue staining was used to analyze cell death. Young branches from transformed *T. hispida* plants treated with 150 mM NaCl for 2 h were used for DAB, NBT, and Evans blue staining. (D–H) Analysis of H$_2$O$_2$ and MDA content, SOD and POD activity, 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 H$_2$O$_2$ (D) and MDA (E) content, SOD (F), and POD (G) activity, and electrolyte leakage (H). *Represents a significant difference ($P < 0.05$). **Represents a very significant difference ($P < 0.01$).

development. Moreover, previous studies have shown that OSBZ8 mediates salt and dehydration stress tolerance by binding to the ABRE motif (Narusaka et al., 2003), and AtMYB44 inhibits oxidative damage and hypersensitivity to abiotic stresses by binding to MBSs to activate the expression of related downstream genes (Persak and Pitzschke, 2014).

When the results of the analysis of cis-elements in ThSOS gene promoters and the phylogenetic analysis were combined,
ThSOS3 was shown to contain abundant abiotic and hormone stress-related elements and to be closely related to AtSOS3 and PtrSOS3. Therefore, we predict that ThSOS3 might also play roles in responses to salt stress.

Plants produce high levels of ROS in adverse environments. ROS act as signaling molecules to control several physiological processes (Liu et al., 2018). Baxter et al. (2013) found that H$_2$O$_2$ and O$_2^\cdot$ signaling networks are involved in responses to abiotic stimuli. Under salt stress, the staining intensity was lower in OE plants and higher in SE plants than in Con plants. Furthermore, the H$_2$O$_2$ levels were consistent with the DAB and NBT staining results in transgenic *T. hispida* plants. The results showed that overexpression of ThSOS3 resulted in the lowest H$_2$O$_2$ and MDA accumulation. Conversely, compared to ThSOS3 overexpression, RNAi silencing induced the opposite physiological changes among the three differently transformed *T. hispida* plants. Next, Evans blue staining was performed to assess cell death in *T. hispida* plants under salt stress. The results indicated that compared with Con plants under salt stress, OE plants under salt stress presented light blue puncta with smaller areas, while SE plants showed the opposite results. We then measured electrolyte leakage, which did not significantly differ among the three differently transformed *T. hispida* plants under normal conditions. Under salt stress, the relative electrolyte leakage rates of SE plants were the highest at 24 h at 1.14 times those of Con plants; moreover, those of the OE plants were 0.82-fold those of the Con plants. The electrolyte leakage assay further confirmed the Evans blue staining results. Similar to our study, a previous study revealed that overexpression of the wheat TaAQP8 gene can confer salt tolerance on transgenic tobacco plants by maintaining ionic balance, reducing H$_2$O$_2$ accumulation and reducing membrane damage (Hu et al., 2012). In addition,
FIGURE 6 | Salt stress tolerance associated with ThSOS3. (A) Growth comparison between OE and WT plants. Arabidopsis plants grown on 1/2-strength MS medium (control) and 1/2-strength MS medium supplied with 120 mM NaCl were used for growth analysis. (B) Root length and (C) fresh weight were also analyzed in at least 30 seedlings under each treatment. (D) Comparison of growth phenotypes between OE and WT Arabidopsis lines grown in soil. The plants were treated with 150 mM NaCl for 5 days for analysis. Plants grown under normal conditions were used as controls. *Represents a significant difference ($P < 0.05$). **Represents a very significant difference ($P < 0.01$).
ThWRKY4 can improve tolerance to salt and ABA treatment by increasing SOD and POD activity, decreasing O$_2^-$ and H$_2$O$_2$ levels, reducing electrolyte leakage, preventing chlorophyll loss, and protecting cells from death (Zheng et al., 2013).

As ROS levels were significantly altered, we further studied the activity of POD and SOD, which are the two most important ROS-scavenging enzymes. In the absence of stress conditions, the activity levels of these enzymes did not significantly differ among the Con, OE, and SE plants. However, SOD and POD activity levels were significantly increased in OE plants under salt stress. At 24 h, the activity levels of SOD and POD in OE plants were 1.35 and 1.24 times those in Con plants, while the levels in SE plants were 80 and 83.73% of Con plants, respectively. As in our study, ThZFPI was shown to enhance salt and osmotic stress tolerance in a previous study by positively regulating proline accumulation and SOD and POD activity (Zang et al., 2015). In addition, ThNAC7 has been found to induce the transcription of genes associated with stress tolerance to enhance salt and osmotic stress tolerance by increasing osmotic potential and enhancing ROS scavenging (He et al., 2019). In summary, the physiological indicator results suggest that ThSOS3 confers salt stress tolerance by increasing the activity of antioxidant enzymes (SOD and POD), reducing ROS accumulation, and decreasing the MDA content and lipid peroxidation of cell membranes.

**CONCLUSION**

The SOS gene plays important roles in responses to salt stress. However, few studies have evaluated the roles of ThSOSs in salt tolerance in *T. hispida*. In this study, five ThSOS genes were cloned and identified. Their expression patterns in response to different abiotic stresses (NaCl and PEG$_{6000}$ exposure) and hormone (ABA) stress were analyzed using qRT-PCR. The expression levels of most ThSOS genes were significantly altered under NaCl, PEG$_{6000}$, and ABA treatment in at least one organ. Notably, ThSOS3 expression was significantly downregulated under salt stress at 6 h. Furthermore, the role of ThSOS3 in salt tolerance was studied. The results showed that overexpression of ThSOS3 confers salt stress tolerance on *T. hispida* by enhancing antioxidant enzyme activity, improving ROS-scavenging capability and decreasing the MDA content and lipid peroxidation of cell membranes. This study provides a foundation for further elucidation of salt tolerance mechanisms involving ThSOSs in *T. hispida*. However, the molecular mechanism by which ThSOS3 confers salt stress tolerance on *T. hispida* is unclear. Future studies should focus on ThSOS3 mechanisms under salt stress.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

**AUTHOR CONTRIBUTIONS**

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

**FUNDING**

This work was supported by grants from the Province in Heilongjiang Outstanding Youth Science Fund (JC2017004), the National Natural Science Foundation of China (31670679), the Overseas Expertise Introduction Project for Discipline Innovation (B16010), and the Heilongjiang Touyan Innovation Team Program (Tree Genetics and Breeding Innovation Team) to GC.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2020.597480/full#supplementary-material

**Supplementary Figure 1** | (A) Locations of cis-elements in the ThSOS gene promoters. (B) Descriptions of the cis-elements.

**Supplementary Table 1** | Classification of SOS genes in different species.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.