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Overexpression of *PtSOS2* Enhances Salt Tolerance in Transgenic Poplars

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Abstract Protein kinases are major signal transduction factors that have a central role in mediating acclimation to environmental changes in eukaryotic organisms. In this study, we cloned and identified three salt overly sensitive 2 (*SOS2*) genes in the woody plant *Populus trichocarpa*, designated as *PtSOS2.1*, *PtSOS2.2*, and *PtSOS2.3*, which were transformed into hybrid poplar clone T89 (*Populus tremula*× *Populus tremuloides* Michx clone T89) mediated by *Agrobacterium tumefaciens*. Southern and northern blot analyses verified that the three genes integrated into the plant genome, and were expressed at a stable transcription level. Meanwhile, overexpression of all three *PtSOS2* genes did not retard the growth of plants under normal conditions. Instead, it promoted growth in both agar-medium and soil conditions in response to salinity stress. Under salt stress, overexpression of *PtSOS2.1*, *PtSOS2.2*, and *PtSOS2.3* increased the concentrations of proline and photosynthetic pigments, and the relative water content (RWC), and the activity of antioxidant enzymes, and decreased the malondialdehyde (MDA) concentrations in transgenic lines compared to the control. These results suggest that overexpression of *PtSOS2* plays a significant role in improving the salt tolerance of poplars, reducing the damage to membrane structures, and enhancing osmotic adjustment and antioxidative enzyme regulation under salt stress.

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Introduction

Because of their sessile nature, plants must adjust to a multitude of external stimuli and coordinate their growth and development accordingly (Santner and Estelle 2009). High salinity triggers various biochemical and physiological responses in plants, including alterations in gene expression and accumulation of osmolytes and changes in activities of antioxidant enzymes (Fujita et al. 2009). The phosphorylation/dephosphorylation of proteins is a major mechanism for the post-translational regulation of protein activity and the transduction of intracellular signals in eukaryotic organisms (Halford and Hey 2009; Ying et al. 2011). Protein kinases are key elements involved in signal transduction responses to metabolism and biotic and abiotic stresses, including the major environmental factor, salinity (Sanz 2003; Diedhiou et al. 2008). A large superfamily consisting of seven types of serine–threonine protein kinases, including sucrose non-fermenting 1-related protein kinases (SnRK), were recently identified (Hrabak et al. 2003).

Based on sequence similarity and gene structure, SnRKs have been grouped into three subfamilies: SnRK1, SnRK2, and SnRK3 (Coello et al. 2011). SnRK1 plays a major role in the regulation of carbon metabolism and energy status (Cho et al. 2012b), while SnRK2 and -3 have been implicated in stress and abscisic acid (ABA)-mediated signaling pathways (Coello et al. 2012). SnRK2 and SnRK3 are unique to plants. Overexpression of SnRK2 was reported to enhance multiple stress tolerance in many species, such as *Arabidopsis* (Boudsocq et al. 2004; Umezawa et al. 2004), rice (Kobayashi et al. 2004), and wheat (Du et al. 2013). SnRK3s are thought to be calcium-dependent because they interact

with calcineurin B-like (CBL) calcium-binding proteins (Guo et al. 2001), and for this reason are also known as CBL-interacting kinases (CIPKs) (Luan et al. 2002).

In SnRK3s, the salt overly sensitive (SOS) signal-transduction pathway is important for ion homeostasis and salt tolerance in plants, which consists of three main components, SOS1, SOS2, and SOS3 (Yang et al. 2009; Huang et al. 2012). *SOS1*, encoding a plant plasma membrane Na^+/H^+ antiporter protein, which catalyzes the exchange of Na^+ for H^+ across membranes (Raquel et al. 2009; Yang et al. 2009; Fraile et al. 2010), plays major roles in removing Na^+ from the cytosol or compartmentalizing it in vacuoles for maintenance of low Na^+ concentrations in plants and controls long-distance Na^+ transport (Shi et al. 2002; Ana et al. 2010). Overexpression of the *SOS1* gene was reported to improve plant salt tolerance in *Arabidopsis* (Yang et al. 2009), tomatoes (Olias et al. 2009), and *Populus* (Wu et al. 2007). SOS3, the first SOS gene cloned, encodes an EF-hand $Ca2^+$-binding protein that functions as a calcium sensor for salt tolerance (Ye et al. 2013) and is required for plant survival under K^+ starvation (Liu and Zhu 1997; Zhang et al. 2011). SOS2 is a serine/threonine protein kinase of the SnRK3/CIPK family (Kolukisaoglu et al. 2002) with an N-terminal catalytic domain similar to SNF1/AMPK and a C-terminal regulatory domain whose function is essential for salt tolerance (Liu and Zhu 1998; Halfter et al. 2000). SOS2 physically interacts with SOS3 to modulate the activity of the plasma membrane H+/Na+antiporter SOS1 in a $Ca2^+$-dependent manner (Halfter et al. 2000; Ursula et al. 2000; Batelli et al. 2007). The SOS2/SOS3 kinase complex phosphorylates and activates SOS1 and also upregulates SOS1 gene expression, thus leading to Na^+ extrusion out of the cell (Qiu et al. 2002). SOS2 is a key regulator of ion transporters (Zhu 2002), some of which have been shown to confer increased salt tolerance when overexpressed in transgenic plants (Liu et al. 2000).

Plants have evolved complex mechanisms to overcome salt stress through the synergistic action of various antioxidants and osmoprotectants (Whaibi et al. 2011). Proline, an important soluble osmolyte, plays a pivotal role in osmotic adjustment and cell structure protection to improve plant salt tolerance (Abraham et al. 2010; Hou et al. 2013). Otherwise, salt stress is thought to trigger oxidative stress in plants (Ahmad et al. 2010; Espin et al. 2011). A high concentration of NaCl impairs electron transport and increases the formation of reactive oxygen species (ROS), such as singlet oxygen, H_2O_2, $O_2^{\cdot-}$, and hydroxyl radicals (Mishra et al. 2011; Nounjan et al. 2012). Plants are equipped with an array of enzymatic and non-enzymatic antioxidant molecules to scavenge ROS (Krantev et al. 2008; Mishra et al. 2011), which are created by cell membrane damage (Abdullahil et al. 2010). These mechanisms employ factors such as antioxidant enzymes, including superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and MDA (Dong et al. 2010; Mishra et al. 2011). In *Populus*, transformed poplars accumulated more chlorophyll and proline, improving salt tolerance (Ben et al. 2010; Su et al. 2011). Enhancing the activity of antioxidant enzymes is known to contribute to salt tolerance in plants synergistically with proline (Arbona et al. 2008; Mishra et al. 2011; Cho et al. 2012a).

The *SnRK3* gene family member, *SOS2*, was identified at the molecular level, including the activity of protein kinase and the critical role of the conserved domain to improve plant salt tolerance. No *SOS2* genes have been identified in *Populus*, and no characterizations of the physiological or biochemical properties of poplar *SOS2* have been reported. In this study, three *PtSOS2* genes were cloned and transformed into poplar and the physiological and biochemical effects thus induced were determined. Additionally, the effects of *PtSOS2* overexpression on the antioxidant defense system and osmotic protection against salt stress were evaluated.

Materials and Methods

Plant Materials

Populus trichocarpa was used to isolate total RNA for cloning of *PtSOS2*, and a hybrid poplar clone (*Populus tremula* × *Populus tremuloides* Michx clone T89) was used for genetic transformation. *P. trichocarpa* and a hybrid poplar clone T89 were grown under 16-h light/8-h dark conditions at 25 °C under confined culture room conditions, and 1/2 MS medium was used for plant growth.

Cloning and Sequence Analysis of *PtSOS2*

To identify the *PtSOS2* genes, the cDNA sequence of *Arabidopsis AtSOS2* was used to search against the *Populus* genome database (http://genome.jgi-psf.org/cgi-bin/runAlignment?Db=Poptr1) using the BLAST program. Among the obtained sequences, three with the highest similarity to *AtSOS2* were chosen for further amplification. Primers were designed to amplify the putative cDNA sequences. The PCR products were cloned into the PMD-18 vector for sequencing. The sequences were submitted to GENESCAN@ Prediction (http://genes.mit.edu/GENSCAN.html) for analysis.

After all *Arabidopsis*, rice, maize, wheat, tomato, and *Populus* sequences had been obtained, phylogenetic analyses were performed using the neighbor-joining (NJ) method in MEGA (5.0). Bootstrap analysis was performed using 1,000 replicates in MEGA (5.0) to evaluate the reliability of different phylogenetic groups.

Construction of the Overexpression Vector

Full-length cDNAs of *PtSnRKs* were cloned into PH35GS using Gateway technology. The cDNAs were first cloned into

entry vectors using pENTR/D-TOPO cloning, as per the manufacturer's instructions (Invitrogen, USA) mediated by the BP clonase enzyme (Invitrogen). Then, the reaction mixture was transformed into chemically competent *Escherichia coli* (DH5α), and colonies were selected on LB agar plates with kanamycin (50 mg/l). The recombinant entry vectors were subsequently recombined with the modified destination vector pH35GS at a ratio of 1:1.5 (v/v) in a 15-μl reaction mix mediated including 2 μl of LR clonase II plus enzyme mix (Invitrogen). The reaction was performed at 25 °C for 3 h and terminated with Proteinase K (1 μl). The recombined constructs (Supplemental Data 1) were transformed into *Escherichia coli* (DH5α) and recombinant clones were selected with spectinomycin (50 mg/l) (Vemanna et al. 2012).

Genetic Transformation

T89 poplar stems were excised (1-cm length) and pre-cultured for 3 days on MS1 medium. *Agrobacterium tumefaciens* strain LBA4404 carrying the binary vector was used to infect plants. After co-cultivation in the dark for 4 days, the discs were washed with sterilized water containing 200 mg/l cefotaxime and then transferred to MS2 medium. Regenerated shoots were selected with 20 mg/l hygromycin in 1/2 MS. The culture medium MS1 and screening medium MS2 were adapted from Bi et al. (2009). Ten transgenic putative plants were selected and propagated for PCR and Southern blotting.

Southern and Northern Blotting of Transgenic Plants

Genomic DNA was isolated from the leaves of both the transgenic plants and the wild-type strain using the CTAB method (Riahi et al. 2010). Genomic DNA (10 μg) was digested with EcoRI, size-fractionated on a 1 % (w/v) agarose gel, transferred to a nylon membrane (Hybondn-Amersham, Sao Paulo, Brazil) and hybridized according to the manufacturer's instructions. A digoxygenin (DIG)-labeled *hyg* marker gene cDNA fragment was synthesized by PCR and used as a probe for Southern blotting. The primers used to amplify the *hyg* marker gene were *hyg* F (5′-AGATCGTTATGTTTATCGGCACTT T-3′) and *hyg* R (5′-CAAGCTCTGATAGAGTTGGTCA AGA-3′). Pre-hybridization, washing, and chemiluminescent detection of the blots were performed according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The specific primers designed for northern blotting probes were:

PtSOS2.1-F: 5′-GAAGGATAAGAATCGAAGA CAGG-3′
PtSOS2.1-R: 5′-CTTGCGGTTGCTGCACTTCCC TGC-3′
PtSOS2.2-F: 5′-GAGGAAGAGGGAGGAAAAGA GG-3′
PtSOS2.2-R: 5′-GCAAGAGTTGAATTCTCAGCAGG AG-3′
PtSOS2.3-F: 5′-GCAGGCAGGTTTTGTGAAACG AG-3′
PtSOS2.3-R: 5′-TTAATCCTTCTATAGTTTGATC AG-3′.

Total RNA was extracted from the leaves of transgenic poplars using TRIzol Reagent (QIANGEN, China). RNA gel blotting analysis was performed using DIG-High Prime (Roche Diagnostics).

Transplantation of Plantlets and Growth Evaluation

The plantlets were grown on agar-medium for 4 weeks in a confined culture room. Four to five leaves had grown on the top bud of the plantlet before being moved to a greenhouse. After a 3-day acclimatization, the plantlets were transplanted into the soil. The soil was then mixed with sterilized peat and perlite (2:1). During the first 2 weeks, water-spraying and vinyl membranes were used to maintain humidity.

Salt-Stress Treatment

To assess the performance of *PtSOS2* transgenic lines in agar-medium under increasing salt concentrations, 15 of each transgenic and wild-type poplar strain were cultured in agar-medium containing 0, 17, 51, 85, and 120 mM NaCl for 2 weeks.

To verify the salt tolerance and growth of transgenic poplars, the plants transplanted into pots of soil were irrigated with salt solution for 45 days. During the first week, 17 mM NaCl salt solution was added to the plants every 2 days. The concentration was increased by 17 mM every 2 days up to 85 mM. The amount of watering was consistent and a tray was placed under the plot to maintain the salt content of the soil. After 3 weeks of salinity stress, the growth state and the concentrations of proline, SOD, POD, CAT, and MDA were measured.

Determination of Leaf Relative Water, Chlorophyll, Proline, SOD, POD, CAT and MDA Concentrations

For leaf relative water content measurements, leaf samples were collected after 2 weeks of salt treatment. Fresh leaves at the same position of the poplars were cut to measure the leaf fresh weight (W_f). After soaking in distilled water for 24 h in Petri dishes in the dark, turgid leaf weight (Wt) was measured. The samples were dried at 80 °C for 48 h, and leaf dry weights (W_d) were obtained. Relative water contents (RWC) were calculated as:RWC (%)=$(W_f - W_d)/(W_t - W_d) \times 100$ % (Chakraborty et al. 2012). To analyze chlorophyll a+b contents, one piece of leaf from the same position of the 2-week salt-treated plants

was pooled. Then, the leaves were cut into pieces with a razor blade and immersed into 3-ml N,N-Dimethylformamide (DMF) solution at 4 °C in the dark overnight until completely de-colored. Absorbances at 663.8, 646.8, and 750 nm were then determined using a spectrometer. Chlorophyll (a+b) concentrations were calculated using the following equation: Chlorophyll concentration (μg/ml)=7.12×(A663.8 − A750)+ 17.67×(A646.8 − A750) (Arnon 1949). Superoxide dismutase (SOD, EC1.15.1.1) activity, catalase (CAT, EC1.11.1.6) activity and malondialdehyde (MDA) concentration measurements in transgenic and wild-type poplar were determined according to the methods of Satoh (1978), Stewart and Bewley (1980), and Wang et al. (2012). Peroxidase (POD, E.C. 1.11.1.7) activity was measured using the method of Pagariya et al. (2012) and proline content was determined as described by Lei et al. (2007).

Statistical Analysis

All experiments were performed in triplicate. The data were analyzed using one-way ANOVA, and subsequent post hoc multiple comparisons of Duncan's test by SPSS 13.0 software. The histogram was plotted using Sigma Plot 13.0 software.

Results

Cloning and Sequence Analysis of *PtSOS2*

Using a blast search, full-length cDNAs of *SOS2* genes were cloned and designated *PtSOS2.1*, *PtSOS2.2*, and *PtSOS23*. *PtSOS21* had an open reading frame (ORF) of 1,356 bp encoding 451 amino acids (aa), with a predicted molecular mass of 50.22 kDa and a pI of 9.54. *PtSOS2.2* had an ORF of 1,329 bp encoding 442 aa with a predicted molecular mass of 49.22 kDa and a pI of 9.11. *PtSOS23* had an ORF of 1,320 bp and encoded a 439-aa protein with a predicted molecular mass of 48.89 kDa and a pI of 9.55. The chromosomal locations of these three genes were determined using GENESCAN prediction (http://genes.mit.edu/GENSCAN.html) (Table 1).

Phylogenetic Analysis and Identification of Conserved Motifs

To elucidate the phylogenic relationship between PtSOS, known SOS proteins, and SRK proteins of other plant species, phylogenic analysis was performed using MEGA 5.0. Twenty-seven full-length amino acid sequences from *A. thaliana* (AtSnRK2.1-2.8, AtSOS1-3, AtKIN10), maize (ZmSnRK2.1-2.8, ZmCDPK, ZmPK4), *Oryza sativa* (OSK1, OsPK4), *Solanum lycopersicum* (SlSOS1), and *Triticum aestivum* (TWPK4) were obtained from GenBank. Three groups (I–III) were recognized in the phylogenetic tree (Fig. 1). AtSnRK1.3, AtKIN10, and OSK1 were subdivided into group I, and AtSnRK2.1-2.8 and ZmSnRK2.1-2.8 were clustered together in group II. ZmCDPK, ZmPK4, SlSOS1, TWPK4, and AtSOS1-3 formed group III. Meanwhile, PtSOS2.1, PtSOS2.2, PtSOS2.3, and AtSOS2 were clustered in the same clade.

SOS2 contains a sucrose-non-fermenting protein kinase 1/AMP-activated protein kinase-like N-terminal catalytic domain with an activation loop and a unique C-terminal regulatory domain with a FISL motif that binds to the calcium sensor *Salt Overly Sensitive 3* (Fig. 2a). Alignment of the amino acid sequences of PtSOS2.1, PtSOS2.2, PtSOS2.3*,* AtSOS2, ZmPK4, OsPK4, and TaWPK4 shows that the activation loop and FISL motif of these proteins are highly conserved (Fig. 2b). The activation loop between the conserved DFG and APE motifs (dots) and the conserved residues in all members of the SOS2 subfamily kinase are marked by asterisks (Ser, Tyr, and Thr) (Fig. 2b) and may be phosphorylated by an upstream protein kinase. It was reported that the conserved domain is a critical requirement for activation of protein kinase for plant salt tolerance (Gong et al. 2002a; Gong et al. 2002b). This result indicates that PtSOS2.1, PtSOS2.2, and PtSOS2.3 are quite likely responsive to serine/threonine protein kinases and may play an important role in improving the salt tolerance of poplars through phosphorylation by an upstream kinase in the putative activation loop.

Characterization of Transgenic *PtSOS2* Poplars

To investigate the function of *PtSOS2* in poplars, we constructed an overexpression vector under the control of CaMV35S and transformed it into poplars using an *Agrobacterium* method. In total, 40 *PtSOS2.1* transgenic lines, 35 *PtSOS2.2* transgenic lines, and 30 *PtSOS2.3* transgenic lines were obtained in this study. Gene integration and expression were confirmed by Southern and northern blotting in plants from ten (four lines of *PtSOS2.1*, three lines of *PtSOS2.2*, and three lines of *PtSOS2.3*) putative transgenic lines. The genomic DNA from poplar transformants was isolated, digested by EcoRI, and hybridized with the 580-bp probe fragment derived from hyg. Southern blotting analysis (Fig. 3a) revealed that the three genes *PtSOS2.1*, *PtSOS2.2*, and *PtSOS2.3* had integrated into the genome stably at two to four copies per genome. To explore the expression levels of the three *PtSOS2* genes, northern blotting was performed. As shown in Fig. 3b, the ten lines containing *PtSOS2* genes were expressed in the transgenic poplars to different levels. Among them, L1-60 of *PtSOS2.1*, L2-27 of *PtSOS2.2*, and L3-7 of *PtSOS2.3* were expressed abundantly. Southern and northern blotting suggested that the genes were integrated into the genome and were expressed stably at the transcript level.

To examine the salt tolerance of *PtSOS2* genes, poplar plants were grown on MS agar medium containing 0, 17, 51, 85, and 120 mM NaCl. Under normal conditions (0 mM salt stress), both the transgenic and wild-type poplars maintained

Table 1 Sequence analysis of *PtSOS2*

Gene	GENESCAN@ Prediction			
	ORF	Polypepti	Chromosome location	E value
PtSOS2.1	1,356 bp	451aa	Poptr1_1LG_XI: 8972840-8974195	3.00E-35
PtSOS2.2	1,329 bp	442aa	Poptr1_1LG_XIV: 3989270-3994524	4.00E-51
PtSOS2.3	1,320 bp	439aa	Poptr1_1scaffold_874: 7059-8851	3.00E-44

normal growth (Fig. 4a). Under mild salt stress (17 mM NaCl), there was no obvious growth reduction in the transgenic poplar lines, while the leaves of wild-type poplars turned slightly yellow and only 20 % of wild-type poplars survived (Fig. 4b). This showed that under mild salt stress, transgenic lines could maintain normal growth while that of the wild-type was reduced. Under moderate salt (51 mM NaCl) stress, most leaves of wild-type poplars appeared withered and yellow, and after 10 days the plants stopped growing (Fig. 4a). However, the edge of the leaves of transgenic lines turned yellow but their growth remained more efficient in moderate salt stress (Fig. 4a). When treated with high salt stress (85 mM), the wild-type could not survive but all transgenic lines remained unaffected until day 16. Some of the transgenic lines stopped growth. Under severe salt stress (120 mM NaCl), all the transgenic lines were maintained for only 7 days.

To investigate the damage caused by salt stress in the poplars, we investigated the survival rate of both wild-type and transgenic poplars on agar medium under mild (17 mM NaCl), moderate (51 mM NaCl), high (85 mM NaCl), and severe (120 mM NaCl) salt stress conditions. As shown in Fig. 4b, the survival rate of transgenic lines was markedly improved compared to wild-type poplars in agar medium. Under mild salt stress, the survival rate of wild-type poplars was decreased to 20 %, but all transgenic lines maintained normal growth. Under moderate and severe salt stress, there was almost no survival in wild-type poplars. However, 80 % of *PtSOS2.1* and *PtSOS2.2* transgenic lines survived,

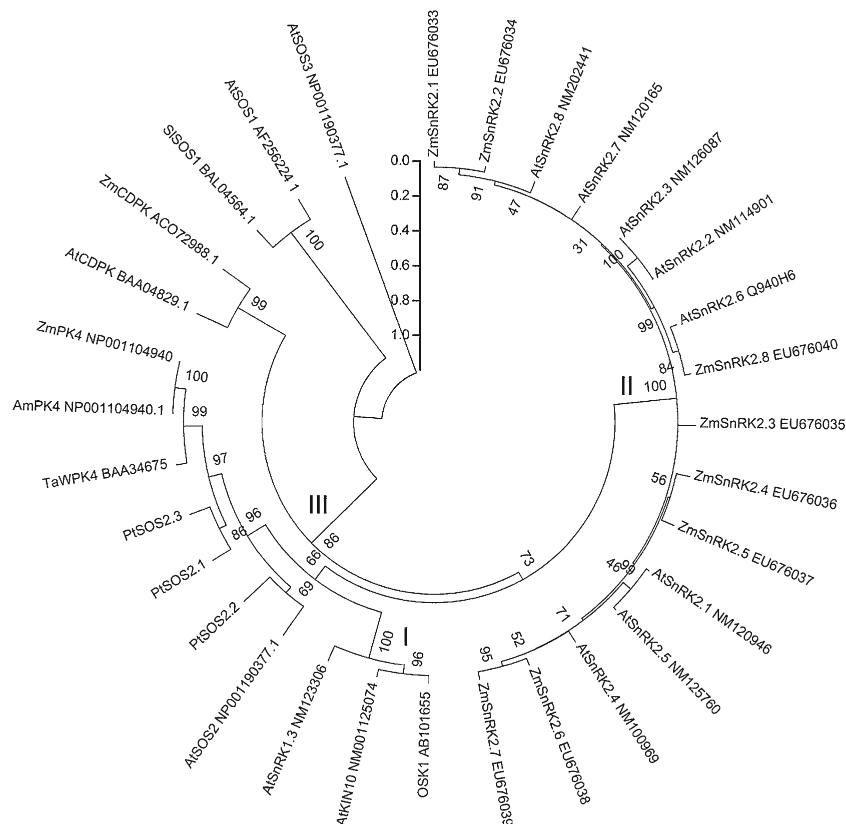


Fig. 1 Phylogenetic analysis of the SOS gene from *Arabidopsis*, rice, maize, and wheat using the amino acid sequence alignment function in the MEGA (5.0) program. Bootstrap analysis was performed using 1,000 replicates (5.0) to evaluate the reliability of the various phylogenetic groups. GenBank Accession numbers: AtSOS1 AF256224, AtSOS2 NP_198391.1, AtSOS3 NP_197815.1, wheat WPK4 AB011670, ZmPK4 NP_001104940, OsPK4 BAA83688, SlSOS1 BAL04564.1, AmPK4 NP001104940.1, AtSnRK2.3 NM126087, AtSnRK2.2 NM114901, AtSnRK2.6 Q940H6, AtSnRK2.8 NM202441, AtCDPK BAA04829.1, ZmCDPK ACO72988.1, AtSnRK1.3 NM123306, OSK1 AB101655, AtKIN10 NM001125074, AtSnRK2.1 NM120946, AtSnRK2.4 NM100969, AtSnRK2.5 NM125760, AtSnRK2.7 NM120165, ZmSnRK2.1 EU676033, ZmSnRK2.2 EU676034, ZmSnRK2.3 EU676035, ZmSnRK2.4 EU676036, ZmSnRK2.5 EU676037, ZmSnRK2.6 EU676038, ZmSnRK2.7 EU676039, ZmSnRK2.8 EU676040

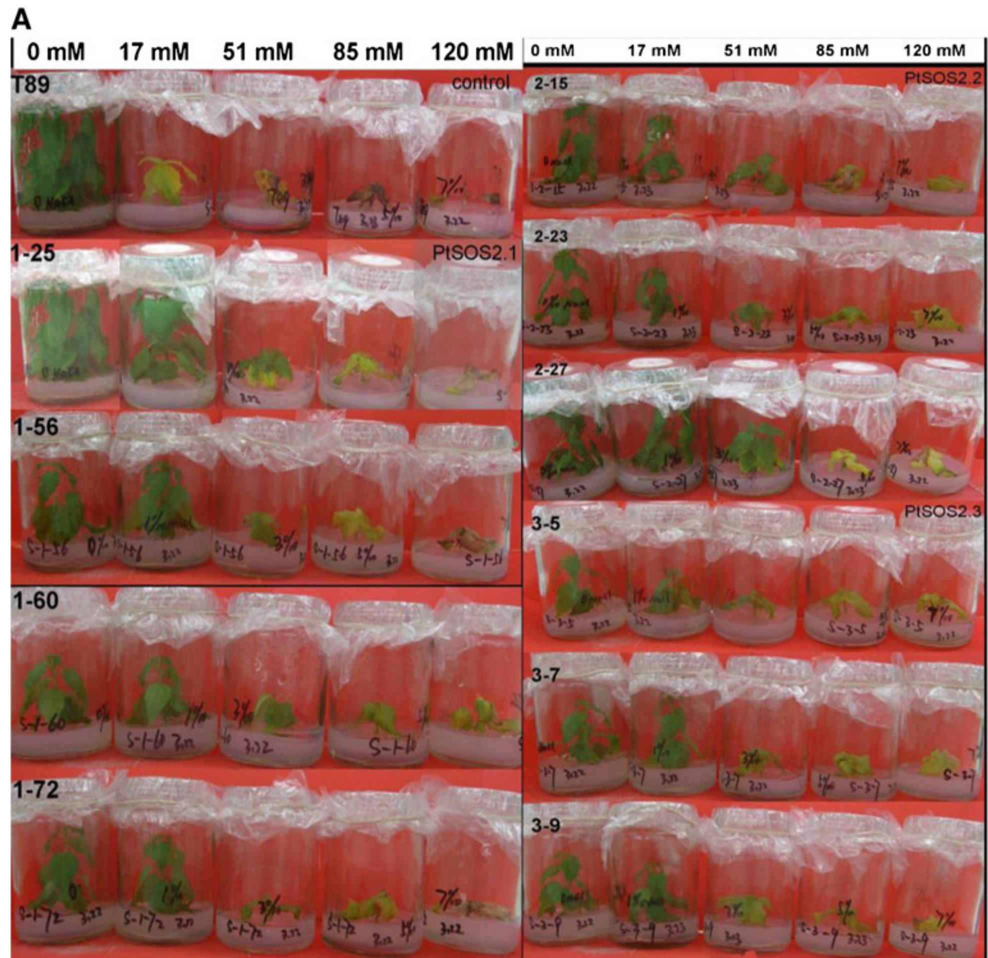
although part of the leaves turned yellow without affecting rooting under moderate salt stress, while 75 % of *PtSOS2.3* transgenic lines grew well under moderate salt stress. Under severe salt stress, the survival rate of *PtSOS2.1* and *Pt SOS2.2* transgenic lines was 60 %, but the growth of plants appeared to be suspended after 16 days. Only 50 % of *PtSOS2.3* transgenic lines could survive under severe salt stress. This suggests that as salt stress increased, both transgenic and wild-type poplars were affected by salt stress. However, the transgenic lines had higher survival rates and lower salt damage than the wild-type in agar-medium. This result indicates that transgenic lines conferred higher salt tolerance than wild-type in agar medium under salt stress.

Fig. 2 Alignment of PtSOS2.1, PtSOS2.2, and PtSOS2.3 with ZmPK4, OsPK4, WPK4, and AtSOS2. **a** A schematic diagram of the domain structure for SOS2. *FISL* FISL motif. **b** Alignment of the SOS2, ZmPK4, OsPK4, WPK4, and AtSOS2 activation loops. The N-terminal kinase catalytic domain is highly conserved. The C-terminal regulatory domain contains the conserved FISL motif (*marked*). The marked sequence is the activation loop between the conserved DFG and APE motifs (*dots*) and the three conserved residues in all members of the SOS2 subfamily kinase are marked by *asterisks* (Ser Tyr and Thr) and a *dot* (Thr), which may be phosphorylated by an upstream protein kinase (+). Conserved residues are indicated by *asterisks* and similar residues are indicated by a *colon* or *dot*

Evaluation of the Stress Tolerance of *PtSOS2* Poplars Under Greenhouse Conditions

To investigate the growth of *PtSOS2* poplars under salt stress in soil, poplars were irrigated with 85 mM NaCl for 45 days.

Fig. 3 Molecular identification of regenerated *PtSOS2* transgenic poplar plants. **a** Southern blotting analysis of *PtSOS2*-transgenic poplars; *P* amplification of a ~20,000-bp band from PH35GS plasmids. *N* qild-type (non-transgenic) poplars. *1–10* bands in 1–25, 1–56, 1–60, 1–72, 2–15, 2–23, 2–27, 3–5, L3–7, and 3–9 transgenic plants, respectively. **b** Northern blotting of three overexpressed *PtSOS2* genes in both transgenic and wild-type poplars. *N* wild-type poplar. *1–3 PtSOS2.1*-overexpressing transgenic lines 1–25, 1–56, and 1–60, respectively; *5–7 PtSOS2.2*-overexpressing transgenic lines 2–15, 2–23, and 2–27, respectively; *8–10 PtSOS2.3*-overexpressing transgenic lines 3–5, 3–7, and 3–9, respectively. Total RNA was isolated from the leaves of 3-week-old poplars grown on agar plates. Each lane was loaded with 20 μg total RNA. The RNA was fractionated on a 1 % agarose gel, blotted onto a nylon membrane, and hybridized with digoxigenin-labeled *hyg* cDNA as probes



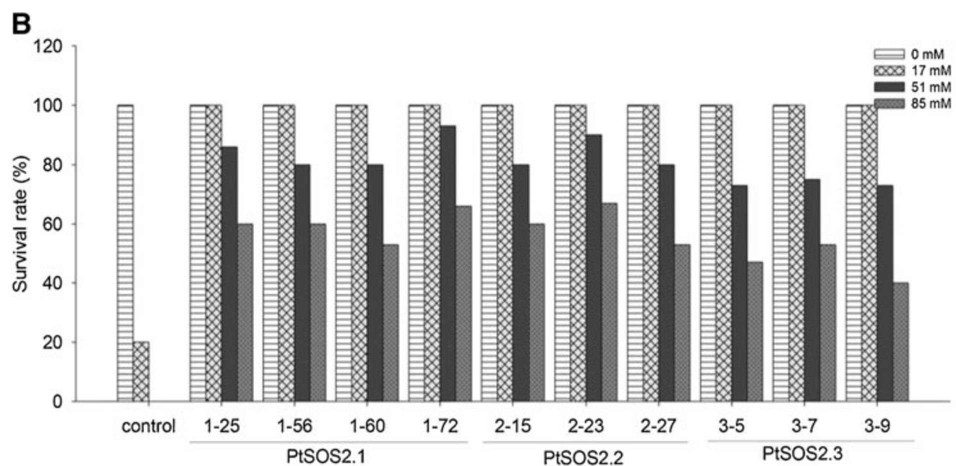


Fig. 4 *PtSOS2*-overexpressing transgenic lines and the wild-type grown in agar medium containing various NaCl concentrations for 2 weeks. **a** Poplars were cultured in ½ MS agar medium containing 0 17, 51, 85, or 120 mM NaCl for 2 weeks. **b** Survival rate of *PtSOS2*-overexpressing transgenic lines and the wild-type under 0, 17, 51, 85, or 120 mM NaCl. T89 is non-transgenic poplar. *1–25*, *1–56*, *1–60*, and *1–72* denote *PtSOS2.1*-overexpressing transgenic lines; *2–15*, *2–23*, and *2–27* denote *PtSOS2.2*-overexpressing transgenic lines; *3–5*, *3–7*, and *3–9* denote *PtSOS2.3*-overexpressing transgenic lines

As shown in Fig. 5a, the growth of the wild-type poplars was suppressed and the leaves shriveled after 2 weeks of salt treatment, while the transgenic lines maintained normal growth. These results indicate that growth of poplars overexpressing *PtSOS2* under salt stress was superior to that of the wild-type.

As shown in Fig. 5b, under both the normal and salt-stressed conditions, there were no significant differences in plant height between the transgenic lines and control poplars during the first 20 days. From 30 to 45 days, the relative plant height of the transgenic and control lines appeared to differ between salt-treated and untreated conditions. Most transgenic lines grew faster than wild-type poplars under both salt treatment and no treatment, but some transgenic lines—such as 1–72 of *PtSOS2.1* and 3–7 of *PtSOS2.3*—showed no growth rate advantage

in plant height (Fig. 5b). In comparison with non-transgenic poplar, all transgenic lines exhibited a greater ability to resist salt damage and most transgenic lines grew faster than wild-type poplars under salt stress treatment.

Determination of Proline, Leaf Relative Water, Chlorophyll, SOD, POD, CAT and MDA Contents

Proline is an important osmoprotectant that protects cells from damage under salt stress. As shown in Fig. 6a, proline activity was significantly higher in *PtSOS2*-overexpressing than control plants under salt stress. As shown in Fig. 6b, under non-stress conditions, the RWC of all transgenic lines did not change significantly compared to the controls. However, when treated with 85 mM saline, all transgenic lines and controls exhibited a decreased RWC, while the dampness of the transgenic lines was significantly lower than that of the control. Water loss in the control was up to 24.88 %, while in transgenic lines water loss ranged from 5.08 to 12.16 %. These results showed that under salt stress, transgenic lines had higher RWC and sustained less water loss than did controls. As shown in Fig. 6c, under normal conditions, the chlorophyll concentrations of transgenic lines were similar to the control (T89). However, under salt stress, chlorophyll concentrations decreased significantly in both the transgenic and control lines by approximately 23.47 and 39.96 %, respectively. As shown in Fig. 6d, POD activity in transgenic lines increased under both normal and salt treatment conditions. In the presence of salt, POD activity in *PtSOS2.1*-, *PtSOS2.2*-, and *PtSOS2.3*-overexpressing poplars increased significantly compared to the control. As shown in Fig. 5e, 1–25, 2–15, 2–23, and 2–27 transgenic lines did not show significantly increased activity compared to the control. When subjected to salt stress, all transgenic lines exhibited significantly increased SOD activity compared with the control; furthermore, the relative increase was more marked than that in the control after salt treatment. CAT activity increased by 4.11 % under salt stress compared to non-stressed conditions (Fig. 6f). In transgenic lines, the CAT activity increased by 16.91–24.37 %, a 4.1-fold increase compared to the control. Moreover, under both the stressed and normal conditions, the CAT activity of all transgenic lines increased significantly compared to the control. Thus, the effect of the transgenic lines on CAT activity was significant compared to the control. Based on Fig. 5g, under normal conditions, *PtSOS2.1*- and *PtSOS2.2*-overexpressing plants had significantly lower MDA concentrations than the control, while those of *PtSOS2.3*-overexpressing transgenic lines did not differ significantly from the control. After salt treatment for 2 weeks, the MDA concentrations in *PtSOS2.1*-, *PtSOS2.2*-, and *PtSOS2.3*-overexpressing transgenic lines were significantly increased.

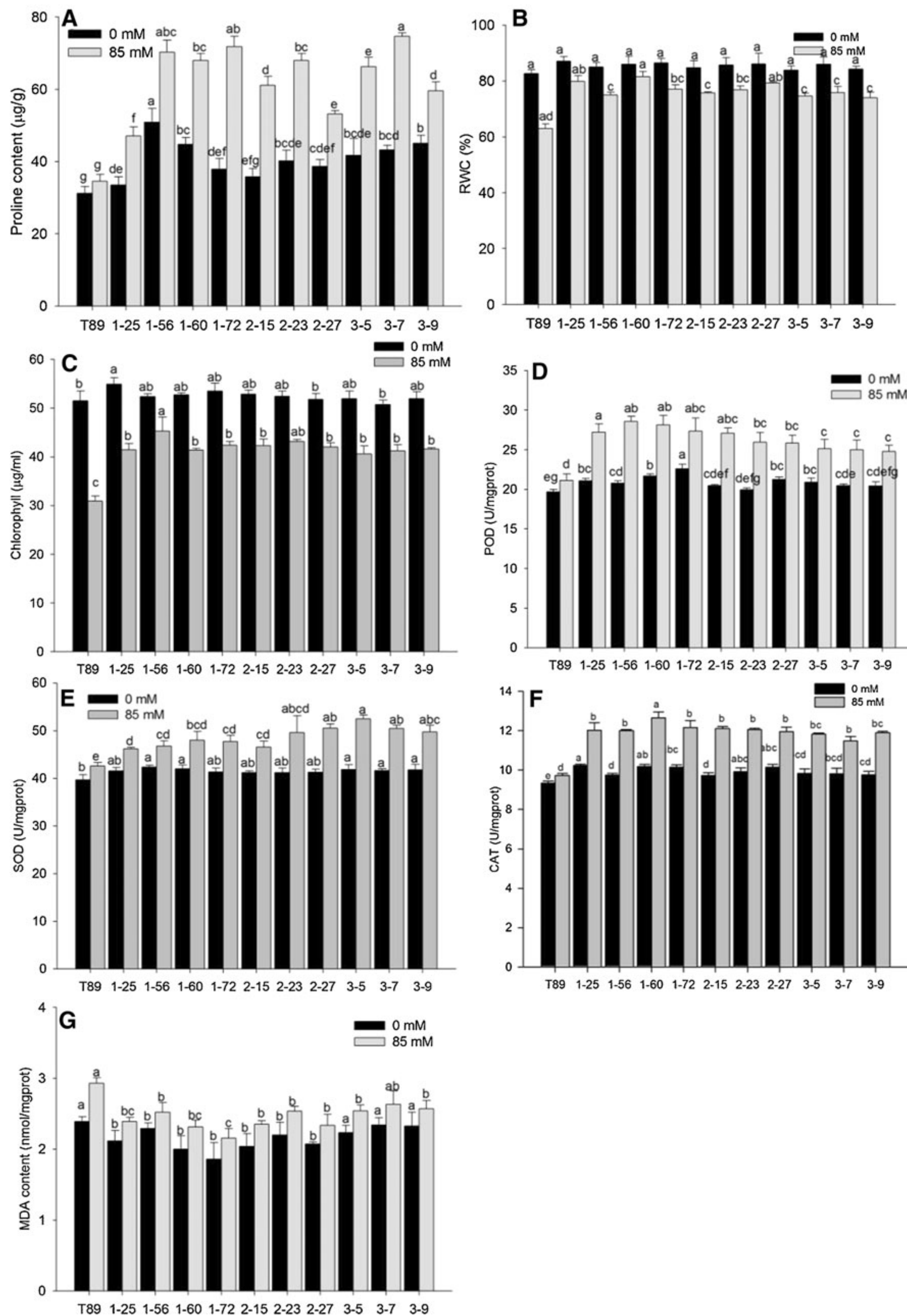
Fig. 5 Phenotype and plant height of poplar lines in soil. **a** Phenotype of *PtSOS2*-overexpressing lines in soil under salt treatment. Both transgenic and wild-type poplars were irrigated with 85 mM NaCl over 45 days. T89 is non-transgenic poplar. *1–25*, *1–56*, *1–60*, and *1–72* denote *PtSOS2.1*-overexpressing transgenic lines; *2–15*, *2–23*, and *2–27* denote *PtSOS2.2*-overexpressing transgenic lines; *3–5*, *3–7*, and *3–9* denote *PtSOS2.3*-overexpressing transgenic lines. **b** Plant height of *PtSOS2*-overexpressing poplars in soil under normal conditions and salt stress (85 mM) over 45 days. a: Plant height of *PtSOS2.1*-overexpressing poplars under non-treated conditions. **b** Plant height of *PtSOS2.1*-overexpressing poplars under 85 mM NaCl stress. **c** Plant height of *PtSOS2.2*-overexpressing poplars under non-treated conditions. **d** Plant height of *PtSOS2.2*-overexpressing poplars under 85 mM NaCl stress. **e** Plant height of *PtSOS2.3*-overexpressing poplars under non-treated conditions. **f** Plant height of *PtSOS2.3*-overexpressing poplars under 85 mM NaCl stress. Data represent the means±SD of three independent replicates. Values with different *letters* are significantly different at $P<0.05$

Discussion

Sucrose non-fermenting-1-related protein kinase 3 (*SnRK3*) has been identified and characterized in many species, such as wheat (Zhang et al. 2011), rice (Chae et al. 2007), maize (Ying et al. 2011), and *Arabidopsis* (Umezawa et al. 2004; Yoshida et al. 2006) is known to be involved in ABA signaling pathways and is activated by abiotic stress via direct phosphorylation of various downstream targets. Recent studies have found that the sucrose non-fermenting-1-related protein kinase gene (*SnRK*) family can improve stress tolerance and increase yield (Halford and Hardie 1998; Coello et al. 2011). However, the cloning and functional analysis of the *SnRK* gene family in woody plants remains limited. Here, we isolated three full-length cDNAs from poplars. Analysis of the putative amino acid sequences revealed that the three genes contained conserved protein kinase activation loop and FISL domains. The N-terminal protein-kinase domain has a high similarity to SNF-like kinases from other organisms. The FISL domain is a unique C-terminal region of serine-threonine protein kinases required and sufficient for the interaction with calcineurin B-like Ca^{2+} binding protein in the Ca^{2+} signaling machinery of plant cells in response to multiple stressors (Shi et al. 1999; Albrecht et al. 2001). The structural analysis suggested that *PtSOS2.1*, *PtSOS2.2*, and *PtSOS2.3* harboring the NAF/FISL domain belonged to the *SnRK* gene family and participated in the interaction with calcineurin B-like Ca^{2+}–binding protein (CBL) in the Ca^{2+} signaling pathway, and revealed that *PtSOS2* protein kinase could be phosphorylated via an upstream protein kinase and then mediate plant salt tolerance.

In maize, overexpression of *ZmSAPK8* in *Arabidopsis* significantly improved growth and development under salt-treated conditions, whereas the wild-type showed severe developmental and physiological inhibition under salt stress (Ying et al. 2011). To evaluate the effect of *PtSOS2* overexpression on poplar growth, salt stress experiments were

A
T89
1-25
1-56
1-60
1-72
2-15
2-23
2-27
3-5
3-7
3-9
B
a
PtSOS2.1
T89
1-25
1-56
1-60
1-72
Plant height (cm)
1 d
10 d
20 d
30 d
45 d
0 mM
b
PtSOS2.1
T89
1-25
1-56
1-60
1-72
Plant height (cm)
1 d
10 d
20 d
30 d
45 d
85 mM
c
PtSOS2.2
T89
2-15
2-23
2-27
Plant height (cm)
1 d
10 d
20 d
30 d
45 d
0 mM
d
PtSOS2.2
T89
2-15
2-23
2-27
Plant height (cm)
1 d
10 d
20 d
30 d
45 d
85 mM
e
PtSOS2.3
T89
3-5
3-7
3-9
Plant height (cm)
1 d
10 d
20 d
30 d
45 d
0 mM
f
PtSOS2.3
T89
3-5
3-7
3-9
Plant height (cm)
1 d
10 d
20 d
30 d
45 d
85 mM



performed in both agar and soil media. Results (Figs. 5, 6) suggested that, in both agar and soil media, the growth state of all the transgenic lines was significantly higher than that of the control. Thus, overexpression of all three *PtSOS2* genes not

Fig. 6 Determination of proline, RWC, chlorophyll, SOD, POD, CAT and MDA contents in poplar lines under normal conditions and salt treatment. **a** Concentration of free proline in control (T89) and transgenic lines. **b** RWC of the leaves of the transgenic lines and the control (T89) under salt treatment. Changes with salt treatment in mean relative water content±SD. **c** Changes in chlorophyll contents of transgenic non-transgenic lines (T89) under non-stress and salt-stressed conditions. **d** POD activity in *PtSOS2*-overexpressing plants and the control under normal conditions and salt treatment. **e** Changes in SOD activity in salt-treated plants under normal and salt-stressed conditions. *Bars* standard errors of triplicate samples. **f** Changes in CAT content of the transgenic lines and CK under untreated conditions and 0.5 % salt treatment. **g** Effect of salt treatment on MDA concentrations in transgenic and wild-type poplars. *Bars* standard errors of triplicate samples. Values with different *letters* are significantly different at $P<0.05$

only maintained poplar growth but also improved the survival rate under salt stress.

Accumulation of osmolytes in plants is important for osmotic adjustment, which affects the physiological machinery by which plants tolerate salt stress. Proline is an osmoprotectant that plays a role in regulation of gene expression in plants under salt stress (Bursy et al. 2007). Therefore, proline accumulation could increase osmotic adjustment and elevate salt stress tolerance. In our study, whether under normal or salt-treatment conditions, the increase of proline content in transgenic lines was higher than that in control plants (Fig. 6a), whereas the decrease of RWC and chlorophyll content (Fig. 6b, c) in transgenic lines was less than that in control plants. Therefore, the osmotic-adjustment capacity in transgenic lines was enhanced compared to the control line. This may explain why *PtSOS2* overexpression improved the osmotic-adjustment ability by maintaining RWC and chlorophyll content to restore the growth of plants in response to salt stress.

The activities of SOD, POD, and CAT, and the concentration of MDA are typically used to measure oxidative damage to membranes in response to salt stress (Mittova et al. 2004). At the biochemical level, under salt stress, the activities of SOD, POD, and CAT increase significantly, while MDA concentrations decrease significantly (Song et al. 2006; Bhagat et al. 2011). In our study, POD, SOD, and CAT activities increased significantly (Fig. 6d–f), and MDA concentrations (Fig. 6g) decreased significantly in transgenic lines compared to the control. This suggests that *PtSOS2* overexpression in poplars reduces oxidative damage to membranes and increases the resistance to salt stress.

Under normal conditions, all *PtSOS2.1*-overexpressing transgenic lines exhibited an SOD activity similar to that of the control. All *PtSOS2.2*-overexpressing transgenic lines had similar POD activities, and no *PtSOS23*-overexpressing transgenic lines showed a significantly altered MDA concentrations. This suggests that *PtSOS2.1*, *PtSOS2.2*, and *PtSOS2.3* perform different functions in the oxidative stress response, possibly associated with different physiological or biochemical roles for SOS2 involved in the ROS detoxification pathway (Zhu et al. 2007).

In conclusion, we cloned *PtSOS2.1*, *PtSOS2.2*, and *PtSOS2.3* from poplars. Molecular analysis confirmed that these three genes integrated into the genome and were expressed stably at the transcript level. *PtSOS2* overexpression mediated osmotic protection and induced antioxidant enzyme systems, resulting in improved salt tolerance.

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