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Transgenic Expression of *Prunus persica* Salt Overly Sensitive 2 (*PpSOS2)* in the *atsos2* Mutant Imparts Salt Tolerance in Arabidopsis

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Cite This: https://doi.org/10.1021/acsagscitech.1c00276

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ABSTRACT: Salinity is one of the main environmental stresses that negatively affect crop productivity. Almond trees are sensitive to salt stress; however, salinity-tolerant rootstocks can significantly enhance crop production under saline conditions. This work reports the functional complementation of the *Prunus persica* Salt Overly Sensitive 2 (*PpSOS2*) gene from the almond rootstock "Nemaguard" in the *Arabidopsis thaliana atsos2* mutant. Two transgenic lines of *PpSOS2* developed using constitutive (PpSOS2.OE3.5) and endogenous (PpSOS2NP.2.7) promoters showed significantly higher germination, survival rates, and dry weight than *atsos2* under 90 mM NaCl treatment. The *atsos2* mutant displayed the inhibition of primary and lateral roots under 50 mM NaCl. The root growth inhibition was restored by *PpSOS2* complementation. Both transgenic lines showed a significant decrease in electrolyte leakage compared to *atsos2* under 50 mM NaCl. The expression analysis of six K^+-rectifying channel genes and a reactive oxygen species-specific gene revealed the differential expression of *AtCHX14* and *AtCHX13* genes in transgenic lines compared to *atsos2* 24 h after the 50 mM NaCl treatment. These observations suggest that *PpSOS2* modulates and restores salt tolerance in *atsos2*. Also, in *Prunus*, the SOS pathway is conserved, suggesting that the exclusion of Na^+ is an important component trait for salt tolerance.

KEYWORDS: *salinity, SOS signaling, salt tolerance, almond, peach, Prunus, potassium signaling, SOS2, PCR-polymerase chain reaction, ROS-reactive oxygen species, qRT-PCR-quantitative reverse transcription-PCR*

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1. INTRODUCTION

Soil salinity is one of the major environmental stresses that affect plant growth and development.[1] By 2050, the world population is estimated to reach 9.1 billion. Thus, increasing food production by 70% to feed the additional 2.3 billion people (http://www.fao.org) will be a major challenge for the agricultural sector. Currently, 71% of the Earth's surface is covered with water, with saline ocean water (30 g of NaCl per liter) accounting for approximately 96.5% of Earth's water (https://www.usgs.gov). Due to excessive groundwater pumping for crop irrigation, seawater can intrude coastal groundwater bodies and affect the land and crops irrigated with this water of increased salinity. Soil is considered saline when its electric conductivity (EC_e) is 4 dS m^{-1} or higher.[2] Soil salinity affects more than 800 million hectares of total world land,[3,4] directly impacting the global agriculture industry, worth around 2.4 trillion U.S. dollars (https://croplife.org). There are natural and anthropogenic factors that affect the salt composition of the soil. Natural factors leading to increased salinity include long-term salt accumulation that results in salt lakes and salt marshes, the weathering of rocks, salt precipitations, a shallow water table, and wind-borne salt from dunes and oceans.[1] Anthropogenic factors affecting soil salinity include, but are not limited to, (i) the increased demand for food and animal feed, both leading to the ever-increasing use of fertilizers (many as Na and Cl salts), (ii) excessive crop irrigation, and (iii) the elimination of natural forests that permanently alters the rain cycle of the deforested area.[5]

This salt-induced stress causes various physiological and metabolic changes in plants and inhibits their growth and development.[6,7] Excessive salt in soils affects plants in two steps. Initially, it induces the osmotic stress that adversely affects water uptake, leading to a physiological drought. Osmotic stress is the initial effect of salinity and induces various physiological changes such as damage to membranes, inability to detoxify reactive oxygen species (ROS), nutrient imbalance, reduction in the stomatal aperture, and photosynthetic rate.[8] If the salt stress persists, a second step or phase sets in as the ionic toxicity caused by the accumulation of salt ions (e.g., Na^+ and Cl^-) in plant tissues. Na^+ and Cl^- accumulation may cause a severe ion imbalance that disrupts various metabolic and physiological processes.[8] Higher Na^+ and Cl^- concentrations inhibit the uptake of K^+ and NO_3^-, respectively, which are essential nutrients for the growth and development of plants.[7] While Earth is a salty planet, plants have developed various mechanisms to deal with excessive salt. Some plant species have evolved mechanisms to tolerate high

Received: December 8, 2021
Revised: January 21, 2022
Accepted: January 24, 2022

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salinity levels in water and soil and are known as halophytes. However, most species, mainly our staple crops, are salt-sensitive and are known as glycophytes. Some glycophytes, such as alfalfa and spinach, can tolerate soils with EC_e over 4.0 dS m^{-1} or irrigation water with EC_{iw} of 6.0 dS m^{-1} or higher and can still maintain a sufficient mineral composition for growth, development, and biomass accumulation regardless of their high salt accumulation.[9−13]

Salt tolerance in plants is a complex trait that involves an intricate network of genes. These include genes involved in the reduced uptake of salt ions, exclusion of these ions from plant tissues, sequestration of ions in vacuoles, regulation of ion transport from root to shoot, tissue tolerance, and ion homeostasis.[12−15] The exclusion of Na^+ from the root cytoplasm is carried out by the salt overly sensitive (SOS) pathway.[16] The SOS pathway consists of three members: SOS1, SOS2, and SOS3. Under salt stress, SOS3—a calcium-binding protein known as a Ca^{2+} sensor—perceives the increase in cytosolic Ca^{2+} caused by the excessive Na^+ uptake in the cytoplasm.[14] Once SOS3 binds Ca^{2+}, it activates SOS2—a SnRK3 family protein kinase (sucrose nonfermenting-1-related protein kinase-3).[17,18] SOS2 is a Ser/Thr protein kinase with an N-terminal catalytic domain and a C-terminal regulatory domain; both are essential for its salt-tolerance role.[18] The C-terminal regulatory domain of SOS2 contains a 21-amino acid autoinhibitory sequence motif called FISL.[19] SOS3 physically interacts with the FISL motif to activate SOS2. The SOS3−SOS2 complex then phosphorylates a Na^+/H^+ antiporter (SOS1) located on the plasma membrane and activates it, leading to extensive Na^+ exclusion from the cytoplasm.[20] The constitutively active SOS2 can be created by changing Thr^{168} to Asp in the putative activation loop of the SOS2 catalytic domain or by removing the FISL motif or the whole C-terminal regulatory domain.[19]

Besides being a central player in the SOS signaling pathway, SOS2 also activates many other proteins. It is known that SOS2 activates tonoplast-localized Na^+/H^+ exchangers (NHXs) during salt stress.[21] Furthermore, SOS2 is also reported to interact with CHX1, a vacuolar H^+/Ca^{2+} antiporter independent of SOS3[22] and a vacuolar H^+-ATPase.[23] Also, SOS2 interacts with nucleoside diphosphate kinase 2 (NDPK2), indicating its potential role in salt stress and H_2O_2 signaling.[24] SOS2 directly interacts with two negative regulators of ABA response, ABI1 and ABI2, illustrating the SOS2 cross-talk with ABA signaling.[25] SOS2 also regulates the ethylene signaling pathway by interacting with ethylene insensitive 3 (EIN3) and modulating salt tolerance.[26] Thus, SOS2 could independently interact with various proteins of the SOS pathway and regulate many transporters. Several studies revealed that the SOS pathway is functionally conserved in higher plants such as tomato, rice, maize, and poplar.[27−30] The overexpression of constitutively active *AtSOS2*, *SlSOS2*, and *PtSOS2* enhanced the salt tolerance of transgenic Arabidopsis, tomato, and poplar plants, respectively.[31−33] This study is focused on the functional characterization of the *PpSOS2* gene from a peach (*Prunus persica*)-based rootstock used in almond (*Prunus dulcis*) commercial cultivation.

The United States tops almond production worldwide by producing more than 80% of the total share.[34] California alone accounts for 99% of the total almond production in the U.S. The total export value of almonds reached $4.5 billion in 2017−18.[35] Although the almond crop requires more water than the other competitive cash crops in California, its economic and nutritional value makes it one of the preferred crops in the state.[36] The almond crop environmental footprints could be reduced significantly through the use of treated municipal recycled wastewaters. However, these waters are higher in salts, and almond trees decrease their yield with a slight increase in irrigation-water salinity.[37] A prolonged drought hit California from 2012 to 2017. After a short break, a new drought starting in 2020 has forced farmers to use groundwaters high in salt for irrigating their orchards with adverse effects on almond production. The growth and development of almond plants drastically reduced to 50% when EC_{iw} increased from 1.5 dS m^{-1} to 4 dS m^{-1},[38] indicating their high sensitivity to salt.

Similar to many other stone fruit crops, rootstocks play a vital role in almond cultivation. Most almond rootstocks are peach-based, plum-based, or composed of complex hybrids of peach, apricot, cherry, almond, and plums.[39] The functional complementation of *PpHKT1* genes from the almond rootstock "Nemaguard" was shown to impart salt tolerance in the *athkt1* Arabidopsis mutant.[40] A recent study on 14 different rootstocks revealed the significant role of Na^+ and Cl^- exclusion in the salt tolerance of rootstocks.[39] In addition, gene expression analysis revealed the highly induced expression of *PpSOS2* in the roots of most salt-tolerant rootstocks.[39]

The primary goal of this study was to examine the function of the *PpSOS2* gene from the peach (*P. persica*)-based rootstock 'Nemaguard' in response to salinity stress. The approaches used to study the function of a gene during growth, development, and in response to various stresses are loss-of-function and overexpression. Because loss-of-function mutant lines are not available for peach and the generation of overexpression lines for a specific peach gene *in planta* would take several years, we expressed the *PpSOS2* gene in the *Arabidopsis thaliana atsos2* mutant under constitutive and endogenous promoters to demonstrate its role in salt tolerance.

2. MATERIALS AND METHODS

2.1. Plant Material and Growth Conditions. The *atsos2* mutant line Salk_056101 for AT5G35410 (*AtSOS2*) was purchased from Arabidopsis Biological Resource Center (Ohio State University; http://www.arabidopsis.org/). The T-DNA insertion is reported to be in the promoter region of the *SOS2* gene. The seeds were surface-sterilized with 70% ethanol for 5 min, followed by 30% bleach + 0.25% Triton 100 for 5 min. Sterilized seeds were spread on 1/2-strength Murashige and Skoog (MS) media, 1.5% sucrose, and 6% agar and were vernalized for 4 days at 4 °C. The seeds were then moved to a growth chamber (Conviron, Model CMP 4030, Winnipeg, Manitoba, Canada) set at 21 °C/18 °C day/night temperatures with the day/night lengths of 16 h/8 h. The light intensity was 200 μmol m^{-2} s^{-1}, and the relative humidity (R.H.) was approximately 50%. After 1 week of germination, seedlings were transferred to the Metro mix (SUNGRO, Horticulture Distribution, Inc., Bellevue, WA, USA), and leaf samples were collected 2 weeks later. Genomic DNA was extracted from leaf samples. The mutant was screened for homozygous lines using Salk_56101_LP/Salk_056101_RP and Salk_056101_LP/LBb1.3 primers (Table S1). Primers were designed using SIGNAL iSect Tools (http://signal.salk.edu/isects.html). The gene expression of homozygous mutant lines was checked by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses.

2.2. Cloning of *PpSOS2*. The almond rootstock 'Nemaguard' (*P. persica*) was used for cloning the *SOS2* gene. The nucleotide sequence of the *SOS2* gene (Prupe.7G244500.1) was used to amplify the coding sequence (CDS), cDNA, and promoter region from root tissues. The 2000 bp upstream to the start codon was screened for the promoter site using promoter prediction software (http://www.

softberry.com/berry.phtml?topic=tssp&group=programs&subgroup=promoter). The region selected to clone the promoter region was 1,000 bp upstream from the start codon and was then selected for cloning the promoter region. The CDS and 3′UTR (three-prime untranslated region) were amplified from cDNA using primers PpSOS2_Start_F and PpSOS2_3′UTR_R (Table S1). The native promoter region of 1 Kb was amplified from the genomic DNA extracted from roots using primers PpSOS2_PromB1 and PpSOS2_cDNA_R to include a part of the coding region (CDS) around the start codon (Table S1). RNA was extracted using a Spectrum Plant Total RNA Kit (Sigma-Aldrich, Carlsbad, CA, USA), and cDNA was synthesized using oligo dT_{18} with a PrimScript 1st strand cDNA synthesis kit (Takara, Bio USA, Mountain View, USA). Reverse transcription was carried out at 65 °C for 5 min and 42 °C for 1 h. The native promoter was fused to cDNA containing 3′UTR by overlapping PCR, where both the amplicons (cDNA and native promoter) have an overlap in the coding region around the start codon. Both amplicons were mixed and used as templates. The fused fragment was amplified using forward primer PpSOS2_promB1 specific to the 5′ end of the native promoter amplicon and reverse primer PpSOS2_promB2 specific to 3′ end of the cDNA containing a 3′UTR amplicon and Q5 High fidelity DNA polymerase (New England Biolabs, Ipswich, MA, USA). The obtained gene constructs of CDS and native promoter + cDNA were cloned in pDONR207 (Gentamycin) Gateway entry vector (Invitrogen Corporation, Carlsbad, CA, USA). Selected clones were sequenced. The CDS construct from pDONR207 was transferred to the Gateway destination vector pMDC32 for overexpression under the 2X 35S promoter. The native promoter + cDNA construct in pDONR207 was transferred to the Gateway destination vector pMDC99 (a promoterless vector) for complementation studies under the native promoter.

2.3. Development of Transgenic Lines. The *SOS2-cds::pMDC32* and *SOS2-NPcDNA::pMDC99* were transformed to *Agrobacterium tumefaciens* strain GV3101 by electroporation. Arabidopsis transformation was carried out in the *atsos2* mutant (Salk_56101) by the floral dip method in 5% sucrose and 0.025% of Silwet-77.[41] The transgenic plants were selected on hygromycin at 25 μg/mL on 1/2-strength MS media. Integration of the construct was confirmed by PCR analysis of hygromycin (25 mg L^{-1})-resistant T_1 generation plants. Hygromycin-resistant T_2 plants from five lines per construct were used for transgenic expression analysis by qRT-PCR using *PpSOS2* specific primers PpSOS2qPCR_2F/2R (Table S1). One line per construct with high transgenic expression was taken for further analysis. T_3 progenies were used for further experiments.

2.4. Salt Tolerance Assay of Transgenic Arabidopsis Lines and Effect of Salt on Seed Germination. Wild-type (WT) Arabidopsis plants, *atsos2* mutant line, and T_3 transgenic lines PpSOS2.OE3.5 and PpSOS2.NP2.7 were surface sterilized and germinated on a 1/2—strength MS medium in a growth chamber set at 23 °C and 50% R.H. with a 16 h/8 h photoperiod. Twelve plants of each line were grown in soil for 3 weeks and then treated with 1/8-strength MS media as control and 1/8-strength MS media supplemented with 50 mM NaCl and 90 mM NaCl for 18 days. NaCl concentration was increased stepwise from 50 mM NaCl to a higher concentration after each treatment on alternate days until the target salinity was reached. The plants were photographed 18 days after the start of the treatment. The 90 mM NaCl treatment was considered for further experiments in soil. The WT, *atsos2* mutant and transgenic lines were used to test the effect of the *SOS2* gene on seed germination under salinity conditions. Seeds were surface sterilized with 70% ethanol, followed by 30% bleach in distilled water. Surface-sterilized seeds were washed with distilled water four times to remove any residual ethanol or bleach solution. Petri dishes with 40 ml of 1/2-strength MS media were used as control, and 1/2-strength MS with 90 mM NaCl were used as salinity treatment for the seed germination assay. Petri dishes were divided into four equal quarters, and 15 surface-sterilized seeds of each genotype were placed in four quarters separately for both control and salinity treatments. Three replicates of each treatment were used, and seed-plated Petri dishes were placed at 4 °C. After 4 days, the Petri dishes were moved to a growth chamber set at 16/8 h light/dark and corresponding temperatures of 22 °C (light) and 16 °C (dark). After 6 days, the germination percentage was recorded, and two-way ANOVA and Tukey HSD test (0.05) were used to depict the statistical differences among different lines.

2.5. Electrolyte Leakage Measurement. The electrolyte leakage was determined by measuring ion conductivity according to a previously described method with minor modifications.[42] One-week-old seedlings of Arabidopsis WT, *atsos2* mutant, T_3 transgenic lines PpSOS2.OE3.5 and PpSOS2.NP2-7 from 1/2-strength MS media were transferred to the soil. Three-week-old plants were irrigated with 1/8-strength MS (control) or 1/8 strength MS with 50 mM NaCl (saline) for 2 weeks on alternate days. After 10 days of treatment, leaf disks of 5 mm diameter were collected from five plants of each line and divided into four replicates (three disks per replicate). Leaf disks were rinsed in deionized water to remove surface ions and incubated in 10 ml of deionized water overnight. The conductivities of each sample (C1) were measured with a model 1056 digital conductivity meter (Amber Sciences, Eugene, Oregon, USA). The samples were then autoclaved to release all ions and cooled down to room temperature. The conductivities (C2) were measured. The relative electrolyte leakage was calculated using the formula: $(C1/C2) \times 100\%$. The whole assay was repeated three times, and the data were analyzed using the ANOVA single factor, and the Tukey HSD test (0.05) was used to depict the statistical differences among different lines.

2.6. Relative Water Content. The relative water content (RWC) of the transgenic lines was determined following a method previously described.[42] Two leaves from three plants of each transgenic line, *atsos2* mutant and WT, were harvested after 10 days under the 90 mM NaCl treatment, were harvested, and the fresh weight (FW) was determined. These samples were then incubated in 10 ml of deionized water for 12 h at room temperature, and the turgid weight (TW) was determined. Then, all samples were dried at 65 °C for 48 h, and the dry weight (DW) was determined. The RWC was calculated using the formula $[(FW - DW)/(TW - DW)] \times 100$. The experiment was repeated twice.

2.7. Root Growth and FW Determination. Primary root growth and lateral root growth were determined using a previously established method with a slight modification.[43] Arabidopsis WT plants, *atsos2* mutant line, and T_3 transgenic lines PpSOS2.OE3.5 and PpSOS2.NP2.7 were germinated on 1/2-strength MS media. Four 10-day-old seedlings were transferred to 1/2-strength MS media or 1/2-strength MS media containing 50 mM NaCl with three replications. The photographs were taken 10 days after the transfer, and the FW of each seedling was determined. The length of primary and lateral roots was measured using ImageJ (https://imagej.nih.gov/ij/). The experiment was repeated three times. ANOVA single factor was used to analyze data, and the Tukey HSD test (0.05) was applied to depict the statistical differences among different lines.

2.8. Quantitative Reverse Transcription-PCR. The expression analyses of *PpSOS2* and *AtSOS2* genes were done using qRT-PCR. One-week-old Arabidopsis seedlings WT, *atsos2* mutant, and transgenic lines expressing *PpSOS2* were transferred to 1/2-strength MS media with and without 50 mM NaCl. Whole seedlings, leaf, and root samples were collected after 24 h, and RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). DNase treatment was done to remove DNA contamination in RNA samples using the DNase I enzyme (Thermo Scientific, Waltham, MA, USA). The expression analyses were carried out in the BioRad CFX96 System using an iTaq Universal SYBR Green One-Step Kit (Bio-Rad Laboratories, Hercules, CA, USA). Primers specific to *PpSOS2* and *AtSOS2* were developed using the Prime quest tool of IDT (https://www.idtdna.com/Primerquest/Home/Index). The reaction was carried out in a total volume of 10 μL containing 25 ng of RNA, 0.25 μM of each primer, 0.125 μL of iScript Reverse Transcriptase enzyme (BIO-RAD, Hercules, CA, USA), and 5 μL of 2X one-step SYBR Green Reaction mix (BIO-RAD, Hercules, CA, USA). The PCR program was as follows: 50 °C for 10 min, 95 °C for 1 min, then 40 cycles of denaturation at 95 °C for 10 s, and annealing and

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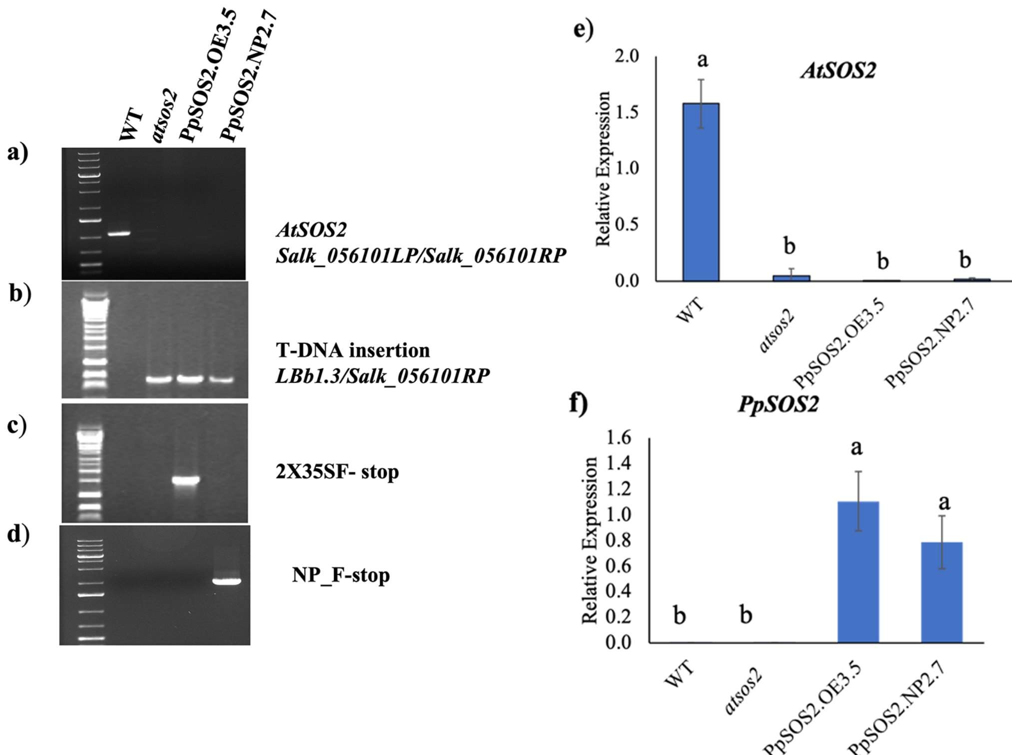

Figure 1. Selection of transgenic lines (a) Screening of the WT plant by amplifying the 1 kb band with Salk_056101LP/Salk_056101RP set of primers. No amplification was seen in the mutant and transgenic lines. (b) Verification of the *atsos2* mutant background by amplifying with LBb1.3/Salk_056101RP set of primers to obtain 500 bp band in T-DNA insertion. The mutant and transgenic lines show the presence of the band. This fragment band was not amplified in the WT plants. (c) Verification of the PpSOS2.OE3.5 transgenic line by amplifying the 1.4 kb band using 35S-promoter forward/Stop codon reverse primers. Only the overexpressing line shows amplification. (d) Verification of the PpSOS2.NP2.7 transgenic line by amplifying the 2 kb band with native promoter forward/Stop codon reverse primers. (e) Expression of *AtSOS2.* The *AtSOS2* gene was expressed in WT, but no expression was observed in the *atsos2* mutant plant and PpSOS2 transgenic seedlings, which are in the mutant background. (f) Expression of *PpSOS2.* The *PpSOS2* gene was expressed in both transgenic lines compared to the *atsos2* mutant but absent in the *atsos2* mutant and WT. Three seedlings per replicate and three replicates were used in the study. Genotypic means followed by the same letters are not significantly different according to the Tukey HSD test ($P < 0.05$). Error bars represent standard errors.

extension at 60 °C for 20 s. Arabidopsis ubiquitin and actin were used as reference genes in this study (Table S1). Quantification of the relative gene expression was performed using the $2^{-\Delta\Delta CT}$ method.[44] The experiment was repeated twice. The statistical analysis was done by the ANOVA-single factor and Tukey HSD test.

2.9. Sequence Analyses of *SOS2* Genes from Different Species. Arabidopsis SOS2 protein sequence was used in Basic Local Alignment Search Tool (BLAST) analyses on the NCBI website to identify corresponding orthologs in 11 other species with varying levels of salt tolerance (Table S1). All protein sequences were aligned using the Neighbor-Joining method of MEGA6.0[45,46] using the MUSCLE alignment.[47] The Poisson correction method was used and represented as the number of amino acid substitutions per site to compute evolutionary distances.[48]

2.10. Subcellular Localization of PpSOS2 Protein. Because of the unavailability of any genome-specific subcellular localization prediction tool for *P. persica*, seven different generalized plant localization prediction tools Plant-mPLoc,[49] LOCALISER,[50] BaCelLo,[51] Yloc,[52] PredSL,[53] Plant-mSubP,[54] and MultiLoc2[55] were used. Besides these, an Arabidopsis-specific tool AtSubP[56] was also used. *PpSOS2* (XP020424233.1) and *AtSOS2* (NP198391.1) sequences retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) were used for localization.

3. RESULTS

3.1. Cloning of the *PpSOS2* Genes and Development of Transgenic Lines in the *atsos2* Mutant. To study the role of *PpSOS2* under salt stress, two different types of transgenic lines of *PpSOS2* were developed in the Arabidopsis *atsos2* mutant background. In one of the lines, the *PpSOS2* CDS was overexpressed (PpSOS2.OE3.5), and in the other, the *PpSOS2* CDS was expressed under the native *PpSOS2* promoter (PpSOS2.NP2.7). At least 10 transgenic lines for each construct were screened by genotyping and *PpSOS2* gene expression analyses, and one transgenic line for each construct was selected (Figure 1). Genotyping analysis using Salk_056101_LP/Salk_056101_RP primers amplified a 1 kb band in WT plants but not in mutant or transgenic lines (Figure 1a). The T-DNA specific band was missing in WT but was present in the mutant and transgenic lines, confirming the mutant background of the transgenic lines (Figure 1b). The 35S promoter-specific 1.4 kb band was present in the PpSOS2.OE3.5 overexpression line (Figure 1c) and a native promoter-specific 2.0 kb band was observed in the native promoter transgenic line, PpSOS2.NP2.7 (Figure 1d). The *AtSOS2* gene was not expressed in the Arabidopsis *atsos2* mutant or PpSOS2 transgenic lines (which were in the Arabidopsis mutant background), compared to WT (Figure 1e). The *PpSOS2* gene was not expressed in the *atsos2* mutant or WT Arabidopsis plants but was highly expressed in both PpSOS2.OE3.5 and PpSOS2.NP2.7 transgenic seedlings (Figure 1f).

3.2. Effect of *PpSOS2* Complementation on the Salt Tolerance of the Transgenic Lines. The evaluation of

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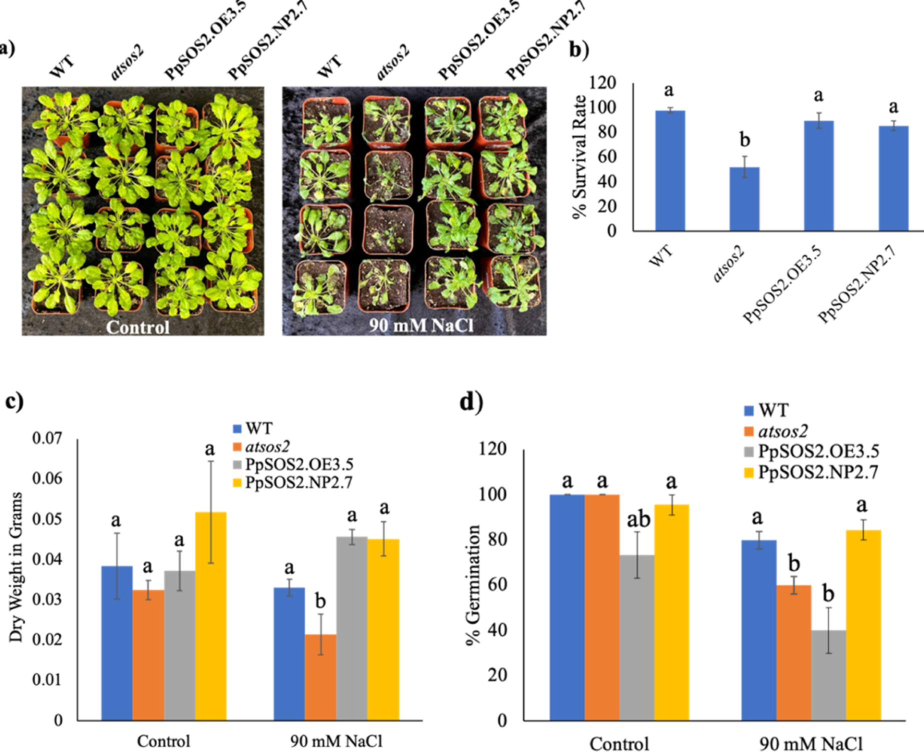

Figure 2. Evaluation of salinity tolerance of transgenic lines. (a) Soil-grown WT Arabidopsis, mutant Arabidopsis *atsos2*, PpSOS2.OE3.5, and PpSOS2.NP2.7 transgenic lines treated with control and 90 mM NaCl in 1/8 strength Murashige and Skoog media for 21 days. (b) Survival rates of WT, *atsos2*, PpSOS2.OE3.5, and PpSOS2.NP2.7 transgenic lines. (c) Dry weight of WT, *atsos2*, PpSOS2.OE3.5, and PpSOS2.NP2.7 transgenic lines. (d) Germination % of WT, *atsos2*, PpSOS2.OE3.5, and PpSOS2.NP2.7 transgenic lines. Genotypic means followed by the same letters are not significantly different according to the Tukey HSD test ($P < 0.05$). Error bars represent standard errors.

transgenic lines PpSOS2.OE3.5 and PpSOS2.NP2.7, WT, and *atsos2* mutant plants at three concentrations of NaCl: 50, 90, and 120 mM showed that the roots of *atsos2* mutant plants were sensitive to the NaCl treatment when grown on 1/2 MS agar plates (data not shown). Even at 50 mM NaCl treatment, seedling root growth was inhibited completely. However, when these lines were grown in soil and irrigated with water containing 50 mM NaCl for 3 weeks, they showed no effect on the growth and development of plants. Treatment with 120 mM NaCl killed mutant plants within 1 week of treatment and inhibited the growth of WT and transgenic lines (data not shown). Based on these studies, we selected 50 mM NaCl and 90 mM NaCl concentrations for various treatments. After 21 days of treatment at 90 mM NaCl on soil, PpSOS2OE3.5 and PpSOS2.NP2.7 transgenic and WT plants were green and healthy (Figure 2a). About 50% of the mutant plants died after 21 days. The survival rate of transgenic plants after 21 days was the same as that of WT plants (Figure 2b). The dry weight of the *atsos2* mutant plants, after 21 days of treatment, decreased significantly compared to that of transgenic and WT plants (Figure 2c). Mean values of germination percentage showed a significant decrease (Tukey's HSD test at 0.05) in the *atsos2* mutant under the 90 mM NaCl salt treatment. Only 60% of *atsos2* mutant seeds germinated on salt as compared to the control. Functional complementation of *PpSOS2* under the native promoter restored germination so that 80% of the seeds germinated under salt treatment. However, the PpSOS2.OE3.5 transgenic line (overexpressing *PpSOS2*) had only 40% gemination under 90 mM NaCl (Figure 2d).

The root growth assay revealed that the primary and lateral root growth was inhibited in the *atsos2* mutant when seedlings were transferred to 50 mM NaCl (Figure 3a). Expression of *PpSOS2* in the *atsos2* background under both native and constitutive promoters restored the WT phenotype (Figure 3a). The transgenic lines had denser roots than the *atsos2* mutant seedlings. The primary root length was inhibited in the *atsos2* mutant seedlings compared to WT, even under control conditions (Figure 3b). The PpSOS2.NP2.7 transgenic seedlings had significantly longer primary roots than the WT and PpSOS2.OE3.5 transgenic seedlings under control (Figure 3b). Under 50 mM NaCl, both transgenic lines had significantly higher primary root lengths than *atsos2* (Figure 3b). On similar lines, the lateral root length was significantly inhibited in the *atsos2* mutant seedlings compared to the WT seedlings grown on 1/8-strength MS media with 50 mM NaCl for 10 days (Figure 3c), while the transgenic lines PpSOS2.OE3.5 and PpSOS2.NP2.7 had significantly longer lateral roots than the *atsos2* mutant seedlings under saline conditions (Figure 3c).

Electrolyte leakage is another parameter to test the salinity tolerance of a plant. We calculated the percent electrolyte leakage in all lines after the 50 mM NaCl treatment for 10 days. The electrolyte leakage analysis revealed a significant increase of electrolytes in the *atsos2* mutant plants compared to the WT and PpSOS2.OE3.5 and PpSOS2.NP2.7 plants (Figure 3d), although the RWC analysis of transgenic lines, WT, and *atsos2* mutant had no significant differences (Figure 3e).

3.3. Salt-Induced Gene Expression in Transgenic Lines. The expression of *PpSOS2* was monitored in both the transgenic lines PpSOS2.OE3.5 and PpSOS2.NP2.7 and also in the *atsos2* mutant plants. The *PpSOS2* gene was not

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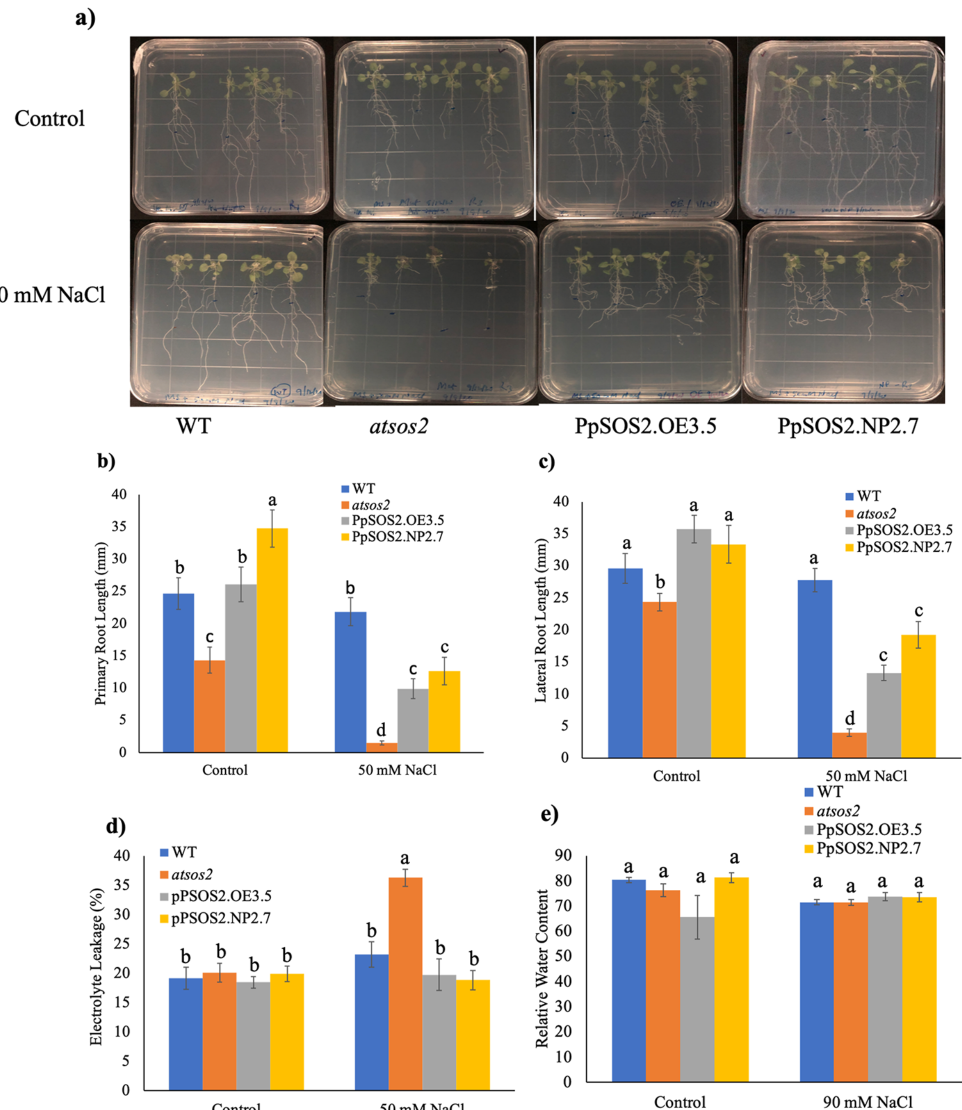

Figure 3. Root growth assay. (a) Primary and lateral root growth of 10-day-old seedlings of WT, *atsos2* mutant, PpSOS2.OE3.5, and PpSOS2.NP2.7 on the 1/2 MS-media plates with and without 50 mM NaCl. Photos were taken 10 days after transferring plants to the plates. The *atsos2* mutants had an inhibition of primary and lateral root growth on 50 mM NaCl 1/2 MS plates. (b) Primary root length. Both transgenic lines had significantly longer primary roots than *atsos2* mutants under 50 mM NaCl. (c) Lateral root length. Both transgenic lines had significantly longer lateral roots than *atsos2* under 50 mM NaCl treatment. (d) Electrolyte leakage. The *atsos2* mutant plants had significantly lower electrolyte leakage percentage than WT after 10 days under 50 mM NaCl treatment. (e) Relative water content (RWC). No significant difference was observed in RWC after 10 days after the 90 mM NaCl treatment. Genotypic means followed by the same letters are not significantly different according to the Tukey HSD test ($P < 0.05$). Bars represent standard errors of the means.

expressed in the *atsos2* mutant either in the leaf or the root (Figure 4a). *PpSOS2* was expressed in both transgenic lines, in leaves and roots; however, the expression was significantly higher in the roots than in the leaves (Figure 4a). Expression of *PpSOS2* was analyzed in both transgenic lines along with the *atsos2* mutant under 50 mM NaCl 24 h after treatment. The expression of *PpSOS2* was significantly higher in the PpSOS2.OE3.5 transgenic line under a salinity stress of 50 mM NaCl, while there was no significant change in the gene expression of *PpSOS2* in the PpSOS2.NP2.7 transgenic line under either treatment or control (Figure 4b).

Compared to transgenic and WT plants, the *atsos2* mutant plants had a significant increase in electrolyte leakage percentage under the 50 mM NaCl treatment. It is well known that the *SOS2* locus plays a significant role in K^+ homeostasis. Therefore, we analyzed the expression of six K^+ rectifying channel genes involved in K^+ homeostasis during stress and the ROS-specific gene NADPH oxidase *AtRbohD*. The gene expressions of shaker type K^+ channels, *AtAKT1*, *AtAKT2*, *AtSKOR*, and *AtGORK*, plasma membrane K^+ transporters, *AtCHX13* and *AtCHX14*, and NADPH oxidase *AtRbohD* were analyzed 24 h after whole seedlings were treated with 50 mM NaCl.

No significant changes were observed between control and salinity treatments in the expression of *AtAKT1*, *AtAKT2*, and *AtGORK* (Figure S1a−c). Expression of *AtCHX13* was significantly higher in transgenic lines under both control and salt conditions when compared to the *atsos2* mutant (Figure 5a). There was no significant difference in control versus salt for individual genotypes. The gene expression of *AtCHX14* was significantly induced in the seedlings of PpSOS2.OE3.5 transgenic line under salt treatment compared

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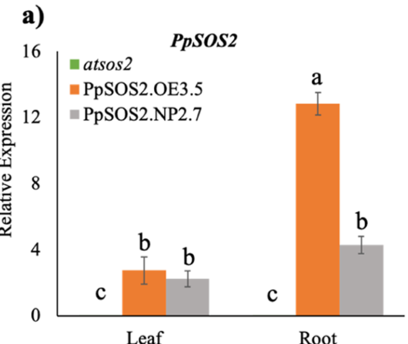

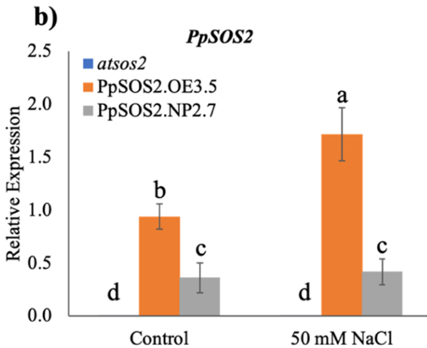

Figure 4. Expression of *PpSOS2* in the *atsos2* mutant and the two transgenic lines. (a) Relative expression of *PpSOS2* in leaves and roots. *PpSOS2* was expressed in leaves and roots of both transgenic lines. In the PpSOS2.OE3.5 transgenic line, the expression level was significantly higher in roots than leaves, while in PpSOS2.NP2.7, the expression level was similar in roots and leaves. (b) Relative expression of *PpSOS2* under control and 50 mM NaCl. The relative expression of *PpSOS2* was significantly induced in seedlings of PpSOS2.E3.5 after 24 h after the 50 mM NaCl treatment. Genotypic means followed by the same letters are not significantly different according to the Tukey HSD test ($P < 0.05$). Bars represent standard errors of the means.

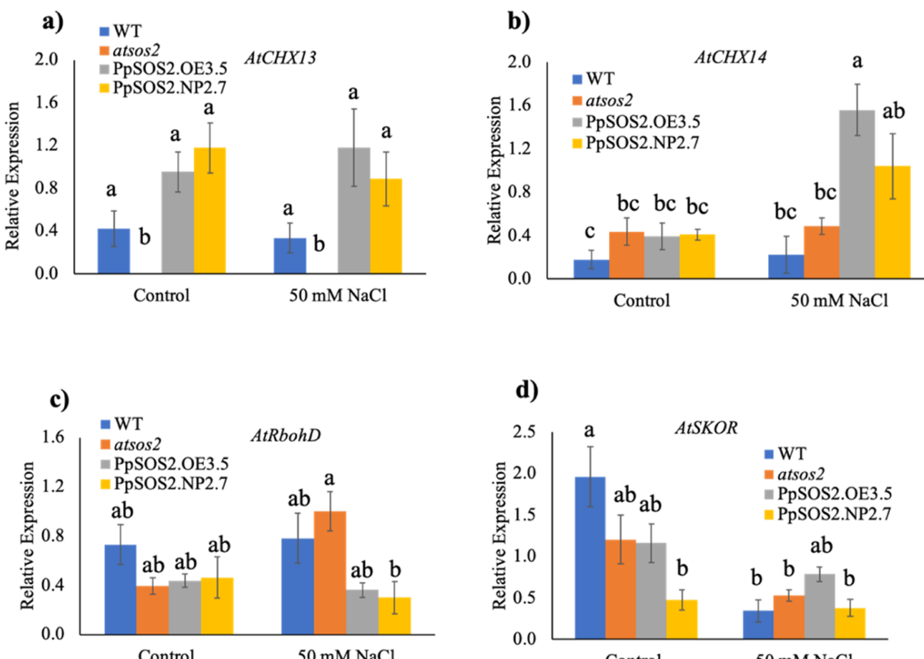

Figure 5. Gene expression analysis of K^+ rectifying channels and of ROS-specific genes in WT Arabidopsis (WT), *atsos2* mutant, and PpSOS2.OE3.5 and PpSOS2.NP2.7 transgenic seedlings after 24 h under 50 mM NaCl. Genetic expression of (a) *AtCHX13*, (b) *AtCHX14*, (c) *AtRbohD*, and (d) *AtSKOR*. Genotypic means followed by the same letters are not significantly different according to the Tukey HSD test ($P < 0.05$). Bars represent standard errors of the means. Two biological replicates and two technical replicates for each biological replicate were used in the study.

to control and was significantly upregulated compared to *atsos2* under salinity (Figure 5b). *AtRbohD* expression significantly reduced in the PpSOS2.NP2.7 transgenic line compared to the *atsos2* mutant under salinity (Figure 5c). The expression of the *AtSKOR* gene was downregulated in WT under salinity compared to control. However, none of the other three genotypes showed downregulation of *AtSKOR* (Figure 5d).

3.4. Phylogenetic Analysis of the *SOS2* Gene in Different Species. A phylogenetic analysis was conducted to have an insight into the evolutionary relationship among SOS2 proteins from 12 plant species with varying levels of salinity stress tolerance (Figure 6 and Table S2). *P. persica* was grouped with its closely related species *P. dulcis* in a separate cluster in group II. Both species are relatively sensitive to salinity.[57] Similarly, salt-sensitive *Phaseolus vulgaris* (French bean) and *Cajanus cajan* (pigeon pea) were grouped in a separate cluster in group II.[58,59] Only *Punica granatum* (pomegranate), a salt-tolerant species, clustered in group II with all salt-sensitive species. All the cereals (sorghum, maize, rice, and barley) clustered together in group I. Highly salt-tolerant species such as *Phoenix dactylifera* (date palm) and *Helianthus annuus* also clustered in group I but showed high divergence with their group members (Figure 6).

3.5. Subcellular Localization. Subcellular localization tools resulted in multiple locations of both AtSOS2 and PpSOS2 proteins (Table S3). Most of the tools localized the two proteins in the cytoplasm and nucleus. However, at least one tool predicted the location in mitochondria or plastids (Table S3).

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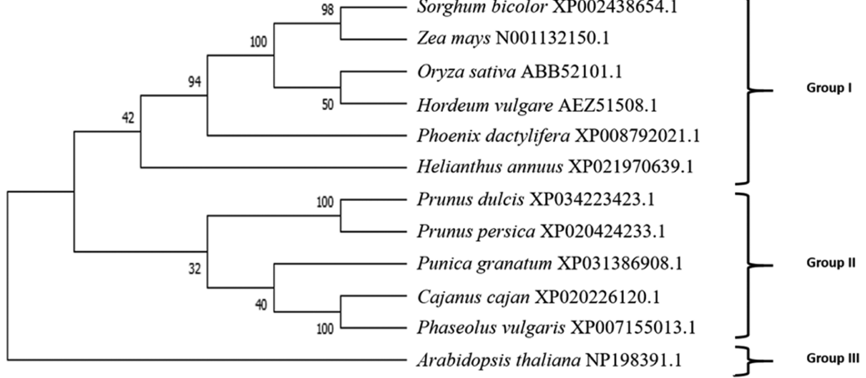

Figure 6. Phylogenetic analysis of the *SOS2* gene of the indicated plant species.

4. DISCUSSION

Salinity is one of the main environmental stresses that negatively affect agricultural production.[60] The predominant ions in saline water and soil (Na^+ and Cl^-) often affect plant growth in glycophytes. One of the plant's responses to salt stress is maintaining cellular ion homeostasis by excluding Na^+ and Cl^- ions.[6] The SOS signaling pathway is a well-defined pathway for excluding sodium ions at the cellular level.[17] The SOS pathway has three components, SOS1, SOS2, and SOS3. The loss-of-function mutation of all three genes causes hypersensitivity to NaCl.[17] *SOS2* is reported to play a significant role in salt tolerance in plants.[18,32,33,61] Although little is known about its role in *Prunus*, a recent study showed the highly induced expression of the *PpSOS2* gene in roots of four salt-tolerant almond rootstocks under salt treatment.[39]

In this study, we investigated the role of the *PpSOS2* gene in salt tolerance. We transformed the *PpSOS2* gene from the peach-based almond rootstock 'Nemaguard' in the Arabidopsis *atsos2* mutant under either a constitutive promoter or a native promoter. The *AtSOS2* gene showed negligible expression in the *atsos2* mutant and *PpSOS2* transgenic lines in the mutant background (Figure 1e). Both transgenic lines, overexpressing *PpSOS2* under the 2X35S promoter and the native promoter of *PpSOS2*, showed higher tolerance to salt than the *atsos2* mutant (Figure 2). The PpSOS2.OE3.5 and PpSOS2.NP2.7 transgenic lines had a significantly ($P \leq 0.05$) higher survival rate and dry weight than *atsos2* mutant plants under salinity (Figure 2b,c). The 90 mM NaCl treatment significantly inhibited the germination of the *atsos2* mutant. This germination inhibition was reversed by the functional complementation of *PpSOS2* under the native promoter but not under the 2X35S promoter (Figure 2d). Reduction in germination in the *atsos2* mutant indicates that the SOS2 protein plays a role in seed germination under salinity stress conditions. The role of *SOS2* on seed germination inhibition under salinity was reported previously.[26] The reduction of germination percentage in overexpressed lines may be due to a possible inhibitory effect of the excess quantity of the SOS2 protein. Some reports also suggest that CaMV 35S promoters are not expressed in all cell and tissue types or during the early stages of development.[62] Thus, there may be another reason for the low germination rate of lines containing *PpSOS2* under the 2X35S promoter lines in both control and salt treatments compared to WT and *PpSOS2* under the native promoter.

It has been shown earlier that the *atsos2* mutant is hypersensitive to salt as it accumulates more Na^+, retains less K^+ in plant tissues under salinity than control, and is defective in the regulation of K^+/Na^+ transport.[17] The *SOS2* locus is essential for salt tolerance and potassium nutrition, and thus, cellular K^+ content above the threshold level needs to be maintained by plants in the presence of excess Na^+ for plant growth and development.[17] In this investigation, the functional complementation of the *PpSOS2* gene in the *Arabidopsis atsos2* mutant enhanced the survival rate and dry weight under salt stress and restored the salt tolerant phenotype (Figure 2b,c). An earlier study reported that the overexpression of SOS pathway genes increased salt tolerance in transgenic *Arabidopsis*.[63] The ectopic expression of the active form of *AtSOS2* in Arabidopsis transgenic lines enhanced salt tolerance.[31] The overexpression of *AtSOS2* partially rescued the salt hypersensitivity of *sos2* and *sos3* mutants, while expression of the SOS2 protein in WT *A. thaliana* conferred increased salt tolerance.[31] When subjected to salt treatment, the poplar *PtSOS2* gene improved both plant survival and health of transgenic poplar plants.[33,61] The improved salt tolerance was associated with a decreased Na^+ accumulation in the leaves of transgenic plants and higher plasma membrane Na^+/H^+ exchange activity and Na^+ efflux in the transgenic plants. Also, the transgenic plants showed improved ROS scavenging capacity.[33]

The root is the first organ that senses the salinity stress and sends a signal to shoot tissues.[64] Salt stress is known to affect the root architecture severely.[17] Salt stress induces the reduction of primary root growth by reducing cell cycle activity at the root meristem.[64] The endodermal ABA signaling initiates the lateral root growth quiescence upon salt stress. In the later stages of stress, ABA signaling also leads to lateral root growth recovery.[64] We found that both primary and lateral root growth were severely inhibited under the 50 mM NaCl treatment in *atsos2* mutant lines. However, *Arabidopsis* transgenic plants expressing *PpSOS2* in *atsos2* background had a significant improvement in primary and lateral root lengths compared to *atsos2* under salinity (Figure 3a,c,d). These observations suggested that *PpSOS2* regulates gene networks involved in primary and lateral root growth under salinity. It was demonstrated that SOS signaling plays a significant role in lateral root initiation and is essential for lateral root emergence in response to low salt stress.[65] Low NaCl concentration (30 mM, water potential −0.15 MPa) did not affect the primary root growth but significantly affected lateral root growth in SOS mutants.[65] However, similar iso-osmotic concentrations of mannitol did not have any effect on lateral root growth. These results show that the SOS signaling

https://doi.org/10.1021/acsagscitech.1c00276
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pathway regulates the ionic effect of NaCl on lateral root development.[65]

Electrolyte leakage is a hallmark of the salt stress response in a plant cell and is an important parameter to evaluate plant salt tolerance.[66] The transgenic lines, PpSOS2.OE3.5 and PpSOS2.NP2.7, had significantly lower electrolyte leakage after 10 days under 50 mM NaCl stress than the *atsos2* mutant plants (Figure 3e). Salt stress induces membrane permeability to K^+ and counterions such as Cl^-, HPO_4^{2-}, NO^{3-}, and $C_4H_4O_5^{-2}$ (malate) that move to balance the efflux of K^+ but do not disturb membrane integrity.[67] Both transgenic lines showed less electrolyte leakage than the *atsos2* mutant under salt stress, indicating that the transgenic lines maintain membrane permeability and reduce the efflux of K^+ and counterions under salt stress. Soil salinity and salt toxicity are mainly associated with Na^+ and Cl^- toxicity and are the main focus of salt stress studies.[8,39,67] K^+ also plays a significant role in abiotic and biotic stress responses. Emerging studies showed the significant role of K^+ signaling in a plant's adaptive response to the environment.[68,69] SOS2 acts as a regulatory component controlling potassium nutrition as *atsos2* mutants could not grow on a culture medium low in K^+ [17]. The expression of *PpSOS2* was significantly higher in the PpSOS2.OE3.5 transgenic line under the 50 mM NaCl treatment, while there was no significant change in the gene expression of *PpSOS2* in the PpSOS2.NP2.7 transgenic line with or without salt treatment (Figure 4b). The expression analysis of the *PpSOS2* gene was carried out at 24 h post-treatment, which is a very early stage of ionic stress during salt stress. That could be a reason why we could not observe the induction of *PpSOS2* in the PpSOS2.NP2.7 transgenic line.

AtCHX13 is a cation-proton exchanger and reported to be a high-affinity K^+ transporter.[70] The expression of *CHX13* was reported to be upregulated in roots during salt stress and required for early root halotropism under low K^+ availability conditions.[71] Our study found that functional complementation of the *atsos2* mutant with *PpSOS2* induces the expression of *AtCHX13* in transgenic seedlings compared to *atsos2* both under control and salinity conditions (Figure 5a). However, there is no significant difference seen in the expression of *AtCHX13* in transgenic lines on salt treatment compared to control (Figure 5a). No expression of *AtCHX13* was observed in the *atsos2* mutant either under control or treatment (Figure 5a). Though the CHX13 protein has a possible role in K^+/Na^+ transport during salt stress, its mechanism is not fully understood. Our results indicate that *PpSOS2* may play a significant role in the regulation/induction of CHX13 in K^+/Na^+ transport during salt stress.

The expression of *AtCHX14*—a plasma membrane cation/H^+ exchanger a close homologue of *AtCHX13*—was significantly increased in overexpressing the transgenic line after 24 h of salt treatment (Figure 5b). Interestingly, the expression of *CHX14* was relatively lower in WT plants than in transgenic lines and was supported by a study carried out in Arabidopsis.[70] Though not much work has been done on the role of CHX14 in salt tolerance, our results suggest a significant involvement of the CHX14 channel and its close homologue AtCHX13 in salt tolerance and the SOS signaling pathway to maintain ion homeostasis under salt stress. Further studies are required to dissect the role of *PpSOS2* not only in Na^+ toxicity but also in K^+ signaling in plant response and tolerance to salt.

Phylogenetic analysis revealed that the studied species grouped into three major clusters based on the divergence in SOS2 protein sequences (Figure 6). SOS2 from *P. persica* showed higher similarity to *P. dulcis*, as expected, due to the close relationship between the species. Despite being moderately tolerant, sorghum[71] was grouped with maize,[72] which is moderately salt-sensitive. Similarly, barley,[73] a salt-tolerant crop, is grouped with highly salt-sensitive rice,[74] indicating that, although there are significant differences among different cereal species in terms of salt tolerance, the SOS2 protein sequence is relatively conserved. It was interesting to observe higher divergence in the protein sequences of salt-tolerant species (*P. dactylifera*, *Helianthus annus*, and *P. granatum*).[75−77] These observations suggest the involvement of several players in the regulation of salt tolerance, emphasizing the complexity of the salt tolerance mechanism in *Prunus*.

Subcellular localization, using different tools, predicted that AtSOS2 and PpSOS2 are located in different organelles inside the cell. The multiple predicted locations of the SOS2 protein can be justified by the fact that it is involved in the activation of various transporters, antiporters, and channels. It aligns with the cytosolic and nuclear fluorescence observed by the GFP-tagged SOS2 protein previously.[78] Localization in multiple cell organelles strengthens the argument about the multifunctional roles of PpSOS2 protein kinase, which interacts with different proteins to regulate different pathways involved with salinity tolerance.

5. CONCLUSIONS

Our results indicate that *PpSOS2* can functionally complement the *atsos2* mutant and restore its salt tolerance. The PpSOS2.OE3.5 and PpSOS2.NP2.7 transgenic lines expressing *PpSOS2* under a constitutive promoter and the endogenous promoter had higher germination, percent survival, and higher dry weight under salinity than the *atsos2* mutant. Both transgenic lines exhibited lower electrolyte leakage than *atsos2*-mutant plants after 10 days under the 50 mM NaCl treatment, indicating that they could maintain cell membrane integrity and avoid electrolyte leakage better than *atsos2*. Differential expressions of *AtCHX13 and AtCHX14* were seen in transgenic lines under salinity but not in *atsos2* mutant seedlings. Expression of *AtCHX14*—a plasma membrane cation/H^+ exchanger, more specifically a K-efflux transporter—was significantly increased in overexpressing transgenic lines 24 h after salt treatment. However, additional work is needed to confirm its previously reported role in K^+ homeostasis and thus salt tolerance. Expression of *AtCHX13*, a close homologue of *AtCHX14*, was significantly reduced in the *atsos2* mutant. The complementation of the *atsos2* mutant with *PpSOS2* induced a significant increase in the expression of the *AtCHX13* gene in transgenic lines irrespective of salt treatment. CHX13 was shown to play a possible role in K^+/Na^+ homeostasis. As SOS2 is known to regulate the expression of both *AtCHX13* and *AtCHX14* during salt stress, SOS2 may play an essential role in K^+ signaling besides being a central regulator of SOS signaling. Divergence in the SOS2 orthologs of salt-tolerant species indicates the possible variation in SOS2 protein functions and opens an avenue for future studies focusing on the SOS signaling pathway in other species. Further studies on *PpSOS2* and its role in salt tolerance will help to understand salt tolerance in *Prunus* and the potential

https://doi.org/10.1021/acsagscitech.1c00276
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use of *PpSOS2* as a candidate gene for gene editing to develop salt-tolerant almonds rootstocks.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsagscitech.1c00276.

List of primers used in the study; list of the plant species studied for the phylogenetic analysis of SOS2 orthologs; subcellular localization *of A. thaliana* (NP198391.1) and *P. persica* proteins (XP020424233.1); and gene expression of K^+ rectifying channels (PDF)

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Author Contributions

D.S. and A.K. conceptualized, designed, and supervised the experiments. AK developed genetic material and evaluated transgenic lines. A.K., V.S., M.D., B.A., and BN carried out salt tolerance and gene expression assays. A.K., A.L., J.F.S.F., and DS analyzed and interpreted the data. The manuscript was written jointly with contributions from all the authors. All authors have read and approved the manuscript.

Funding

This study was supported by a funded grant from the Almond Board of California (18.Hort26.Sandhu-COC) (https://www.almonds.com/) to Devinder Sandhu (PI) and Jorge Ferreira (Co-PI). This research was also supported by the faculty start-up funds to AK from the Department of Plants, Soils, and Climate, USU. Marco Duenas was supported and funded by Award Number T34GM062756 from the National Institutes of Health.

Notes

The authors declare no competing financial interest.
All relevant data are within the manuscript.

■ ACKNOWLEDGMENTS

Acknowledgements are due to Dr. Kiran Mysore, Noble Research Foundation, Ardmore, Oklahoma, for providing Gateway Cloning Vectors and Dr. Manju Pudussery for technical help.

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