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ORIGINAL ARTICLE

Soybean Na^+/H^+ antiporter *GmsSOS1* enhances antioxidant enzyme activity and reduces Na^+ accumulation in Arabidopsis and yeast cells under salt stress

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Abstract Our previous work revealed that the soybean *GmsSOS1* enhances salt tolerance in Arabidopsis. In this work, we studied the physiological mechanisms by which the GmsSOS1 confers salt and oxidative stress tolerance in Arabidopsis and yeast cells. Under salt stress condition, the *GmsSOS1*-expressing Arabidopsis plants displayed larger leaf area, lower leaf relative electrolytic leakage, less accumulation of H_2O_2, superoxide anion radicals ($O_2^{\cdot-}$), and malondialdehyde compared with wild type. In consistent with these observations, the activities of antioxidant enzymes catalase, ascorbate peroxide, and peroxidase in the GmsSOS1-expressing plants were higher than those in wild type under salt stress. Combined salt and oxidative stresses caused more damage and higher accumulation of H_2O_2 and Na^+ than single stress condition in both wild type and the *GmsSOS1*-expressing plants. However, the *GmsSOS1*-expressing Arabidopsis plants could maintain significantly lower levels of H_2O_2 and Na^+ and exhibited better growth than wild type under either single or combined stress. The GmsSOS1 complemented the yeast plasma membrane-localized Na^+/H^+ antiporter and enhanced salt tolerance by reducing Na^+ accumulation in yeast cells. Our results suggest that the soybean GmsSOS1 can alleviate the primary Na^+ toxicity by limiting Na^+ accumulation and mitigate the secondary oxidative stress through improving antioxidant enzyme activity.

Keywords *GmsSOS1* · Arabidopsis · Salt stress · Antioxidant enzymes · Na^+ and K^+ contents

Introduction

Soil salinization is an increasingly problem for the irrigated lands, and high salinity in soil adversely affects plant growth and development thus reduces crop quality and yield (Yadav et al. 2012; Ma et al. 2014a, b; Volkov 2015). Salt stress causes primary injuries, including osmotic effects at an early phase and ionic toxicity at a later phase of plant growth (Sanadhya et al. 2015). This is often followed by secondary damages involving nutritional imbalance and oxidative stress due to accumulation of reactive oxygen species (ROS), such as $O_2^{\cdot-}$ and H_2O_2 (Manchanda and Garg 2008; de Oliveira et al. 2013; He et al. 2015). However, plants have evolved diverse mechanisms to mitigate the damaging effects of salt stress through morphological, physiological, biochemical, and molecular adjustments (Qiu et al. 2014; Schmidt et al. 2013). In soil, sodium (Na^+) is a common soluble ion deleterious to plants, including all major crops. Na^+ is not an essential ion for most plant growth, thus excessive Na^+ outside of or accumulation in plant cells results in disturbance or imbalance of intracellular osmotic, ionic, and oxidative homeostasis (Adem et al. 2014). Therefore, preventing Na^+ accumulation and maintaining appropriate K^+/Na^+ ratio in the cytoplasm, which are largely regulated via Na^+ transporters, are crucial for plant survival and growth in saline

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environments (Xue et al. 2014; Liu et al. 2015; Sanadhya et al. 2015; Volkov 2015; Wu et al. 2015).

The SOS (salt-overly sensitive) pathway consisting of three components, the plasma membrane-localized Na^+/H^+ antiporter SOS1, the cytoplasmic protein kinase SOS2, and the Ca^{2+} sensor SOS3, is the most extensively studied salt tolerance mechanisms in plants (Ma et al. 2014a; Zhou et al. 2014). The *A. thaliana* SOS1 protein (AtSOS1) is the first described plasma membrane-localized Na^+/H^+ antiporter mediating Na^+ efflux and controlling long-distance Na^+ transport from roots to shoots (Shi et al. 2002). Oh et al. (2010) further revealed the roles of SOS1 in vacuolar morphology, pH homeostasis, and membrane trafficking, in addition to conferring salt tolerance in roots during early stages of salt stress. Up to now, a number of *SOS1* homologous genes have been identified and functionally analyzed from different plant species, including halophytes, such as *Thellungiella salsuginea*, *Populus euphratica*, *Mesembryanthemum crystallinum*, *Salicornia europaea*, *Suaeda salsa* and *Sesuvium portulacastrum*, xerophyte as *Zygophyllum xanthoxylum* and crop plants, such as rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), wheat (*Triticum aestivum* and *Triticum durum*), and soybean (*Glycine max* and *Glycine soja*) (Ma et al. 2014b; Martínez-Atienza et al. 2007; Olías et al. 2009a; Feki et al. 2014; Zhou et al. 2015; Nie et al. 2015). Transgenic studies suggest that SOS1 as an Na^+ efflux transporter could increase salt tolerance in different plant species (Quintero et al. 2002; Yadav et al. 2012; Feki et al. 2014).

SOS1 mRNA in Arabidopsis is inherently instable at normal growth conditions, but its stability is elevated by salt or oxidative stress (Chung et al. 2008; Jiang and Shi 2008). ROS signals were shown to be required for the regulation of Na^+/H^+ antiporter activity (Zhou et al. 2014). Interestingly, mutations in the gene render *atsos1* mutants more tolerant to oxidative stress caused by the treatment with MV (methyl viologen), indicating that AtSOS1 may play a negative role in oxidative stress tolerance (Chung et al. 2008). In addition, under salt or oxidative stress, AtSOS1 could interact through its long cytoplasmic C-terminal tail with RCD1 (Radical-Induced Cell Death1, a regulator of oxidative stress responses), thus the cross-talk between ion homeostasis and oxidative stress response might be involved in plant salt tolerance (Katiyar-Agarwal et al. 2006). Our previous work has shown that the plasma membrane Na^+/H^+ antiporter gene (named *GmsSOS1*) from *G. max*, *G. soja,* and their hybrid conferred salt tolerance in the Arabidopsis *atsos1-1* mutant and wild-type plants (Nie et al. 2015). In this work, we studied the physiological mechanisms of salt tolerance conferred by the *GmsSOS1* gene in Arabidopsis and yeast cells by examining Na^+ and ROS accumulation and ROS scavenging enzymes. We found that *GmsSOS1* not only conferred salt tolerance, but also elevated oxidative stress tolerance in the transgenic plants. Notably, we observed that oxidative stress treatments promoted Na^+ accumulation under salt stress condition, while *GmsSOS1* expression mitigated such enhanced Na^+ accumulation, which suggests a possible link between oxidative stress and Na^+ influx and/or efflux transport systems. Our results provided further evidence on the important role of the plasma membrane-localized Na^+/H^+ antiporter in salt and oxidative stress tolerance in plants.

Materials and methods

Plant materials, growth conditions, and stress treatments

A. thaliana (Columbia *glabrous1* ecotype) wild type (WT) and *GmsSOS1*-expressing line (T_3 generation, Nie et al. 2015) were used in this study. For seed germination test, seeds were surface-sterilized and stratified at 4 °C for 2–4 days. The seeds were then germinated and grown on MS medium supplemented with 3% sucrose (w/v) and 0.8% agar with or without 150 mM NaCl. After growth for 7 days, seed germination rate was calculated by counting the percentage of cotyledon greening (Li et al. 2015), 30 seeds per petri plate each with three replications. For stress response at seedling stage, surface-sterilized and stratified seeds were sowed in soil consisting of sterilized peat moss and vermiculite mixture in plastic pots (diameter = 7 cm), cultured with 1/2 Hoagland nutrition solution in a growth chamber (16 h light (22 ± 2 °C)/8 h dark (18 ± 2 °C) cycle at 60–70% relative humidity). The 5-week-old seedlings were divided into two groups for different treatments: one group was used for single salt stress (150 mM NaCl in 1/2 Hoagland nutrition solution) treatment and the other group was used for salt stress (120 mM NaCl), oxidative (3 μM MV or 4 mM H_2O_2), and combined stress (120 mM NaCl + 3 μM MV or 120 mM NaCl + 4 mM H_2O_2 in 1/2 Hoagland solution, respectively). The seedlings watered with 1/2 Hoagland nutrition solution only were used as the controls. After 7 days of treatments, the seedlings were photographed and sampled for measurements of leaf area and relative electrolytic leakage (REL), contents of $O_2^{\cdot-}$ and malondialdehyde (MDA), activities of antioxidant enzymes [catalase (CAT), peroxidase (POD), ascorbate peroxide (APX), and superoxide dismutase, i.e., SOD] in leaves and roots and for DAB (3,5-diaminobenzidine) staining of leaves or plants.

Measurements of leaf area and REL

Leaf area was measured by photographing the leaf samples which were then analyzed using ImageJ according to Maloof et al. (2013). REL in leaves or shoots was assayed as described by Tian et al. (2014). Briefly, the fresh samples (0.5 g) were added to a tube with 20 mL of deionized water, and the conductance of the deionized water (C_W), the leaf tissues before boiling (C_1), and the leaf tissue after boiling (C_2) was measured using a conductance meter (DDS-307, Shanghai, China). REL was calculated as $(C_1 - C_W)/(C_2 - C_W)$.

Determination of $O_2^{\cdot-}$ and MDA contents

$O_2^{\cdot-}$ content was measured by following the method by Gajewska and Sklodowska (2007) with some modifications. Fresh leaf and root tissues (0.5 g) were homogenized in 3 mL of 65 mM phosphate buffer (pH 7.8) and then centrifuged at 10,000*g* for 10 min. The supernatant (0.5 mL) was mixed with 0.5 mL of 65 mM phosphate buffer (pH 7.8) and 0.1 mL of 10 mM hydroxylamine chlorhydrate, and the mixture was incubated at 25 °C for 1 h. 1 mL of 58 mM sulfanilamide and 1 mL of 7 mM α-naphthylamine were then added, and the mixture was further incubated at 25 °C for 20 min. The absorbance was measured at 530 nm, and $O_2^{\cdot-}$ contents were calculated from a standard curve of $NaNO_2$.

MDA content was measured according to the method described by Jouve et al. (2007). A total of 0.5 g of fresh materials was ground in 5 mL of 5% (*w/v*) trichloroacetic acid (TCA), and 2 mL of supernatant was mixed with 2 mL of 0.67% (w/v) thiobarbituric acid (TBA) in 5% (w/v) TCA and incubated at 100 °C for 30 min. After centrifuging, the optical density was measured at 450, 532, and 600 nm, respectively. The amount of MDA was calculated from the following formula: $C = 6.45(A_{532}–A_{600}) - 0.56A_{450}$, C represents the concentration of MDA in supernatant and expressed as μmol L^{-1}, A_{532}, A_{600}, and A_{450} represent the absorbance values at 532, 600, and 450 nm, respectively. The final MDA content was expressed as nmol g^{-1} FW.

Assay of antioxidant enzyme activities

Enzyme extractions were performed according to the method of Meng et al. (2014). Fresh shoots and roots (0.5 g) were homogenized in a mortar and pestle with 2 mL of 50 mM ice-cold phosphate buffer (pH 7.0) containing 1 mM EDTA Na_2 and 0.5% PVP (w/v). The homogenate was centrifuged at 12,000*g* for 20 min at 4 °C. The supernatant was used as the enzyme extracts for assays of APX, CAT, POD, and SOD activities. CAT activity was assayed according to Aebi (1984). The 3-mL reaction mixture contained 0.1 mL of enzyme extracts, 0.1 M phosphate buffer (pH 7.0), deionized water, and 20 mM H_2O_2. The decomposition of H_2O_2 was measured by following the decrease in absorbance at 240 nm for 3 min and quantified by its molar extinction coefficient (39.4 mmol L^{-1} cm^{-1}). One unit of CAT activity was defined as a change in absorbance of 0.1 units min^{-1} caused by addition of the enzyme extracts. POD activity was measured with guaiacol as the substrate according to Nakano and Asada (1981). The reaction mixture (3 mL) consisted of 100 mM sodium acetate buffer (pH 5.4), 10 mM guaiacol solution, 0.1 mM H_2O_2, and 10 μL enzyme extracts. The increase in absorbance due to oxidation of guaiacol was measured at 470 nm for 1 min. One unit of POD activity was defined as a change in absorbance of 0.1 unit min^{-1}. SOD activity was assayed using the photochemical nitroblue tetrazolium (NBT) method (Beauchamp and Fridovich 1971). The reaction mixture contained 100 mM phosphate buffer (pH 7.8), 130 mM methionine, 750 μM NBT, 20 μM riboflavin, 1 mM EDTA Na_2, deionized water, and 80 μL enzyme extracts in a 3 mL volume. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction monitored at 560 nm. APX activity was assayed in a reaction mixture of 3 mL containing 100 mM phosphate (pH 7.0), 100 mM ascorbic acid, 0.1 mM H_2O_2, deionized water, and 0.1 mL enzyme extracts. The reaction was started by adding enzyme extracts to the mixture. Enzyme activity was quantified by following the decrease in absorbance at 290 nm for 3 min. One unit of APX activity was defined as an absorbance change of 0.01 units min^{-1} (Nakano and Asada 1981).

DAB staining

H_2O_2 in plant tissues was detected in situ by DAB staining as described by Li and Cai (2015). The leaves or plants were vacuum-infiltrated with a solution of DAB (1 mg mL^{-1}, pH 3.8), then placed in a 5-cm erlenmeyer flask wrapped with aluminum foil, and incubated for 6 h at 100 rpm in a shaker. After discarding the DAB staining solution, clearing solution (ethanol:acetic acid:glycerol = 3:1:1) was added to the samples and incubated in a boiling water bath (95 °C) for 15 min, and the clearing process was repeated twice. The DAB stained leaves or plants were then photographed. Each sample with at least ten leaves or six plants was conducted.

Measurements of Na^+ and K^+ contents

Roots and shoots of seedlings under NaCl, oxidative, or combined stress were fully rinsed in distilled water, fixed at

105 °C for 5 min, and were dried to constant weight at 80 °C. Dry matter was ground and screened with a 60-mesh sieve, then 100 mg of each sample was added to a tube (25 mL) and 20 mL of deionized water was added. Samples were boiled for 2–3 h, then filtered and deionized water was added to make a final volume of 50 mL. K^+ and Na^+ contents were estimated using a flame spectrophotometer (AP1200 type, Shanghai Aopu Analytical Instrument Co., China), and measurements were calibrated using NaCl or KCl solutions of known concentrations (Wei et al. 2015).

GmsSOS1 expression in yeast mutant and salt treatment test

Yeast (*Saccharomyces cerevisiae*) strain G19 (Δ*ena1::HIS3::ena4*), and its mutants ANT3 (Δ*ena1::HIS3::ena4*, Δ*nha1::LEU2*) and GX3 (Δ*ena1::HIS3::ena4*, Δ*nhx1::TRP1*) have been described elsewhere (Song et al. 2012). The entire soybean *GmsSOS1* open reading frame (Nie et al. 2015) was cloned into the yeast expression vector pYES2 under the control of the constitutive promoter *GAL1* (Halley et al. 2010). The yeast expression vector containing *GmsSOS1* gene was, respectively, transformed into NHA1- or NHX1-deleted yeast mutant strains *ena1 nha1* or *ena1 nhx1* using PEG/LiAc procedure and transformants were identified by PCR-based method (Gietz 2014). The yeast mutant *ena1* was used as a positive control. Ten-fold serial dilutions (starting at OD_{550} ≈0.5) of each sample were plated on YPD (1% yeast extract, 2% peptone, and 2% dextrose), YPG (1% yeast extract, 2% peptone, and 2% galactose) medium, or YPG medium supplemented with hygromycin B (HygB) (50 mg L^{-1}) or arginine-phosphate medium (AP medium: 10 mM L-arginine, 8 mM H_3PO_4, 2 mM $MgSO_4$, 0.2 mM $CaCl_2$, 2% glucose, vitamins and trace elements, and pH 6.5) plus NaCl (70 mM) and KCl (1 mM), and the plates were incubated at 28 °C for 60 h and then photographed (Song et al. 2012). For Na^+ and K^+ content measurements, the yeast cells were grown in the liquid AP medium plus NaCl (70 mM) and KCl (1 mM) and collected at exponential growth phase (OD_{550} ≈0.2) (Quintero et al. 2002). The Na^+ and K^+ contents in the yeast cells were determined using a flame spectrophotometer as above-described. Each strain was repeated with three independent cultures.

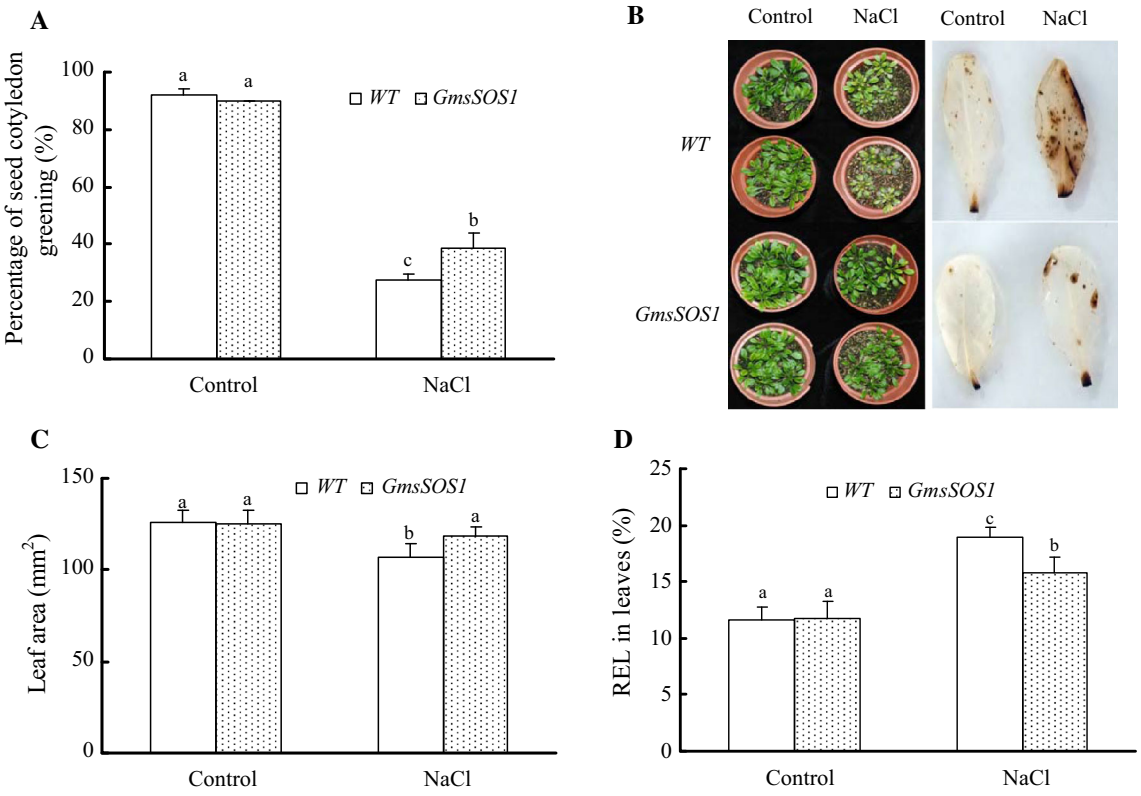

Fig. 1 Growth response of *GmsSOS1*-expressing and WT plants to salt stress. **a** Effects of 150 mM NaCl treatment on the percentage of cotyledon greening. **b** Growth phenotype of 5-week-old seedlings after salt stress treatment for 7 days (*left panel*) and DAB staining of the leaves (*right panel*, *brown* color indicates H_2O_2 accumulation). **c** Leaf area. **d** REL

Statistical analysis

Data were expressed as mean ± SD of at least three replications besides the above special instructions, and were analyzed using one-way analysis of variance (ANOVA) with Duncan's multiple range test at the $P < 0.05$ level of significance in the SPSS 19.0 software.

Results

Expression of *GmsSOS1* in Arabidopsis enhances seed germination and seedling growth under salt stress

When seeds of *WT* and *GmsSOS1*-expressing *A. thaliana* were grown in MS agar medium without NaCl, the percentages of seed cotyledon greening in both genotypes exceeded 90% and did not show significant difference ($P > 0.05$). When germinated in MS agar medium plus 150 mM NaCl, the percentages of seed cotyledon greening of both genotypes were remarkably declined as compared to the controls, while the cotyledon greening of *GmsSOS1*-expressing Arabidopsis was significant higher than that of *WT* ($P < 0.05$) (Fig. 1a). When the seedlings grown in soil were treated with 150 mM NaCl solution for 7 days, the growth of both genotypes was visibly inhibited, accompanied with significant decrease in leaf area and increase in REL. However, the salt-induced changes in leaf area and REL in *GmsSOS1*-expressing Arabidopsis were less than those in WT (Fig. 1b-left, c, d). DAB staining is a common method to visualize in situ H_2O_2 accumulation in tissues as a reddish-brown precipitate (Fujibe et al. 2004). DAB staining indicated a lower accumulation of H_2O_2 in the leaves of *GmsSOS1*-expressing plants than that of WT after salt stress treatment (Fig. 1b-right).

Expression of *GmsSOS1* in Arabidopsis reduces $O_2^{-\cdot}$ accumulation and MDA content under salt stress

When cultured under normal conditions, the contents of O_2^- and MDA in leaves and roots of *WT* and *GmsSOS1*-expressing plants did not show significant differences ($P > 0.05$). After treated with 150 mM NaCl solution for 7 days, the contents of $O_2^{-\cdot}$ and MDA in leaves of *WT* increased 2.71 and 2.31 times of the controls, respectively ($P < 0.05$), while $O_2^{-\cdot}$ and MDA contents in leaves of *GmsSOS1*-expressing plants were only 1.18 and 1.35 times increases when compared with the controls. In roots, salt stress treatment resulted in a slight elevation of MDA content in wild type but not in *GmsSOS1*-expressing plants. Salt stress did not cause changes in $O_2^{-\cdot}$ contents in roots of both *WT* and *GmsSOS1*-expressing plants (Fig. 2).

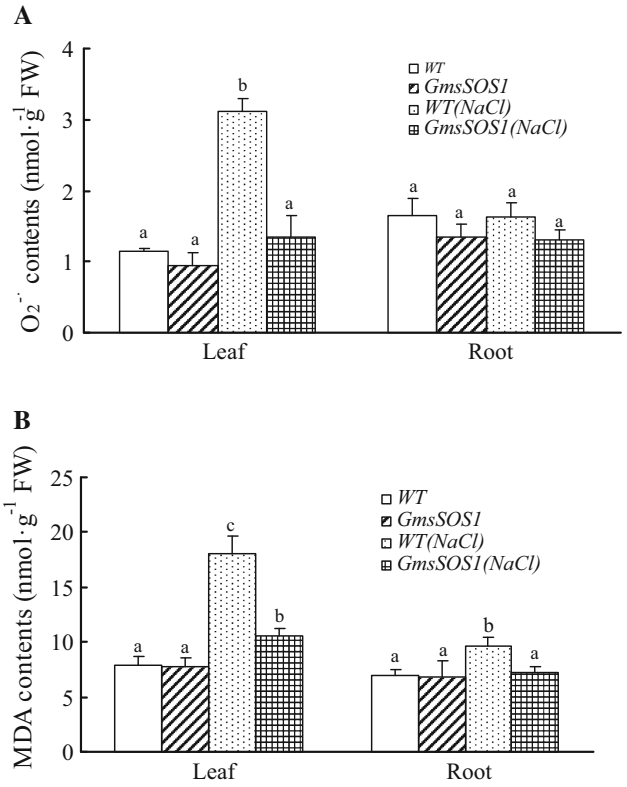

Fig. 2 Changes in the contents of $O_2^{-\cdot}$ (**a**) and MDA (**b**) in leaves and roots of 5-week-old *GmsSOS1*-expressing and WT seedlings after 150 mM NaCl stress for 7 days

Expression of *GmsSOS1* in Arabidopsis elevates the activities of antioxidant enzymes under salt stress

When cultured under non-stress condition, the activities of CAT, POD, SOD, and APX did not show significant difference between *WT* and *GmsSOS1*-expressing seedlings ($P > 0.05$) (Fig. 3). When exposed to 150 mM NaCl for 7 days, the activities of CAT in leaves and roots and APX in leaves of *WT* and *GmsSOS1*-expressing seedlings were remarkably decreased in comparison with the control plants, but the decreases in *GmsSOS1*-expressing seedlings were significantly lower than those in *WT*. The *GmsSOS1*-expressing seedlings displayed 1.51, 1.71, and 1.97 times higher activities of CAT in leaves and roots and APX in leaves than those in WT. Under salt stress, the POD activity in leaves of *GmsSOS1*-expressing seedlings increased significantly ($P < 0.05$) when compared with that in WT. The SOD activity in *GmsSOS1*-expressing seedlings did not increase when compared with WT under normal growth conditions or after salt treatment (Fig. 3). Overall, stronger activity of antioxidant enzymes could be maintained in *GmsSOS1*-expressing Arabidopsis seedlings than *WT* under saline condition.

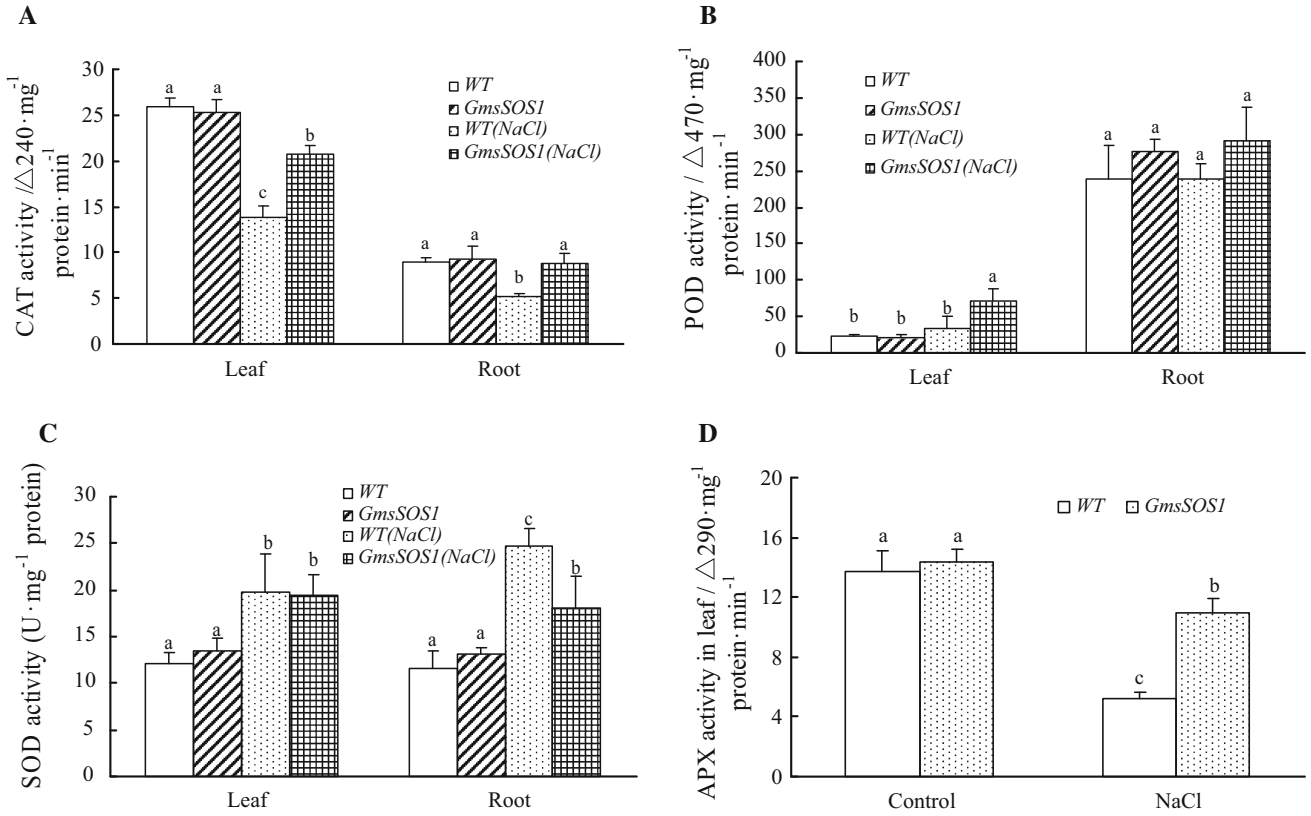

Fig. 3 Changes in the activities of CAT (**a**), POD (**b**), SOD (**c**) in leaves and roots and leaf APX (**d**) of 5-week-old *GmsSOS1*-expressing and WT seedlings after 150 mM NaCl stress treatment for 7 days

Effects of NaCl, oxidative and combined stress on growth and H_2O_2 accumulation in *WT* and *GmsSOS1*-expressing Arabidopsis

Under salt treatment with 120 mM NaCl for 7 days, the growth of *WT* seedlings was more inhibited and the seedlings displayed more severe salt stress phenotypes, such as yellowish and pigmented leaves than *GmsSOS1*-expressing seedlings. Treatment with MV (3 μM) or H_2O_2 (4 mM) caused weaker growth inhibition and stress-injury symptoms than NaCl treatment in both genotypes, but *GmsSOS1*-expressing seedlings displayed higher tolerance to these stress conditions than WT. A combination of salt and oxidative stress treatment (NaCl plus MV or H_2O_2) for 7 days resulted in more severe growth inhibition and stress damage than the single stress treatment in both genotypes, while the *GmsSOS1*-expressing seedlings performed visibly better than WT (Fig. 4a, upper panel). Improved tolerance to salt and oxidative stress by *GmsSOS1* was also evidenced by lower REL in the shoots of *GmsSOS1*-expressing seedlings than in WT (Fig. 4b). DAB staining indicated lower H_2O_2 accumulation in *GmsSOS1*-expressing seedlings than in WT under single (NaCl, MV, or H_2O_2) or combined stress conditions (Fig. 4a-lower panel).

Effects of NaCl, oxidative, and combined stress on Na^+ and K^+ contents in *WT* and *GmsSOS1*-expressing Arabidopsis

NaCl treatment increased the Na^+ contents in both leaves and roots of both genotypes, but *GmsSOS1*-expressing seedlings accumulated significantly lower Na^+ than WT. Oxidative stress (MV or H_2O_2) also caused a slight increase in Na^+ content, and *GmsSOS1*-expressing shoots had lower Na^+ content than WT after H_2O_2 treatment. Interestingly, the combined treatments (NaCl plus MV or H_2O_2) significantly increased Na^+ accumulation than NaCl treatment alone, and *GmsSOS1*-expressing seedlings accumulated less Na^+ than WT under these combined stress conditions (Fig. 5a, b). In addition, greater Na^+ reductions in *GmsSOS1*-expressing shoots than roots under NaCl or NaCl-oxidative combined stress were observed, which may account for improved salt tolerance of *GmsSOS1*-expressing plants. Overall, *GmsSOS1*-expressing plants did not show significantly changes in K^+ contents when compared with WT under single or combined stress conditions, except that H_2O_2 treatment resulted in a slight increase in K^+ content in the shoots of *GmsSOS1*-expressing plants (Fig. 5c, d).

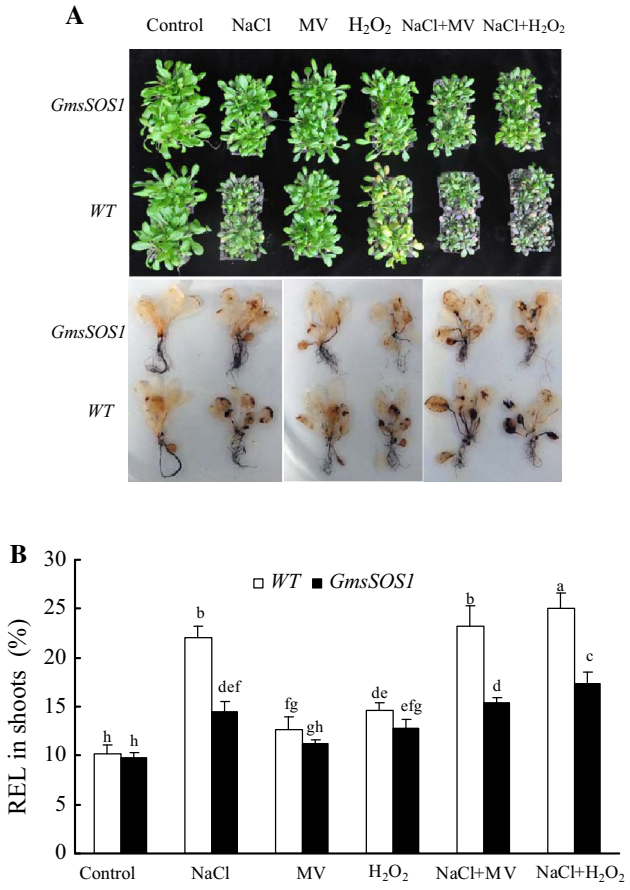

Fig. 4 Effects of 120 mM NaCl, oxidative stress (3 μM MV or 4 mM H_2O_2), and the combined stress (120 mM NaCl + 3 μM MV or 120 mM NaCl + 4 mM H_2O_2) treatment for 7 days on plant growth (**a**, *upper panel*), ROS accumulation (DAB staining, **a**, *lower panel*), and REL in shoots (**b**) of 5-week-old *GmsSOS1*-expressing and WT seedlings

GmsSOS1 complements the yeast *NHA1*

Yeast (*S. cerevisiae*) cells possess the plasma membrane Na^+/H^+ antiporter NHA1 for Na^+ efflux and the vacuolar membrane Na^+/H^+ antiporter NHX1 for Na^+ sequestration and conferring HygB resistance (Shi et al. 2002). To determine the capacity of GmsSOS1 to substitute for yeast NHA1 or NHX1 antiporters, mutant complementation tests were performed. Yeast mutant strains *ena1*, *ena1 nha1* and *ena1 nhx1* could grow normally in YPD or YPG medium, even when transformed with *GmsSOS1* (+*GmsSOS1*). When cultured in the AP medium plus 70 mM NaCl and 1 mM KCl, both *ena1 nha1* and *ena1 nhx1* mutants exhibited more growth inhibition than *ena1* mutant. Heterologous expression of *GmsSOS1* in *ena1 nha1* or *ena1 nhx1* restored cell growth in the presence of NaCl to the level equivalent to that mediated by the native NHA1 and NHX1 antiporters (strain *ena1*), but it could not rescue the sensitivity of *ena1 nhx1* to hygromycin B (Fig. 6a). This result indicates that *GmsSOS1* imparts specific tolerance to Na^+ through complementing the functions of the plasma membrane Na^+/H^+ antiporter NHA1. Na^+ and K^+ measurements revealed that *GmsSOS1* overexpression in *ena1 nha1* cells reduced cellular Na^+ accumulation. While *GmsSOS1* overexpression in *ena1 nhx1* did not significantly change the cellular Na^+ contents (Fig. 6b). *GmsSOS1* overexpression in the yeast mutants did not affect cellular K^+ accumulation (Fig. 6c).

Discussion

As an important member of the major salt tolerance *SOS* pathway, SOS1 has also been implicated in ROS detoxification or ROS-mediated signaling initiated by salt stress (Katiyar-Agarwal et al. 2006; Chung et al. 2008; Jiang and Shi 2008; Zhou et al. 2014). There are a number of research works showing that overexpression of *SOS1* in various plant species could confer salt tolerance by reducing Na^+ in roots, or retrieving Na^+ from the xylem or recirculating from leaves of transgenic plants (Olías et al. 2009a, b; Shi et al. 2003). Post-transcriptional gene silencing of *ZxSOS1* gene demonstrated that ZxSOS1 is important not only in long-distance transport and spatial distribution of Na^+ and K^+, but also in maintaining Na^+, K^+ homeostasis in salt-treated *Z. xanthoxylum* plants (Ma et al. 2014b). In this study, the *GmsSOS1*-expressing Arabidopsis showed clear alleviation in salt injury symptoms and enhanced salt tolerance when compared with WT (Figs. 1, 4). Salt tolerance conferred by GmsSOS1 could be attributed to reduced Na^+ accumulation and maintaining ion homeostasis in the transgenic plants under salt stress (Fig. 5). We also observed that *GmsSOS1*-expressing Arabidopsis seedlings had reduced accumulation of $O_2^{-\cdot}$ and H_2O_2 (Figs. 2a, 4a), which is coincided with stronger activities of antioxidant enzymes, such as APX, CAT, and POD (Fig. 3). Lower ROS (including H_2O_2 and $O_2^{-\cdot}$) levels may account for reduced damages in *GmsSOS1*-expression plants under salt stress.

To determine whether *GmsSOS1* provided not only salt tolerance but also oxidative stress tolerance, we compared the responses of *GmsSOS1*-expressing and WT plants to oxidative stress (MV or H_2O_2) and a combined salt-oxidative stress. Our results indicated that *GmsSOS1*-expressing plants grew better than WT especially under the combined stress conditions, which is supported by lower REL in the *GmsSOS1*-expressing plants (Fig. 4). Better performance of *GmsSOS1*-expressing plants is consistent with reduced Na^+ accumulation in shoots and roots of the transgenic plants. Interestingly, oxidative stress treatments (MV or H_2O_2) resulted in increased accumulation of Na^+, and expression of GmsSOS1 alleviated the elevation of

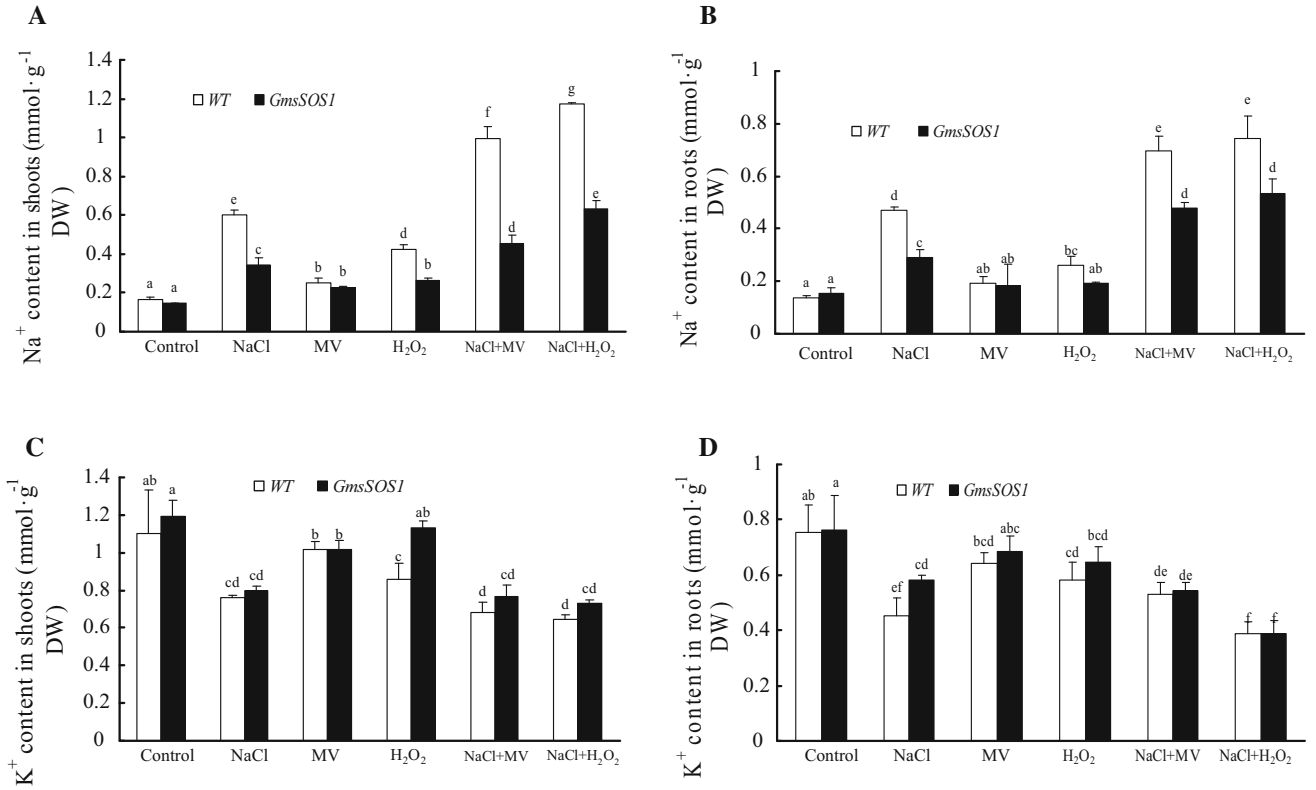

Fig. 5 Effects of 120 mM NaCl, oxidative (3 μM MV or 4 mM H_2O_2) and the combined stress (120 mM NaCl + 3 μM MV or 120 mM NaCl + 4 mM H_2O_2) treatment for 7 days on Na^+ contents in shoots (**a**) and roots (**b**), K^+ contents in shoots (**c**) and roots (**d**) of 5-week-old *GmsSOS1*-expressing and WT seedlings

Na^+ contents caused by oxidative stress (Fig. 5a, b). These results suggest that ROS somehow target and inactivate the Na^+ efflux transporters such as the plasma membrane Na^+/H^+ antiporter SOS1 thus enhances Na^+ accumulation in roots and shoots, while overexpression of GmsSOS1 could compensate the plasma membrane Na^+/H^+ antiporter activity thus reduce Na^+ accumulation in the plants. Katiyar-Agarwal et al. (2006) and Chung et al. (2008) have documented the implications of SOS1 in oxidative stress, and suggested that SOS1 and RCD1 might be important in controlling apoplastic ROS accumulation under salt stress and that physical interaction between the cytoplasmic C-terminal tail of plasma membrane-located SOS1 and the nucleus-localized RCD1 could bridge the cross-talk between ionic homeostasis and oxidative stress pathway in plants exposed to salt or oxidative stress. When compared with Arabidopsis *WT*, *sos1,* and *rcd1* mutant plants were more sensitive to salt stress and apoplastic ROS imposed by H_2O_2, but more tolerant to chloroplastic ROS imposed by MV (Katiyar-Agarwal et al. 2006). When soybean *GmsSOS1* was compared with counterpart genes of the glycophyte *Arabidopsis thaliana* (*AtSOS1*, GenBank accession No. HE802928.1) and its close relative and halophytic species, *T. halophila* (*ThSOS1*, GenBank accession No. EF207775.1), 68.5 and 65.4% identity were shared, respectively, but 87.1% was displayed between *AtSOS1* and *ThSOS1*. This may indicate that *GmsSOS1* is somewhat different with *AtSOS1* functioned under oxidative stress (including apoplastic and chloroplastic ROS). This intriguing observation deserves further investigation.

GmsSOS1 expression in the yeast mutants defective of NHA1 or NHX1 did not affect cellular K^+ uptake, but significantly reduced Na^+ accumulation and restored cell growth of the NHA1 defective mutant under salt stress (Fig. 6). This result further supports that GmsSOS1 functionally complements the yeast plasma membrane Na^+/H^+ antiporter NHA1. *AtSOS1* overexpression in the yeast mutants defective of NHA1 or NHX1 resulted in not only reduction in the Na^+ content but also improvement in the K^+ status (Quintero et al. 2002; Shi et al. 2002), which is not fully consistent with our work on *GmsSOS1*-overexpressing yeast mutants. However, *AtSOS1*-overexpressed yeast mutant cells lacking TRK1 and TRK2 proteins (both comprising the high-affinity K^+ uptake system of *S. cerevisiae*) failed to restore the growth in low external K^+ condition, which suggests the role of AtSOS1 on K^+ uptake is indirect (Quintero et al. 2002). The previous works have indicated that AtSOS1 may play a negative role in oxidative stress tolerance of Arabidopsis plants (Chung et al. 2008). Salt cress (*T. halophila*) is a close relative of

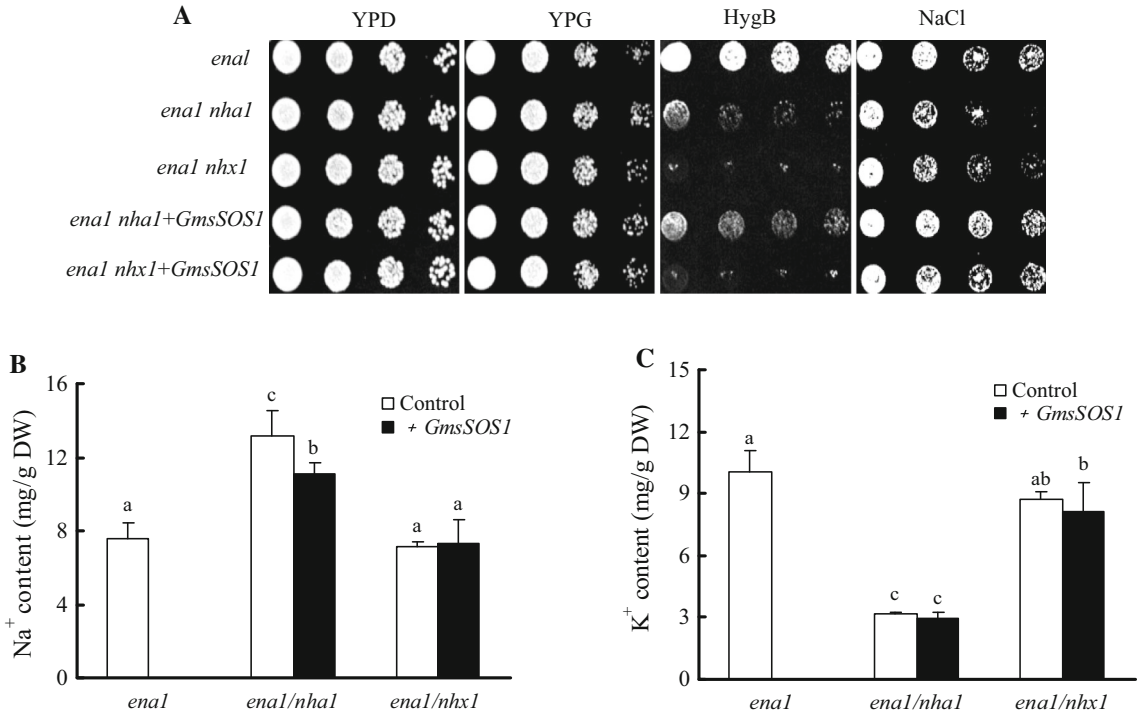

Fig. 6 Complementation of the yeast Na^+/H^+ antiporter NHA1 by GmsSOS1. **a** Cells of *S. cerevisiae* strains *ena1*, *ena1 nha1*, *ena1 nhx1*, and *ena1 nha1*and *ena1 nhx1* transformed with *GmsSOS1* (+*GmsSOS1*) were grown in YPD, YPG, or YPG medium supplemented with HygB (50 mg L^{-1}) or AP medium plus NaCl (70 mM) and KCl (1 mM). Five microliters of serial decimal dilutions were spotted onto agar plates of the same medium. Plates were incubated at 28 °C and photographed after 60 h. **b**, **c** Cells of the above-mentioned *ena1*, *ena1 nha1*, *ena1 nhx1* (control), or *GmsSOS1*-expressing cells (+*GmsSOS1*) were grown in liquid AP medium with NaCl (70 mM) and KCl (1 mM). When cultures reached OD_{550} ≈0.2, cells were collected by filtration, and the Na^+ and K^+ contents were determined. Data shown are the average ± SD of ion contents of three independent cultures of each strain

Arabidopsis but very tolerant to high salinity. The *SOS1* homologous gene in salt cress (*ThSOS1*) is expressed at high levels even in the absence of salt stress, and compared to Arabidopsis, salt cress is also more tolerant to oxidative stress (Taji et al. 2004). In this study, *GmsSOS1*-overexpressing Arabidopsis plants displayed enhanced salt and oxidative stress tolerance than WT. Certainly, *GmsSOS1* gene possesses phylogenetic background different or distant from *AtSOS1* or *ThSOS1* (Gao et al. 2016), and the capacity of salt tolerance of soybean (Zhang et al. 2011) is between salt cress and Arabidopsis. Thus, the physiological variations between the transgenic Arabidopsis plants harboring *GmsSOS1* and *AtSOS1*, and whether these two homologous genes execute their functions in the Arabidopsis genome in the same or different ways, are worthy to be studied in the future.

Salt tolerance is a complex trait involving multiple genes. Classical and molecular-assisted breeding towards salt tolerance in crops have not been very successful due to the complex feature of soil salinity and plant adaptation to saline environments (Zhang and Shi 2013). However, several key players, such as the *SOS* genes, have been identified and well characterized in plants. Natural variations of these salt tolerance genes could provide tools for crop improvement. For example, the *SOS1* variant genes in soybean varieties and wild soybean species may display distinct capability for salt tolerance. Alternatively, the *SOS1* variant genes may have different expression patterns due to evolutionary adaptation to saline environments. Both alternations in the coding regions and regulatory promoters could be utilized for improvement of salt tolerance in soybean by classical or molecular breeding.

In conclusion, compared with *WT*, *GmsSOS1*-expressing Arabidopsis exhibited reduced salt injury and enhanced salt and oxidative stress tolerance not only by reducing Na^+ accumulation, but also by modulating the antioxidant enzyme activities and maintaining relatively lower ROS levels in plant roots and shoots. Heterologous expression of *GmsSOS1* in the yeast mutant defective of NHA1 reduced Na^+ accumulation and increased salt tolerance of the mutant cells. Our results further demonstrate that soybean or other plant SOS1 gene can be considered as a superior salt tolerance determinant that could be utilized in molecular breeding for creating salt tolerant crop varieties.

Author contribution statement Xiufang Zhao, Peipei Wei, and Zhen Liu conducted the experiments, collected, and analyzed the data. BingjunYu and Huazhong Shi

designed the experiments. Bingjun Yu, Huazhong Shi, and Xiufang Zhao interpreted the data and wrote the manuscript. All authors read and approved the final version of the manuscript and have no conflict of interest.

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