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Research article

SIP1, a novel SOS2 interaction protein, is involved in salt-stress tolerance in *Arabidopsis*

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ABSTRACT

A novel salt overly-sensitive 2 (SOS2) interaction protein was identified by yeast two hybrid (Y2H) library and was referred to as SOS2 interaction protein 1 (SIP1). SIP1 belongs to a plant-specific protein family, which contains a conserved domain that corresponds to a putative N-acetyltransferase. The members of this family are tolerant to heavy metals and oxidative stress. Here, SIP1 was identified as a salt-responsive gene. The *sos2* × *sip1-1* double mutant was more sensitive than the *sos2* single mutant upon salt stress, whereas the overexpression of SIP1 gene enhanced the plant salt tolerance, suggesting that SIP1 was involved in plant salt tolerance. We also found that SIP1 increasingly accumulated in response to salt stress, and this accumulation was inhibited in the *sos2* mutant background. This finding suggests that the function of SIP1 upon salt stress was dependent on SOS2 protein. Further investigation suggested that SIP1 improved *Arabidopsis* tolerance to salt stress by reducing the ROS accumulation. Taken together, these findings reveal a novel function of SIP1 in adjusting *Arabidopsis* adaptation to salt stress.

1. Introduction

Salt stress retards plant growth and decreases crop productivity. The response of plants to salinity stress is regulated at the transcriptional and post-transcriptional levels. However, substantial progress has been achieved in understanding the transcriptional process induced by stress signals or regulatory factors, which modulate gene expression. The salt overly-sensitive (SOS) pathway for salt stress signalling has been proposed based on molecular genetic analysis. Several SOS genes were cloned and found to be critical components of ion homeostasis in ion transport and signal transduction. SOS3, which encodes a myristoylated calcium-binding protein, interacts with and activates SOS2, which is a serine/threonine protein kinase (Halfter et al., 2000). SOS3-SOS2 complex regulates the activity of SOS1, which is a salt effector gene that encodes a plasma membrane Na^+/H^+ antiporter by phosphorylation upon salt stress (Qiu et al., 2002). Co-expression of SOS3 and SOS2, together with SOS1, can dramatically enhance the salt tolerance of the yeast mutant (Quintero et al., 2002). SOS2 also interacts with protein phosphatase 2C abscisic acid (ABA) INSENSITIVE 2 (ABI2) (Ohta et al., 2003), a well-known negative regulator of ABA signalling and is important for plant tolerance to several abiotic stresses, including salt, drought and freezing (Leung et al., 1997; Nakashima and Yamaguchi-Shinozaki, 2013).

In addition to ion toxicity, salt stress also leads to the rapid and high production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide (H_2O_2) and hydroxyl radicals (Zhu, 2001). ROS, as an important signal molecule, plays critical roles in response to biotic or abiotic stresses (Mittler, 2002; Apel and Hirt, 2004; Baxter et al., 2013). However, excessive production of ROS could interrupt the cellular redox homeostasis, leading to oxidative damage (Zhu, 2001; Miller et al., 2010). The C-terminal cytoplasmic tail of SOS1 interacts with the radical-induced cell death RCD1, which is a regulator of oxidative-stress responses, to protect the cell against oxidative injuries during salt stress (Katiyar-Agarwal et al., 2006). The *enh1-1* mutant, which was isolated as the enhancer of the salt sensitivity of *sos3*, presented high accumulation of ROS under salt stress, suggesting the possible link between SOS pathway and superoxide metabolism and indicating the function of ENHANCER OF SOS3-1 (ENH1) in the detoxification of ROS that results from salt stress (Zhu et al., 2007). SOS2 interacted with the H_2O_2 signalling protein nucleoside diphosphate kinase 2 (NDPK2) and inhibited its autophosphorylation activity (Verslues et al., 2007). Upon salt stress, the double mutant of *sos2ndpk2* showed more sensitivity than the *sos2*

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single mutant. SOS2 can also interact with CATALASE2 (CAT2) and CATALASE3 (CAT3), further supporting the connection between salt signal and H_2O_2 metabolism. However, more novel components that directly adjust the cytosolic redox status to adapt to salt stress should be investigated.

OXIDATIVE STRESS 3 (OXS3) belongs to a family of proteins that share a highly conserved domain, which corresponds to a putative N-acetyltransferase or thioltransferase catalytic site (Blanvillain et al., 2009). The OXS3 gene family has been wildly found in several plant species, such as *Arabidopsis thaliana*, *Brassica juncea*, *Medicago truncatula*, *Deschampsia Antarctica* and *Oryza sativa*. Recent reports have suggested that these genes are involved in tolerance to heavy metals and oxidative stress. The expression of AtOXS3 from *Arabidopsis* or BjOXS3 from *Brassica juncea* in *Schizosaccharomyces pombe* improved the tolerance to heavy metal (Cd^{2+}, AS (III) and Cu) stress or oxidative stress (Diamide) (Blanvillain et al., 2009). Overexpression of *Oryza sativa* OXS3 like 2 (OsO3L2) also conferred the rice Cd tolerance by reducing the Cd accumulation in OsO3L2 overexpression line (Wang et al., 2016).

We used SOS2 as the prey to perform the yeast two-hybrid screening from the *Arabidopsis* cDNA yeast library and identify an OXS3 gene family protein OXIDATIVE STRESS 3 like 1 (AtO3L1), which can interact with SOS2 protein *in vitro* and *in vivo* experiments. Thus, we refer to this protein as the SOS2 INTERACTION PROTEIN 1 (SIP1). Overexpression of SIP1 significantly improves the tolerance to salt stress. However, the double mutant of *sos2*×*sip1-1* is more sensitive than the *sos2* single mutant. Further investigation suggests that SIP1 improves *Arabidopsis* tolerance to salt stress through improving its stability by interacting with SOS2 protein and reduces the ROS accumulation by an unidentified mechanism. These results reveal a previously uncharacterized novel function of SIP1 in adjusting *Arabidopsis* adaptation to salt stress.

2. Material and methods

2.1. Transgenic materials

SIP1 (AT5g21940) CDS was inserted into pRI101-AN (Takara, Japan) between two restriction enzymes *Nde*I and *Sal*I, and a single copy of HA tag was fused in front of the SIP1 CDS (named as 35S-HASIP1) to develop an SIP1 overexpression vector. To determine the protein cellular location, a green fluorescent protein (GFP) fragment that originated from pEGAD vector was inserted into pRI101-AN in *Nde*I and *Sal*I sites to be the pRI101G vector. The full length fragments of SIP1 and SOS2 (AT5G35410) were subcloned into pRI101G in *Sal*I and *Eco*RI sites, referred to as 35S::SIP1-GFP and 35S::SOS2-GFP. A 2000-bp fragment upstream of the translation start site of SIP1 gene was amplified and inserted into pGreen-CCD-1262 (Wang et al., 2013) to be SIP1pro::GUS to assess the promoter activity of SIP1 gene. After sequencing, these constructs were transformed into *Arabidospsis thaliana* (Col-0 background) mediated by *Agrobacterium* GV3101 according to floral dipping method (Clough and Bent, 1998). At least five individual transgenic lines were selected by using appropriate antibiotics from the T_0 seeds and were confirmed by Western Blot analysis. After two to three generation selection, the homozygous plants were selected out and used to perform phenotype analysis or other experiments.

2.2. Plant treatments and growth conditions

The inhibition effect of NaCl on plant growth was performed by following the description. Surface-sterilized and stratified seeds were sown on square Petri plates that contain solid Murashige and Skoog (MS) medium supplemented with different gradient concentrations of NaCl. Wild-type (WT) and transgenic lines were grown on the same plate to reduce the plate-to-plate variation. Petri plates were incubated vertically in a growth room maintained at 22 °C and 16 light/8 dark cycles. After three-ten days growth, the seedlings were photographed and collected for further analysis.

2.3. Transient expression in tobacco leaves and Arabidopsis protoplasts

For tobacco (*Nicotiana benthamiana*) transient expression, *Agrobacterium* was suspended in infiltration buffer (10 mM MgCl2, 10 mM MES-KOH, pH 5.7) that contains 150 μM Acetosyringone (freshly added before using). *Agrobacterium* suspension solution was infiltrated into the abaxial surface of the healthy and full-expansion leaves using 1 mL syringe without needle. After three days of culture, the infiltrated leaves were collected for further observation.

Transient expression of SIP1 in *Arabidopsis* mesophyll protoplasts was performed according to sheen J (Yoo et al., 2007). *Arabidopsis* protoplasts were prepared from mature leaves, and approximately 10 μg plasmid of SIP1-GFP was transferred into protoplasts mediated with PEG 4000. After co-culturing in weak light for 16 h, transfected protoplasts were used to observe the subcellular localizations under the Olympus FLUOVIEW FV3000 confocal laser scanning microscope (Olympus Co. Tokyo, Japan) at an excitation wavelength of 488 nm and an emission wavelength of 509 nm.

2.4. Phos-tag SDS-PAGE and western blot analysis

Protein samples were prepared using phenol extraction method (Faurobert et al., 2007). Whole seedlings were collected and grinded into powder in liquid nitrogen. A total of 600 μL extraction buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% PVP, 1% Triton X-100, 0.2 mM PMSF, pH7.5) was added into the powder and retained on ice for 30 min. After centrifugation at 4 °C, the supernatants were transferred into a new tube and were mixed with the equal volume of phenol (Tris-HCl pH7.5). After phenol extraction, proteins were precipitated with ammonium acetate in methanol. The pelleted proteins were then dissolved in urea buffer (7 M urea, 2 M thiourea, 2% CHAPS, 4 mM dithiothreitol, pH 7.5), and the protein concentration was measured with BCA protein assay kit (Pierce) prior to SDS-PAGE analysis.

For normal SDS-PAGE, 10 μg of each protein sample was loaded on 12% polyacrylamide gel and was electrophoresed at 120 V. Proteins on gel were transferred onto polyvinylidene fluoride (PVDF) membrane by using a semi-dry electrotransfer apparatus (Bio-Rad). For phos-tag SDS-PAGE, 25 μM Phos-tag™ Acrylamide (AAL-107, Wako, Japan) and 25 μM $MnCl_2$ were added to the separating gel before polymerization. The gel was gently agitated in transfer buffer supplemented with 1 mM EDTA for 10 min before transferring the proteins onto PVDF membrane. Western blot analysis was carried out with anti-HA antibody (Roche Applied Science, Mannheim, Germany) or anti-GFP antibody (Clontech, Japan) according to the manufacturer's instruction.

2.5. Real-time quantitative PCR

Total ribonucleic acid (RNA) was isolated from *Arabidopsis* seedlings using the Trizol (Invitrogen, USA). After treating with DNase I, 1 μg RNA was used to synthesize cDNAs using M-MLV Reverse Transcriptase (Promega, USA). Quantitative PCR was performed using the ABI7500 real-time PCR system (Bio-Rad, USA) and employing SYBR green to monitor dsDNA synthesis. ACTIN2 was used as an internal reference to detect the target gene transcriptional level. All primers used for the assays were listed in Supplemental Table S2.

2.6. Histochemical beta-glucuronidase (GUS) assays

Transgenic seedlings, SIP1pro::GUS, were treated with or without 100 mM NaCl for 6 h and were collected for GUS staining with GUS stain buffer (1 mg/mL X-Gluc in 100 mM sodium phosphate buffer, pH 7.0, 10 mM Na_2EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM

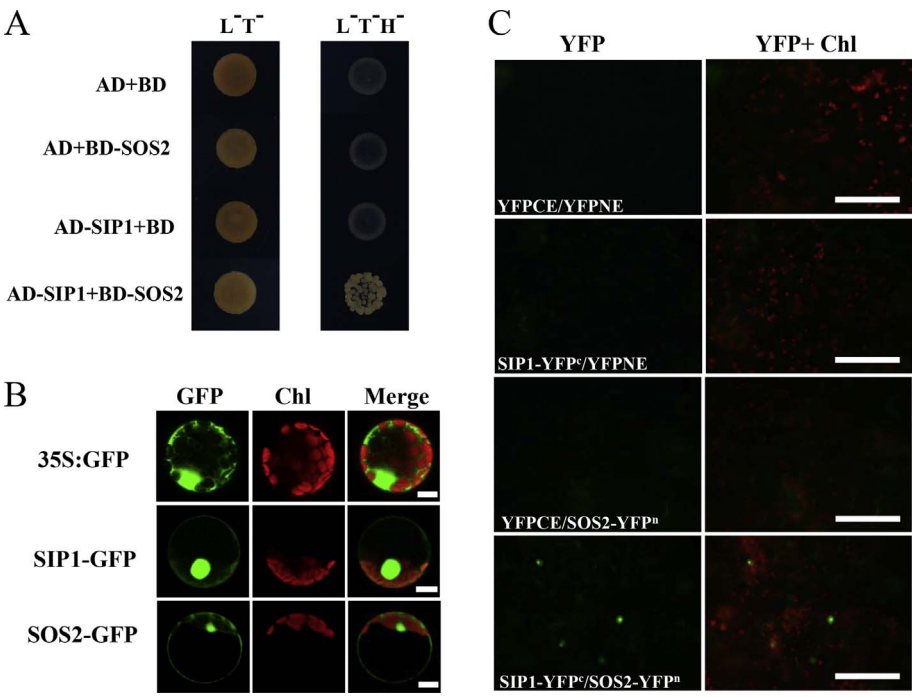

Fig. 1. SIP1 protein physical interacted with SOS2 protein. (A) Yeast two hybrid test for interaction between SIP protein and SOS2 protein; L-T-: SD/–Leu/–Trp with Agar. L-T-H-: SD/–Leu/–Trp/–His with Agar. (B) Cellular location of SIP1 protein and SOS2 protein. The SIP1 gene and SOS2 gene were fused with GFP and transiently expressed in *Arabidopsis* protoplast. The cellular location were observed on Laser Scanning Confocal Microscope, and 35S-GFP was used as control. Chl:Chlorophyll; Merge: overlap of GFP signal and Chl; Scale bars = 25 μm. (C) *In vivo* test interaction between SIP protein and SOS2 protein by BiFC assay in *Nicotiana benthamiana* leaves, and fluorescence signal was observed on fluorescence microscope. Scale bars = 0.4 mm.

potassium ferricyanide and 0.1% [v/v] Triton X-100). After incubation at 37 °C for 6 h in the darkness, the samples were decolorized with 75% (V/V) ethanol and detected on Leica EZ4D Stereomicroscope (Leica, Germany).

2.7. Yeast two-hybrid

Yeast two-hybrid analysis was performed using the Matchmaker Gold Yeast Two-Hybrid System (Clontech, Japan), according to the manufacturer's instructions. The coding region of SIP1 and SOS2 was amplified and inserted into pGADT7 and pGBKT7 vectors to achieve AD-SIP1 and BD-SOS2, respectively. The plasmid BD-SOS2 was transfected into yeast strain Y2HGold, and mated with the yeast strain Y187 that contains AD-SIP1. Medium supplemented with SD-Leu-Trp-His was used to test the protein interaction of SOS2 and SIP1.

2.8. BiFC assay

The coding region of SOS2 and SIP1 was amplified and inserted into 35S-pSPYNE and 35S-pSPYCE to generate SOS2-YFP^{n} and SIP1-YFP^{C}. The *Agrobacterium* that contains SOS2-YFP^{N} or SIP1-YFP^{C} was co-infiltrated into the leaves of *N. benthamiana*, and the empty vectors 35S-pSPYNE and 35S-pSPYCE were used as negative controls. After incubation for three days, the leaves were collected for observation under fluorescence microscope (Leica DM1000, Germany).

2.9. Histochemical detection of superoxide and H_2O_2

Seven-day old seedlings were treated with 1/2 MS liquid medium that contains 0 or 100 mM NaCl for three days, and further underwent Nitroblue tetrazolium (NBT) staining or 3, 3′-Diaminobenzidine (DAB) staining (Yang et al., 2013). For NBT staining, whole seedlings were stained in 2 mM freshly prepared NBT solution in 50 mM phosphate buffer (pH7.5) for 15 min. For DAB staining, whole seedlings were stained in 1 mg/mL freshly prepared DAB in 0.1 M HCl (pH3.8) for 10 min. The seedlings stained by NBT or DAB were transferred to 75% ethanol to remove the chlorophyll, and then transferred into 60% glycerol solution for further photographing under an EZ4D Leica stereomicroscope (Leica, Germany). Each experiment contained at least 10 independent seedlings.

2.10. Determination of H_2O_2 and MDA

Seven-day old seedlings were treated with 100 mM NaCl for 0, 1, 2 and 5 h and were collected for H_2O_2 and MDA content measurement. H_2O_2 was determined by colorimetric method (Eisenberg, 1943). The 1-g treated materials were grinded in 5 mL of precooling acetone. After centrifuging for 5 min with 4000 rpm/min, the supernatant was transferred into a new tube for H_2O_2 determination. The 1-mL extracted liquid was mixed with 5% (W/V) titanium sulphate and ammonia water to form precipitate. After centrifuging with 4000 rpm/min, the precipitate was dissolved in 2 M sulphuric acid for further measurement at 415 nm by a spectrophotometer. MDA content was measured by the thiobarbituric acid method (Hodges et al., 1999). The 0.2-g treated material was grinded in 4 mL of 0.1% (w/v) trichloroacetic acid (TCA) solution on ice, and centrifuged for 5 min at 10,000 rpm. The 0.5 mL aliquot of the supernatant was mixed with 1 mL of 20% (w/v) TCA that contains 0.5% (w/v) TBA. The mixture was boiled for 30 min and cooled on ice immediately. After centrifugation at 10,000 rpm for 10 min, the absorbance of the supernatant was measured at 532 and 600 nm using a spectrophotometer. The MDA concentration was calculated with its extinction coefficient (155 mM^{-1} cm^{-1}).

3. Results

3.1. A new SIP1 is isolated using yeast two hybrid library

SOS2 protein plays critical roles in regulating ion homeostasis under salt stress. To explore the detailed regulatory mechanism of SOS2, we screened *Arabidopsis thaliana* cDNA library by using SOS2 as the bait in the yeast two-hybrid (Y2H) system, and then identified one SIP1. The nucleotide sequence encoding SIP1 (TAIR ID: At5g21940) predicted an open reading frame of 264 amino acids, with a calculated molecular mass of 28.005 kDa. The frame was previously referred to as the AtOX3-like protein 1 (AtO3L1) that belongs to the AtOX3 family that takes part in the *Arabidopsis* response to heavy metal and oxidant stress (Blanvillain et al., 2009). SIP1 (or AtO3L1) is predicted to contain the conserve domain associated with catalytic sites of the putative N-

acetyltransferase domain of a *Staphylococcus aureus* protein (Gene bank accession: 1R57_A gi|47168715) (Blanvillain et al., 2009). We isolated the SIP1 gene from *Arabidopsis* cDNA and cloned into pGADT7, and then confirmed the interaction between SIP1 and SOS2 again in Y2H system. As shown in Fig. 1A, the yeast cell that contained BD-SOS2 and AD-SIP1 could grow appropriately on $Trp^-/Leu^-/His^-$ screening medium, but not those that contained pGBKT7-SOS2 and the empty pGADT7 vector, or AD-SIP1 and the empty pGBKT7 vector, suggesting that SOS2 could physically interact with SIP1.

To further explore the possibility of the interaction of SIP1 and SOS2 proteins *in vivo*, we firstly checked the cellular location of these two proteins. Previous studies indicated that SOS2 protein could locate in plasma membrane, cytosol and in nucleus (Quan et al., 2007a; Kim et al., 2013), consistent with our results in transiently transfected *Arabidopsis* protoplast with SOS2-GFP plasmid (Fig. 1B). We also checked the cellular location of SIP1 in transiently expressed protoplast with SIP1-GFP. The result shows that SIP1 was predominately located in cell nucleus (Fig. 1B), and could be observed in cellular membrane. Then, we examined the interaction of SOS2 and SIP1 in plants via biomolecular fluorescence complementation (BiFC), where the full-length SOS2 protein was fused with the N-terminal yellow fluorescence protein (YFP^n), whereas the full-length SIP1 was fused to the C-terminal yellow fluorescence protein (YFP^c). Co-expression of SOS2-YFP^n and SIP1-YFP^c in mature tobacco leaves mediated by *Agrobacterium* injection resulted in detectable BiFC, whereas the co-expression of SOS2-YFP^n with empty vector 35S-SPYCE did not show any BiFC, as well as co-expression of SIP1-cYFP with empty vector 35S-SPYNE (Fig. 1C). These experiments *in vivo* and *in vitro* demonstrate that SIP1 could physically interact with SOS2 protein.

3.2. Deficiency in SIP1 increased the sensitivity of SOS2 mutant to salt stress

To determine whether SIP1 and SOS2 had similar functions in terms of salt tolerance, we obtained two SIP1 T-DNA insertion lines *sip1-1* (SALK_042877) and *sip1-2* (SAIL_1240_B04), and an SOS2 T-DNA insertion line *sos2* (SALK_016683) from TAIR (http://www.arabidopsis.org). The positions of the T-DNA insertion are shown in Supplemental Fig. 1A. Homozygous T-DNA lines *sip1-1* and *sip1-2* were identified using T-DNA left border primers and SIP1 gene-specific primers designed by T-DNA Primer Design tool (http://signal.salk.edu/tdnaprimers.2.html). To determine whether the transcription of SIP1 gene was affected due to T-DNA insertion in these two lines, the mRNA of SIP1 was evaluated by quantitative RT-PCR analysis. Results show that the transcripts of SIP1 could not be detected in *sip1-1* line, whereas some residuals in *sip1-2* line, which are significantly lower than that in WT, were observed (Supplemental Fig. C). A previous study demonstrated that mutation in SOS2 gene, such as *sos2-1* EMS mutant, causes hypersensitivity to salt stress (Zhu et al., 1998). Here, we monitored *sip1-1*, *sip1-2* and *sos2* mutant growth in response to salt stress. No significant differences were observed among *sip1-1*, *sip1-2*, *sos2* and WT line under normal growth condition (Fig. 2A–C). Increasing the salt concentration to 25 mM severely suppressed the short growth and root elongation of *sos2* mutant line, but no significant differences for *sip1* mutants were observed compared with WT (Fig. 2A and C). Increasing salt concentration to 50 mM completely suppressed the growth of *sos2* lines, but significant differences between *sip1* mutants and WT were not observed (Fig. 2A and C). To investigate the role of SIP1 on SOS2-mediated salt response, we obtained the double *sos2*×*sip1-1* mutant by crossing *sos2* with *sip1-1* mutant. As shown in Fig. 2A, *sos2*×*sip1-1* double mutation did not affect the plant growth on MS medium without salt stress. However, when growing on a medium that contains 25 mM NaCl, root elongation and shoot growth in *sos2* × *sip1-1* mutant was reduced markedly compared with that of WT, and the reduction degree in *sos2*×*sip1-1* mutant was even more pronounced than that in *sos2* single mutant (Fig. 2A–C). Increasing NaCl concentration to 50 mM completely suppressed the shoot growth and root elongation for *sos2*×*sip1-1* mutant. On the contrary, the *sos2* mutant could still partly survive on such condition (Fig. 2A–C). Increasing NaCl concentration to 100 mM completely inhibited the seed germination of *sos2* × *sip1-1* mutant; the *sos2* mutant seeds could partly germinate, though the growth was severely inhibited (Fig. 2D). These results suggest that mutation in SIP1 does not alter the plant salt tolerance, but significantly aggravates the salt sensitivity of *sos2* mutant.

3.3. Overexpression of SIP1 conferred the salt tolerance

To further explore the role of SIP1 in response to salt stress, we constructed an overexpression vector of SIP1 gene driven by 35S promoter in which a single copy of HA-tag was fused to SIP1 (Fig. 3A), and transformed into WT *Arabidopsis*. Several individual SIP1 overexpression lines were screened out by kanamycin, and were further confirmed by western blot (Fig. 3B). Two homozygous SIP1OX lines were used for further salt stress analysis. No significant difference between WT lines and SIPOX lines was observed when growing on normal growth medium (Fig. 3C). The shoot growth was significantly inhibited in the WT and SIP1OX lines with 75 mM NaCl, whereas the inhibition effect on WT was more serious than that on SIP1OX lines (Fig. 3C–E). The increase in salt concentration to 100 mM resulted in a more significant growth inhibition in WT than in SIP1OX lines (Fig. 3C–E). Overexpression of SIP1 gene could also slightly improve the rate of seed germination especially under high concentration of salt stress (Supplemental Fig. 2). These results indicate that the SIP1 play a positive role in improving plant salt tolerance.

3.4. The transcription level of SIP1 gene was upregulated in response to salt stress

The experimental results showed that SIP1 gene played an important role in salt tolerance for plants in response to salt stress. To elucidate the function of SIP1 gene in response to salt stress, the gene expression profile of SIP1 was analyzed by SIP1pro::GUS transgenic *Arabidopsis* and quantitative real-time PCR (qPCR). The tissue-specific expression of SIP1 gene is shown in Fig. 4A and in Supplemental Fig. 3. The SIP1 gene was expressed mainly in leaves, roots and meristem tissues. Salt stress significantly enhanced the expression of SIP1 gene in these tissues as shown by GUS staining (Fig. 4A). The ten-day old WT seedlings treated with 100 mM NaCl were collected at different time points (0, 3, 6 and 12 h) for qPCR analysis of the SIP1 gene expression. Results showed that the transcription level of SIP1 gene was increase significantly, though declined after 3 h of salt treatment (Fig. 4B). These results suggest that SIP1 is a salt responsive gene.

3.5. SIP1 accumulated upon salt stress in post-transcriptional level and is dependent on SOS2 protein

We examined the SIP1 level under salt stress to determine whether it affected the stability of SIP1 *in vivo*. We constitutively expressed the GFP-tagged SIP1 controlled by CaM35S promoter in WT *Arabidopsis* (35S::SIP1-GFP) to distinguish between transcriptional and post-transcriptional regulations of SIP1 levels. The ten-day old 35S::SIP1-GFP transgenic lines were treated with 100 mM NaCl and were collected at the time points of 0, 3, 6, 12 and 24 h for protein and mRNA level analysis. The result of western blot analysis with GFP antibody showed that the level of SIP1-GFP fusion protein increased significantly and was maintained at a high level in response to salt stress (Fig. 5A). The expression level of SIP1-GFP mRNA was measured to confirm whether the protein accumulation was due to upregulation of mRNA level. The qPCR primers for SIP1-GFP mRNA were designed according to the GFP sequence, considering the endogenously produced SIP1 mRNA. The result shows that salt stress did not significantly affect the mRNA level of SIP1-GFP driven by the 35S promoter (Fig. 5A), thereby suggesting

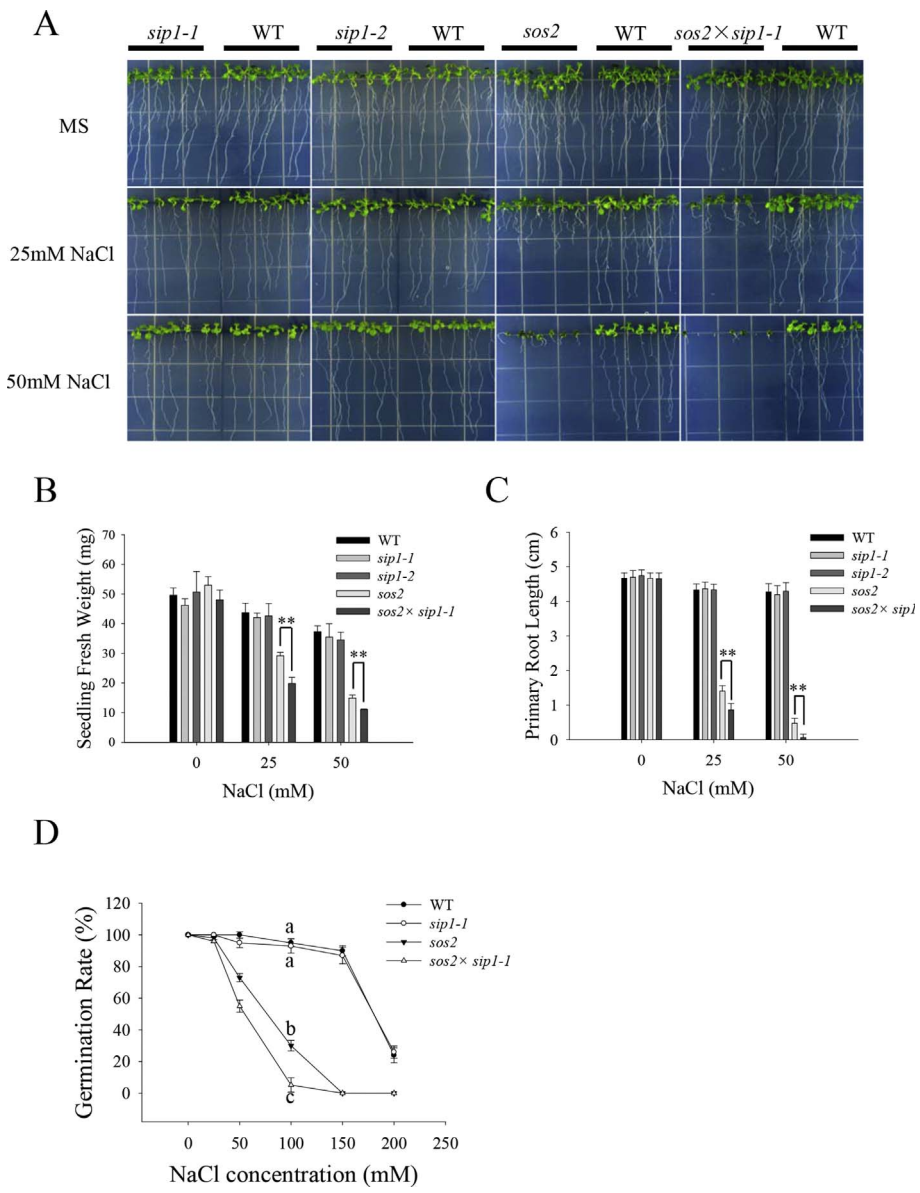

Fig. 2. Mutation in the SIP1 gene cause *sos2* mutant to be more sensitive to salt. (A) Phenotypes of *sip1-1*, *sip1-2*, *sos2*, *sos2*×*sip1-1* double mutant and WT *Arabidopsis* in response to 0, 25, and 50 mM NaCl; Photographs were taken after seven days growth. (B)–(C) Fresh weight and root length of seedlings grown in MS median containing 0, 25, and 50 mM NaCl for seven days are measured. Results shown are the mean (± SD) of measurements from 30 seedlings. Asterisks indicate statistically significant differences between *sos2 and sos2*×*sip1-1* mutants (Student's *t*-test, **P < 0.01). (C) Seed germination rate of *sip1-1*, *sos2*, *sos2* × *sip1-1* double mutant and WT *Arabidopsis* are assessed under 0, 25, 50, 100, 150 and 200 mM NaCl treatment. 100 seeds of each line are used to germination tests, and data are means (± SD) of three replicates. Means of seed germination rate under 100 mM NaCl treatment sharing the same letter are not significantly different from each other (one-way AVONA, P > 0.05).

that the expression of SIP1 gene could also be regulated at post-transcriptional level.

The molecular weight of SIP1 was approximately 28 kDa based on its amino acid composition, whereas the actual molecular weight (between 35 and 50 KDa) was higher than the prediction, as shown in western blot (Fig. 3B). The result suggests that SIP1 was subjected to the protein post-translational modifications. The phosphorylation status of SIP1 in response to salt stress was checked, considering that SOS2 was a protein kinase. Phos-tag™ is a novel phosphate-binding tag that can be used to separate phosphorylated proteins by SDS-PAGE and can be detected easily by western blot (Kinoshita et al., 2009). As shown in Fig. 5B, the SIP1 bands appeared smearing in Mn^{2+}-Phos-tag contained gel compared with that in No-Mn^{2+}-Phos-tag gel, in which only a single band was detected by western blot. The result indicates that SIP1 was regulated by phosphorylation. However, SIP1 protein was phosphorylated even under normal growth condition, suggesting that the phosphorylation status of SIP1 might not be totally dependent on salt stress.

We crossed the 35S::SIP1-GFP line with *sos2* mutant line to explore whether the accumulation of SIP1 upon salt stress was related to SOS2 protein. The homozygous line *sos2* × 35S::SIP1-GFP was treated with 100 mM NaCl solution for 0, 3, 6, 12 and 24 h, and the SIP1-GFP fusion protein was detected by western blot. Interestingly, our data showed that the mutation in SOS2 gene resulted in the disruption of the SIP1 accumulation in response to salt stress (Fig. 5C). Thus, SOS2 protein plays an important role in maintaining high level of SIP1 upon salt stress.

3.6. Deficiency in SIP1 increased the reactive oxygen accumulation of sos2 mutant under salt stress

Salt stress can cause the rapid production of ROS including H_2O_2 and superoxide in plants. We compared the ROS accumulation among the WT, *sos2*, *sip1-1*, *sos2*×*sip1-1* double mutant and SIP1OX lines. All these lines accumulated slightly low level of superoxide radicals (as indicated by nitroblue tetrazolium staining, NBT) under no salt condition (Fig. 6A). However, after treatment with 100 mM NaCl for three days, the mutant lines of *sip1-1*, *sos2* and *sos2*×*sip1-1* accumulated higher level of superoxide radicals than that of WT *Arabidopsis* lines, and the superoxide radical level in *sos2*×*sip1-1* was the highest (Fig. 6A). Much less superoxide radicals accumulated in SIP1OX line were observed compared with WT seedling (Fig. 6A). The WT, *sip1-1*, *sos2*, *sos2*×*sip1-1* mutants and SIP1OX lines accumulated similar levels

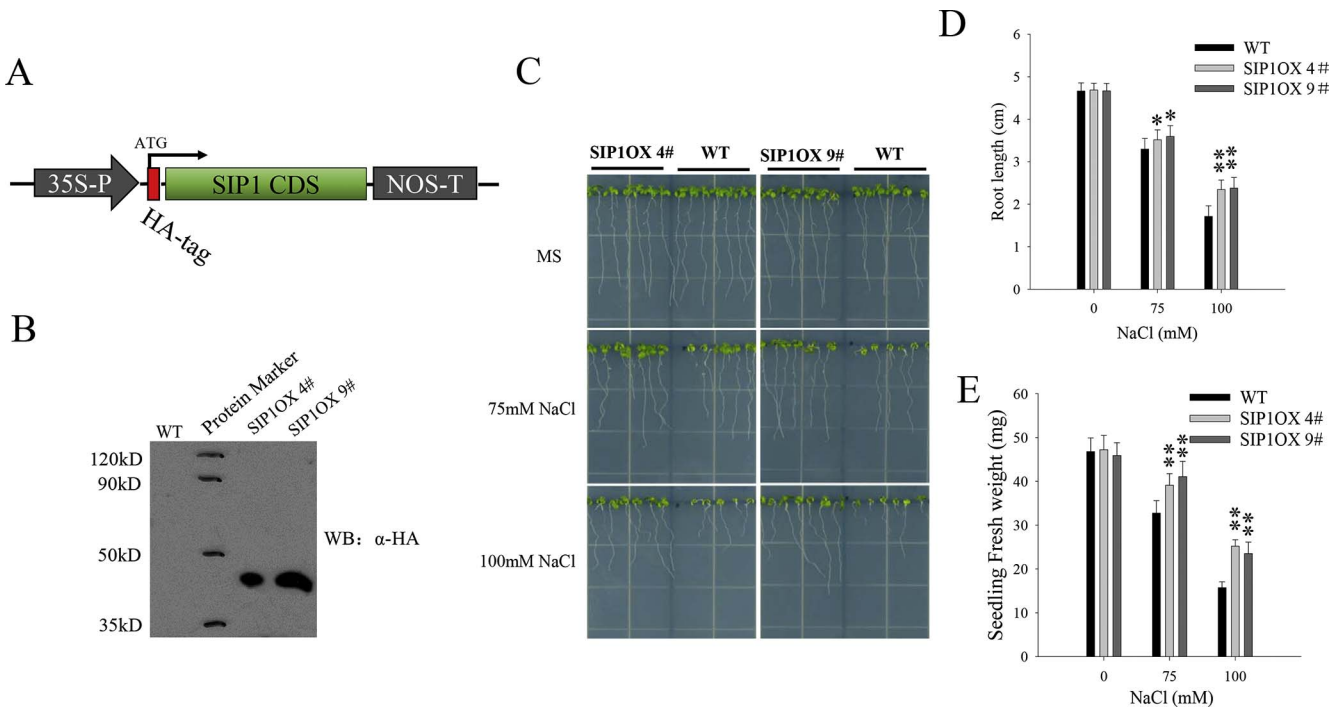

Fig. 3. Overexpression of SIP1 improves the tolerance to salt tress. (A) Schematic diagram of overexpression construction for SIP1 gene. Green box represents the full length of SIP1 CDS, and red box represents one copy of HA-tag just in front of the SIP1 CDS. (B) Western blot against HA of wild-type *Arabidopsis* and two individual transgenic lines constructed as indicated in (A). (C) Phenotypes of two SIP1 overexpression lines and WT *Arabidopsis* grown on MS median containing 0, 75 and 100 mM NaCl; Photographs were taken after seven days growth. (D)–(E) Fresh weight and root length of two SIP1 overexpression lines and WT *Arabidopsis* are measured under conditions as indicated in (C). Results shown are the mean (± SD) of measurements from 30 seedlings. Asterisks indicate statistically significant differences of SIP1 transgenic lines versus wild type *Arabidopsis* under the same NaCl concentration (Student's *t*-test, *P < 0.05, **P < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

of H_2O_2 (as stained by 3,3′-diaminobenzidine staining, DAB) under the mock condition. After three days of treatment with 100 mM NaCl, the H_2O_2 level in *sip1-1*, *sos2* and *sos2*×*sip1-1* increased much higher than that in WT and SIP1OX lines. In these lines, *sos2*×*sip1-1 line* accumulated the highest level of H_2O_2, whereas SIP1OX lines showed the lowest level of H_2O_2 (Fig. 6A).

To monitor the rate of H_2O_2 production, the H_2O_2 content was measured after treatment with 100 mM NaCl for 0, 1, 2 or 5 h. H_2O_2 contents in *sip1-1*, *sos2*, *sos2*×*sip1-1* mutant and SIP1OX lines were increased with salt treatment. More rapid production of H_2O_2 was observed in *sip1-1*, *sos2* and *sos2*×*sip1-1* double mutant compared with that in WT and SIP1OX lines, whereas overexpression of SIP1 significantly lowered the rate of H_2O_2 production (Fig. 6B).

Excessive accumulation of ROS such as H_2O_2 would damage the cellar membrane system. We monitored the rate of the Malondialdehyde (MDA) production to evaluate the membrane integrity of *sip1-1*, *sos2*, *sos2*×*sip1-1* mutant lines and SIP1OX lines in response to salt stress. MDA is one of the most frequently used indicators of lipid peroxidation, and measured by the thiobarbituric acid (Hodges et al., 1999). Salt stress promoted the accumulation of MDA content in all *sip1-1*, *sos2*, *sos2*×*sip1-1* mutant and SIP1OX lines, and high level of MDA content in *sip1-1*, *sos2* and *sos2*×*sip1-1* mutant were detected compared with WT. On the contrary, SIP1 overexpression line accumulated much lower MDA (Fig. 6C). These results clearly indicate that altered expression of SOS2 and SIP1 genes promotes ROS production in response to salt stress, and thus SIP1 is involved in regulating redox status of cell under salt stress.

4. Discussion

We reported a novel SIP1, which was previously referred to as AtO3L1 in *Arabidopsis* (Blanvillain et al., 2009). The SIP1 belonged to plant specific OXS3 protein family, and the overexpression of several members of this family in *Schizosaccharomyces pombe* enhanced the

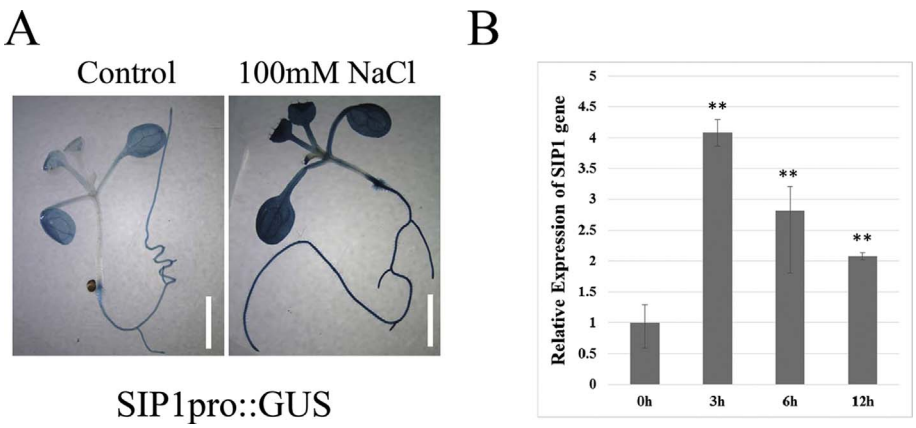

Fig. 4. Salt stress enhances the expression of SIP1 gene. (A) Histochemical GUS staining of SIP1pro::GUS seedlings. Ten-day old seedlings were treated with 100 mM NaCl for 6 h. Scale bars = 3 mm. (B) The expression level of SIP1 gene in response to salt stress was tested by quantitive RT-PCR. ten-day old wild type *Arabidopsis* seedlings were treated with 100 mM NaCl and collected the samples at the time point of 0, 3, 6 and 12 h after NaCl treatment. The showed values were the mean (± SD) of three dependent experiments. Asterisks indicate statistically significant differences compared with untreated seedlings (Student's *t*-test, **P < 0.01).

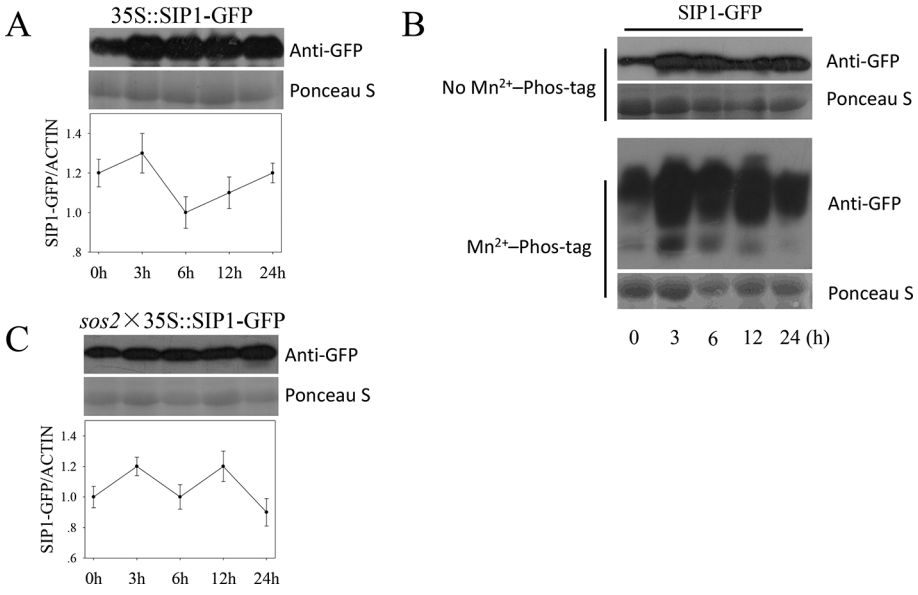

Fig. 5. Accumulation of SIP1 protein upon salt stress is dependent of SOS2 protein. (A)-(B)SIP1 overexpression line (SIP-GFP) or *sos2* × SIP-GFP were treated with 100 mM NaCl for 0, 3, 6, 12 and 24 h, and were harvested at each time point for Western blot using GFP antibody or transcriptional level detection by qPCR. Ponceau staining served as the protein loading control. The SIP1 mRNA level driven by 35S promoter was represented by GFP level using GFP primers. The mean(± SD) of quantified values of three independent experiments were shown.

tolerance to a range of metals and oxidizing chemicals (Blanvillain et al., 2009). Salt stress could activate the expression of SIP1 gene and promote SIP1 accumulation, confirming that SIP1 gene was a salt responsive gene. The overexpression of SIP1 gene significantly improved plant tolerance, whereas *sos2*×*sip1-1* double mutant was more salt sensitive than *sos2* single mutant. These findings strongly implied that the SIP1 played a positive role in salt stress, considering that SOS2 protein is a key component in the SOS pathway. Further explorations suggested that the enhancement of the plant salt tolerance by SIP1 was contributed by lowering ROS production in response to salt stress.

SOS pathway plays a critical role in osmotic adjustment and ion balance for plant cells in response to salt stress (Qiu et al., 2002). Under salt stress, cytoplasm membrane protein SOS3 is activated by binding to increasing Ca^{2+} triggered by excessive Na^+ and recruits the serine/threonine protein kinase SOS2 to form SOS3-SOS2 complex to regulate the downstream ion transporters such as plasma membrane protein SOS1 (Qiu et al., 2002), tonoplast protein Ca^{2+}/H^+ antiporter CAX1 (Cheng et al., 2004) and the Vacuolar proton ATPase (V-ATPase) and tonoplast Na^+/H^+ (Batelli et al., 2007). These events regulated by SOS3-SOS2 complex mainly occur in cytoplasm or cellular member. SIP1 was mainly located in nucleus, and SOS2-SIP1 complex could be detected in nucleus by BiFC assay, thereby implying SOS2 could function as the salt responsive regulator for SIP1 in cell nucleus. The problem was how the salt signal was transferred into the nucleus, and, to date, we had no direct evidence to elucidate the salt signalling transduction. However, salt signal could be transmitted from the cytoplasmic

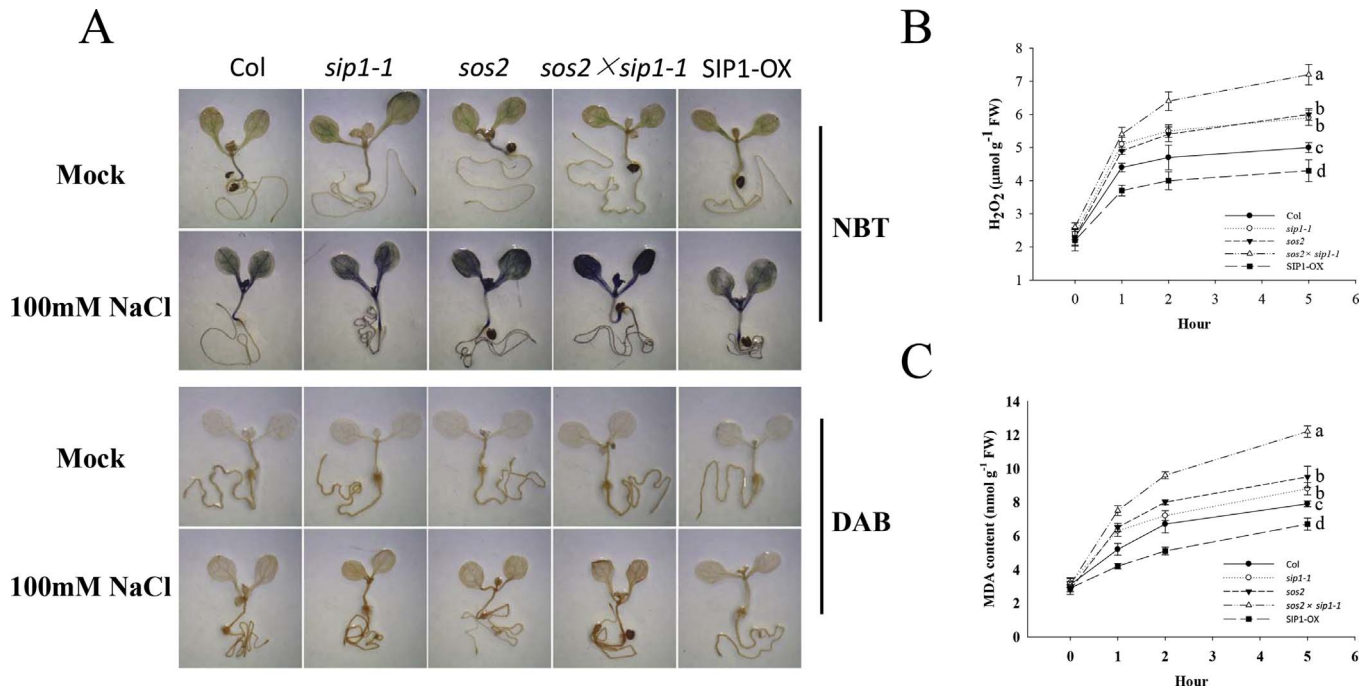

Fig. 6. SIP1 protein enhances ROS scavenging. (A) NBT and DAB staining of seven-day old *sip1-1*, *sos2*, *sos2* × *sip1-1* double mutant, SIP1 overexpression line and WT *Arabidopsis* treated with 100 mM NaCl or mock for 3 days. (B)–(C) H2O2 content and MDA content of seven-day old *sip1-1*, *sos2*, *sos2*×*sip1-1* double mutant, SIP1 overexpression line and WT *Arabidopsis* are evaluated after treatment with 100 mM NaCl for 0, 1, 2 or 5 h. Each value of the indicated time points is the mean (± SD) of three replicates. Means sharing the same letter are not significantly different from each other (one-way AVONA, $P > 0.05$).

information into the cellular nucleus (Guan et al., 2013; Ok et al., 2005). For example, root in salt medium 1 (RSA1), which is a nuclear calcium-sensing protein, responded to salt stress by interacting with bHLH transcriptional factor RITF1 to regulate the expression of genes that are important for detoxification of salt-induced ROS and for Na^+ homeostasis under salt stress (Guan et al., 2013). Thus, screening the possible regulatory factors upstream SOS2 protein is necessary to explain the function of SOS2 in the nucleus.

SIP1 protein accumulated significantly upon salt stress, whereas mutation of SOS2 gene inhibited the accumulation of SIP1, suggesting that SOS2 protein was necessary to stabilize the SIP1 in response to salt stress. We have ever assumed that the stabilization of SIP1 was due to phosphorylation by SOS2 protein. However, we did not detect the phosphorylation of SIP1 by SOS2 protein *in vitro* in our system (data not shown). SIP1 was phosphorylated even under no salt stress condition (Fig. 5B), thereby suggesting that phosphorylation of SIP1 is not dependent on SOS2 protein. The possible reason is that SOS2 in nuclear has no phosphorylation activity due to the absence of upstream protein such as SOS3 or SCABP8 (Halfter et al., 2000; Quan et al., 2007b).

Heavy metal or oxidizing chemical treatments lead to redox status changes in plant cells (Schutzendübel and Polle, 2002; Sharma and Dietz, 2009) and salt stress (Hasegawa et al., 2000). The enhancement of the capability to scavenge over-accumulated ROX improves tolerance to stress in plants. NDPK2 interacted with SOS2 protein and was characterized as a salt response gene (Verslues et al., 2007). The *sos2-2 ndpk2* double mutant was more salt sensitive than the *sos2-2* single mutant. Though mutation of NDPK2 results in H2O2 accumulation under normal condition, the double mutant do not hyperaccumulate H_2O_2 in response to salt stress, suggesting that NDPK2 regulates plant salt tolerance through another signalling pathway rather than reduction of H_2O_2 toxicity (Verslues et al., 2007). Our data showed a positive correlation between SIP1 and free radical scavenging in response to salt stress, suggesting that SIP1 improves plant salt tolerance possibly by regulating the ROS status with the help of SOS2 protein. It is not clear how SIP1 protein regulates the ROS status under salt stress. However, previous studies showed that the OXS3 protein family possesses a putative N-acetyltransferase activity, and triggers gene expression by chromatin remodelling in response to stress (Blanvillain et al., 2009). Therefore, N-acetyltransferase activity of SIP1 should be further determined to identify the genes targeted by SIP1.

Contribution

CT Wang and XY Hu design the experiments; CT Wang, Q Chen, N Xiang, YY Liu and XX Kong performed the experiments; CT Wang, YP Yang and XX Hu analyzed the results; CT Wang wrote the manuscript with the help of YP Yang and XX Hu.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2018.01.018.

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