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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