E3 ligase, the *Oryza sativa* salt-induced RING finger protein 4 (OsSIRP4), negatively regulates salt stress responses via degradation of the OsPEX11-1 protein

Ju Hee Kim[1] · Cheol Seong Jang[1]

Abstract

Key message **OsSIRP4 is an E3 ligase that acts as a negative regulator in the plant response to salt stress via the 26S proteasomal system regulation of substrate proteins, OsPEX11-1, which it provides important information for adaptation and regulation in rice.**

Abstract Plants are sessile organisms that can be exposed to environmental stress. Plants alter their cellular processes to survive under potentially unfavorable conditions. Protein ubiquitination is an important post-translational modification that has a crucial role in various cellular signaling processes in abiotic stress response. In this study, we characterized *Oryza sativa* salt-induced RING finger protein 4, OsSIRP4, a membrane and cytosol-localized RING E3 ligase in rice. OsSIRP4 transcripts were highly induced under salt stress in rice. We found that OsSIRP4 possesses E3 ligase activity; however, no E3 ligase activity was observed with a single amino acid substitution (OsSIRP4^{C269A}). The results of the yeast two hybrid system, in vitro pull-down assay, BiFC analysis, in vitro ubiquitination assay, and in vitro degradation assay indicate that OsSIRP4 regulates degradation of a substrate protein, OsPEX11-1 (*Oryza sativa* peroxisomal biogenesis factor 11-1) via the 26S proteasomal system. Phenotypic analysis of OsSIRP4-overexpressing plants demonstrated hypersensitivity to salt response compared to that of the wild type and mutated OsSIRP4^{C269A} plants. In addition, OsSIRP4-overexpressing plants exhibited significant low enzyme activities of superoxide dismutase, catalase, and peroxidase, and accumulation of proline and soluble sugar, but a high level of H_2O_2. Furthermore, qRT data on transgenic plants suggest that OsSIRP4 acted as a negative regulator of salt response by diminishing the expression of genes related to Na$^+$/K$^+$ homeostasis (*AtSOS1*, *AtAKT1*, *AtNHX1*, and *AtHKT1;1*) in transgenic plants under salt stress. These results suggest that OsSIRP4 plays a negative regulatory role in response to salt stress by modulating the target protein levels.

Keywords OsPEX11-1 · Protein degradation · Rice · RING E3 ligase · Salt stress

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Introduction

Plants are sessile organisms that can be exposed to environmental stresses such as extreme temperatures, soil salinity, drought, and flood. Soil salinity in the root zone affects the performance of plants. Especially, soil salinity reduces plant growth and limits productivity in crops. These problems caused by soil salinity are some of the important issues to be resolved worldwide. Approximately 20% of irrigated agricultural lands are affected by high salinity (Pitman and Läuchli, 2002). Approximately 400 million hectares of the world's land area and 25% of the groundwater used for irrigation has been found to be saline (Anonymous 2008). In the case of India, about 6.727 million hectares are affected by high salinity (Anonymous 2008). Furthermore, the

accelerated expansion of saline soil areas is increasing by 10% each year owing to various reasons, including low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor cultural practices (Jamil et al. 2011).

Salt treatment significantly affects various plant physiological and metabolic processes, such as leaf development, flowering time, root length, plant height, and fruit production (Liang et al. 2018). When a plant is exposed to saline soil, osmotic stress is the first stress experienced and then water uptake, cell elongation, and leaf senescence occur (Munns et al. 2006, 2008; Horie et al. 2011). Ionic stress due to salt exposure occurs because of excessive accumulation of Na^+, which leads to the inhibition of photosynthesis, protein synthesis, and enzyme activity. Ionic toxicity and osmotic stress can cause oxidative stress and a series of secondary stresses. Reactive oxygen species (ROS) in plants are produced by abiotic stress like drought and salinity, and an enhanced level of these ROS causes degradation of proteins, lipids, carbohydrates, RNA, and DNA, and ultimately death of a plant (Mittler 2002; del Río et al. 2006). When the levels of ROS are enhanced during cellular metabolism in plants, oxidative stress leads to imbalance between ROS and antioxidants (Foyer and Harbinson 1994). Thus, plants have developed an antioxidant defense system with efficient enzymatic ROS-scavenging mechanisms to scavenge the excess ROS produced under unfavorable environmental conditions. To maintain redox homeostasis under stress, antioxidant enzymes such as ascorbate peroxidase, catalase (CAT), glutathione reductase, peroxidase (POD), and superoxide dismutase (SOD) are involved in mitigating ROS, and these enzymes are found in all subcellular compartments (Hossain and Dietz 2016).

In addition, when exposed to abiotic stress, plants alter cellular processes via protein level changes. Among the post-translational modifications, protein ubiquitination is known to involve covalent attachment of ubiquitin onto substrate protein, which is recognized by the 26S proteasome system for protein degradation (Callis 2014; McClellan et al. 2019). Ubiquitination in plants influences the biomolecules involved in various cellular signaling processes, such as transcription factors, hormone receptors, and damaged proteins (Moon et al. 2004; Dreher and Callis 2007; Lee et al. 2011; Sadanandom et al. 2012). In ubiquitination, E3 ligase plays a major role in target recognition and transfer of ubiquitin to the substrate (Metzger et al., 1843). E3 ligases are classified into two distinct super families; one superfamily comprises the really interesting new gene (RING), homologous to the E6-AP carboxyl terminus (HECT), and U-box E3 ligase, and the other superfamily comprises cullin4-damaged-specific DNA binding protein1 (CUL4-DDB1), anaphase promoting complex (APC), and skp1-cullin-F-box (SCF) E3 ligases (Zheng et al. 2002; Pazhouhandeh et al. 2011; Chang et al.

2014). RING E3 ligase presents the most abundant type of ubiquitin ligases, which mediates their interactions with the ubiquitin-conjugated E2 (Deshaies and Joazeiro 2009). Recent studies have revealed important roles of RING E3 ligases in response to salt stress. For example, *Oryza sativa* salt-induced RING finger protein 1 (OsSIRP1), *Oryza sativa* drought-induced RING protein 1 (OsDIRP1), *Oryza sativa* drought-, heat-, salt-induced RING finger protein 1 (OsDHSRP1), and *Oryza sativa* salt-, ABA- and drought-induced RING finger protein 1 (OsSADR1) were identified as negative regulators of salt stress response (Hwang et al. 2016; Cui et al. 2018; Park et al. 2018; Kim et al. 2020). In contrast, positive roles of salt-induced RING E3 ligase 2 (OsSIRP2) and novel RING-H2 finger protein (OsRHP1) in rice were reported under salinity stress (Zeng et al. 2014; Chapagain et al. 2018). Furthermore, rice RING finger E3 ligase, RNA interference silencing of *Oryza sativa* stress-related RING finger protein 1 (OsSRFP1), showed increased tolerance to salt stress in rice (Fang et al., 2015). In this study, we showed that *Oryza sativa* salt-induced RING protein 4 (OsSIRP4), which is a membrane-localized C_4HC_3-type E3 ligase in rice, and its gene was highly induced by salt. The functional interaction of *Oryza sativa* peroxisomal biogenesis factor 11-1 (OsPEX11-1) with OsSIRP4 protein was identified using the yeast two-hybrid (Y2H) system. OsSIRP4-overexpressing plants (*35S:OsSIRP4-YFP*) exhibited reduced tolerance to salt stress when compared with the wild type (WT) and mutated OsSIRP4-overexpressing *Arabidopsis* plants. These results indicate that OsSIRP4 acts as a negative regulator of salt stress response.

Materials and methods

Plant materials and stress treatments

Rice (*Oryza sativa* 'Donganbye') seeds were grown in a growth chamber at 30 °C, 70% relative humidity, and a 12 h light/12 h dark cycle. Fourteen-day-old seedlings were treated with 150 mM NaCl, and half-strength Murashige and Skoog (MS) medium was added. Shoot and root tissues from these seedlings were harvested at different time points (6, 12, 24, and 48 h). Plant tissues were frozen instantly in liquid nitrogen and stored at −80 °C in a deep freezer for further studies.

Quantitative RT-PCR analysis

The plants (control and stressed) were ground in liquid nitrogen, and total RNA were extracted using TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA). One microgram of RNA was used as a template for first strand cDNA synthesis using the PrimeScript™ 1st-Strand cDNA

Synthesis Kit (Takara-Bio, Ohtsu, Japan). Quantitative real time PCR (qPCR) was conducted with the CFX ConnectTM Real-Time PCR Detection System (BIO-RADTM), using SYBR® Green TOP real qPCR 2X PreMIX (EnzynomicsTM, Daejeon, Korea). qPCR was performed with an initial denaturation at 95 °C for 10 min followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55–60 °C for 30 s, and extension at 72 °C for 30 s. The experiment was conducted with two biological and three technical replicates. The *ActinII* (Os03g50885) and *AtUBC* (AT5g25760) genes were used as internal controls in rice and *Arabidopsis*, respectively.

In vitro ubiquitination assay

The full-length OsSIRP4 cDNA was amplified using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA) and cloned into a pMAL-c5X vector (New England Bio-Labs, Ipswich, MA, USA). The mutant OsSIRP4 (C269A) was produced by site-directed mutagenesis with mutagenic primers. Recombinant MBP-OsSIRP4 and mutant fusion proteins were expressed in *Escherichia coli*, purified by affinity chromatography using an amylose resin (New England BioLabs). AtUBC10 cDNA (E2) was amplified by PCR and cloned into the 6X His-tagged pET-28a (+) vector (Novagen, Gibbstown, NJ, USA) and then purified using the Ni–NTA purification system (Invitrogen Life Technologies). Purified MBP-OsSIRP4 was incubated in ubiquitination reaction buffer (50 mM Tris–HCl, pH 7.5, 40 mM ATP, 100 mM $MgCl_2$, 40 mM dithiothreitol, 5 μM ubiquitin (Sigma-Aldrich), 100 ng human E1 (Sigma), and 100 ng *Arabidopsis* E2 (UBC10)) at 30 °C for 2 h. The reactions were stopped by boiling in 5X sodium dodecyl sulfate (SDS) sample buffer (0.25 M Tris–HCl, 0.25% bromophenol blue, 0.5 M dithiothreitol, 50% glycerol, 10% SDS) and subsequently analyzed using western blotting. Reaction products were separated by SDS-PAGE and subjected to immunoblot analysis using anti-MBP antibody (New England BioLabs) or anti-ub antibody (Sigma-Aldrich) with a secondary goat anti-mouse or anti-rabbit IgG peroxidase antibody (Sigma-Aldrich). The photographs were obtained using the ChemiDoc™ XRS + imaging system (Bio-Rad, Hercules, CA, USA).

Confocal laser scanning microscope imaging

Rice plants were grown in a growth chamber at 30 °C, 70% relative humidity, and a 12 h light/12 h dark cycle. Using 10-day-old seedlings, the protoplasts from leaf samples were isolated according to the method described by Zhang et al. (2011). The OsSIRP4 or OsPEX11-1 cDNA were amplified using PCR and cloned into the YFP vector as described by Lim et al. (2013). For the bimolecular

fluorescence complementation (BiFC) analysis, their full-length clones were inserted into 35S:HA-SPYCE(M) and 35S:c-Myc-SPYNE(R) vectors, respectively. The plasmids were delivered into protoplasts using a PEG-calcium mediated method described previously (Zhang et al. 2011). The protoplasts expressing the fusion protein after 16 h incubation were observed using a laser confocal microscope (model LSM880 with Airyscan, Carl Zeiss, Oberkochen, Germany).

Yeast two-hybrid screening

The full-length cDNA of the OsSIRP4 was amplified using PCR and cloned into pGBKT7 (BD) (Clontech, Palo Alto, CA, USA). The OsSIRP4-BD was introduced into *Saccharomyces cerevisiae* strain Y2H Gold yeast cells for mating and used as a bait protein. Then, the yeast transformed OsSIRP4-BD strain was mated with the rice salt library as described by Chapagain et al. (2018). The mated strains were grown on the double dropout (DDO) medium lacking leucine and tryptophan with 40 mg mL^{-1} X-α-gal and 42 ng mL^{-1} aureobasidin A (AbA). The blue clones were picked after 5 d and cultured on the medium lacking adenine, histidine, leucine, and tryptophan (QDO), with 40 mg mL^{-1} X-α-gal and 42 ng mL^{-1} AbA (Clontech). To identify protein–protein interactions, the full-length cDNA of OsPEX11-1 was amplified using PCR and cloned into pGADT7 (AD) (Clontech). The OsSIRP4-BD and OsPEX11-1 were co-transformed into the Y2H Gold yeast cells, and incubated on the DDO medium for 3 d at 30 °C. Each of the clones was spotted on the DDO/AbA and QDO/X-α-gal/Aba medium for 3 d at 30 °C.

In vitro pull-down assay

His-Trx-tagged OsSIRP4 and MBP-tagged OsPEX11-1 were expressed. To confirm protein–protein interaction, in vitro pull-down assay was performed using a Pull-Down Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The pull-downed proteins were denatured by boiling with 5X SDS sample buffer at 95 °C for 5 min. Thereafter, proteins were separated by SDS-PAGE. The signals were detected with an anti-Trx (Sigma-Aldrich) or anti-MBP antibody (New England Biolabs).

In vitro protein degradation

In vitro protein degradation assay was performed as described by García-Cano et al. (2014). The OsPEX11-1 cDNA was amplified by PCR and cloned into a pET32a (+)-His-Trx vector and the *E. coli* strain BL21 (DE3) pLysS (Promega). Expressed proteins were purified using the Ni–NTA purification system (Invitrogen Life Technologies).

The total protein extracts were prepared from harvested rice seedlings that were ground in liquid nitrogen. Total protein was extracted from the ground rice powder using a Plant Total Protein Extraction Kit (Sigma-Aldrich). Then, MG132 was used as a proteasome inhibitor and added to a final concentration of 40 μM. The reactions were stopped by boiling in 5X SDS sample buffer and subsequently analyzed using western blotting.

Phenotypic analysis under salt stress

For the salt stress tolerance assay, WT (35S:YFP) and over-expressing *Arabidopsis* plants were cultured on half MS medium under a 16 h (light)/8 h (dark) cycle at 22 °C for 3 d, and then the seedlings were transferred to fresh half MS medium supplied with 150 and 200 mM NaCl and grown vertically for 10 d. The primary root length (root elongation) was measured. These assays were repeated at least thrice.

Assay of antioxidant enzyme activity, and H_2O_2, proline, and soluble sugar content

Ten-day-old *Arabidopsis* plants (0.1 g) were homogenized in 0.2 mL of pre-cooled 50 mM sodium phosphate buffer (pH 7.0) with 2 mM EDTA, 5 mM β-mercaptoethanol, and 4% PVP-40 by grinding. The mixture was centrifuged at 4 °C for 30 min at $12,000 \times g$. The supernatants were used for the determination of activities of antioxidant enzymes. SOD, CAT, and POD activity were determined according to the methods described by Beauchamp and Fridovich (1971), Aebi (1984), and Chance and Maehly (1955), respectively. For determination of H_2O_2 content, plants (0.1 g) were ground in 1 mL of 0.1% trichloroacetic acid at 0 °C. The mixture was incubated on ice for 1 h, and then centrifuged at $12,000 \times g$ for 30 min at 4 °C. To 0.1 mL aliquot of the supernatant, 1 mL mixture containing 25 mM ammonium ferrous (II) sulfate, 0.5 M sulfuric acid, 0.1 M sorbitol, and 0.125 mM xylenol orange were added and the mixture was incubated for 30 min at room temperature in dark condition. After incubation, the absorbance was measured at 560 nm. Proline and soluble sugar content were measured according to Bates et al. (2013) and Ci et al. (2009), respectively.

Results

Expression of *OsSIRP4* is induced by salt stress

We studied the potential role of OsSIRP4 (LOC_Os04g01490) in plant salt stress responses. To investigate the expression pattern of the *OsSIRP4* gene, we treated rice plants with salt and sampled at 6, 12, 24, and 48 h. The expression of *OsSIRP4* was induced in both the shoot

and root under salt treatment, following all time points of watering with 150 mM NaCl (Fig. 1a). For example, under the salt stress condition, *OsSIRP4* was highly induced at 12 h in the root tissue (approximately 26.5-fold); whereas, it was highly induced at 6 h in the shoot tissue (approximately 8.7-fold). *OsSalT* was used as the salt stress marker gene and its expression was highly induced in both the shoot and root tissues under salt condition (Fig. S1). These results showed that the expression of *OsSIRP4* was induced by salt stress, implying that OsSIRP4 might play a role in salt stress responses.

OsSIRP4 is a C_4HC_3-type RING ubiquitin ligase

The *OsSIRP4* gene encodes 495 amino acids with a putative molecular weight of 53.9 kDa. A conserved C_4HC_3-type RING domain was found in alignment with some orthologs, Bradi5g02290 (*Brachypodium distachyon*), GRMZM2G131611 (*Zea mays*), and Sb06g000460 (*Sorghum bicolor*) (Fig. 1b). OsSIRP4 is 66%, 59%, and 58% identical to Bradi5g02290, GRMZM2G131611, and Sb06g000460, respectively (Fig. S2). These structural conservations suggest that OsSIRP4 possesses E3 ligase activity (Fig. 1b, Fig. S2). To test E3 ligase activity of OsSIRP4, MBP-fused OsSIRP4 or -mutated OsSIRP4 (C269A) were expressed and purified. The purified proteins were incubated with human E1, *Arabidopsis* E2 (AtUBC), and ubiquitin at 30 °C for 2 h and subjected to immunoblot analysis with an anti-Ub antibody (Fig. 1c, left) and anti-MBP antibody (Fig. 1c, right). In the presence of E1, E2, Ub, and E3, poly-ub chains were detected, whereas no poly-ubiquitinated products were detected in mutated OsSIRP4 (C269A) or empty MBP vectors (Fig. 1c, lane 1 and 3). Collectively, the results demonstrate that OsSIRP4 protein is a RING E3 ligase.

OsSIRP4 was localized to the cytosol and plasma membrane

To identify another domain of the OsSIRP4 protein, the National Center for Biotechnology Information conserved domain database (CDD; Lu et al. 2020) was used. The OsSIRP4 protein possesses three other distinct domains: the Atrophin-1 and transmembrane, as well as the RING domain (RINGv or RING-CH) (Fig. 2a). TMHMM analysis suggests that OsSIRP4 is an intrinsic membrane protein with a central transmembrane domain (Fig. 2b). To determine subcellular localization of OsSIRP4, YFP-fused OsSIRP4 (35S:OsSIRP4-YFP) or mutated OsSIRP4 (35S:OsSIRP4^{C269A}-YFP) was transiently expressed in rice protoplasts under the control of the cauliflower mosaic virus 35S promoter. The yellow fluorescence of OsSIRP4 or mutated OsSIRP4 was observed in both the cytosol and
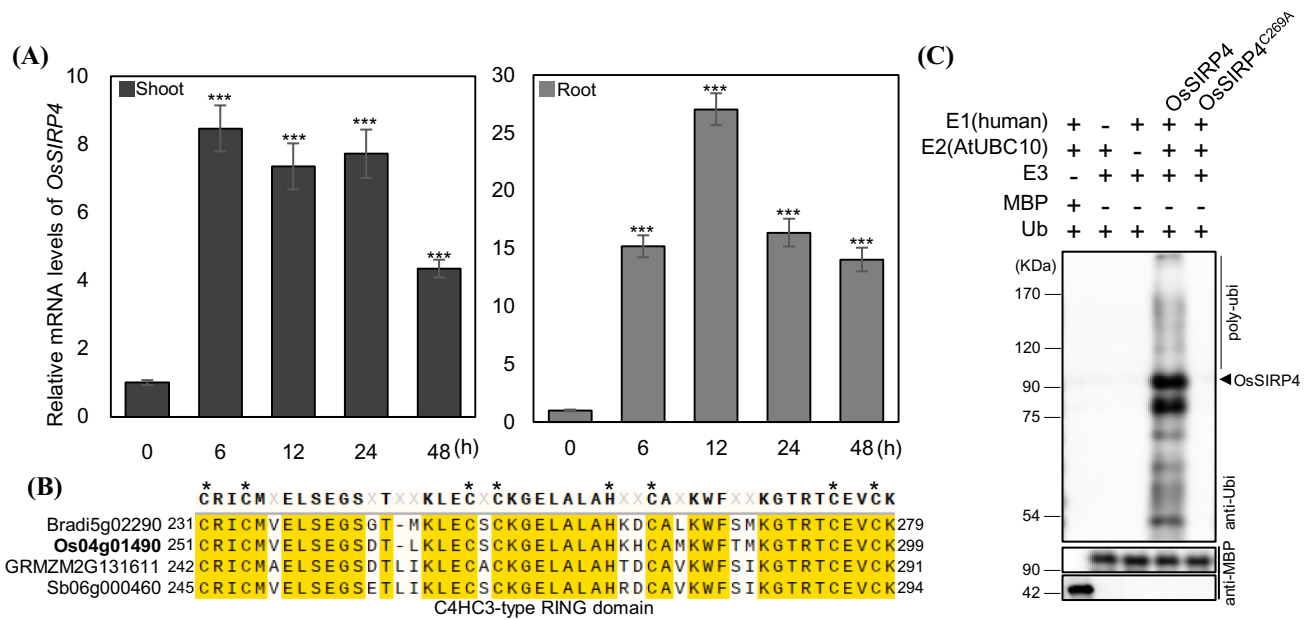
Fig. 1 Gene expression analysis and E3 ligase activity of OsSIRP4. **a** Expression level of the *OsSIRP4* gene under salt stress (150 mM NaCl) in the shoot (left) and root (right). The mRNA expression levels were measured at 6, 12, 24, and 48 h. Data are presented as the mean ± standard deviation (SD), n = 9. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with control (0 h) as 1. *ActinII* was used as the internal standard. **b** Amino acid alignments of the OsSIRP4 RING domain with its orthologs (Bradi5g02290; *Brachypodium* *distachyon*, GRMZM2G131611; *Zea mays*, Sb06g000460; *Sorghum bicolor*). The sequence alignment was performed using the ClustalW2 (https://ebi.ac.uk/clustalw/). **c** E3 ligase activity of the OsSIRP4 protein. MBP-OsSIRP4 and mutated MBP-OsSIRP4 (C269A) fusion protein were incubated at 30 °C for 2 h in the presence or absence of E1 (human), E2 (*Arabidopsis*, UBC10), and/or ubiquitin. Ubiquitinated proteins were detected by immunoblotting with anti-Ub antibody and anti-MBP antibody, respectively

plasma membrane in comparison with the diffused cytoplasmic localization of the YFP control (35S:YFP) (Fig. 2c). Thus, to identify plasma membrane fluorescence, we co-expressed OsSIRP4-YFP or mutated OsSIRP4-YFP with the mCherry-fused plasma membrane marker (PM-rk CD3-1007) in rice protoplasts. The YFP-fused OsSIRP4 or mutated OsSIRP4, as well as the mCherry-fused plasma membrane marker, were perfectly merged. These results imply that OsSIRP4 is associated with the plasma membrane in rice protoplasts. These data suggest that OsSIRP4 is localized in the plasma membrane as well as in the cytosol.
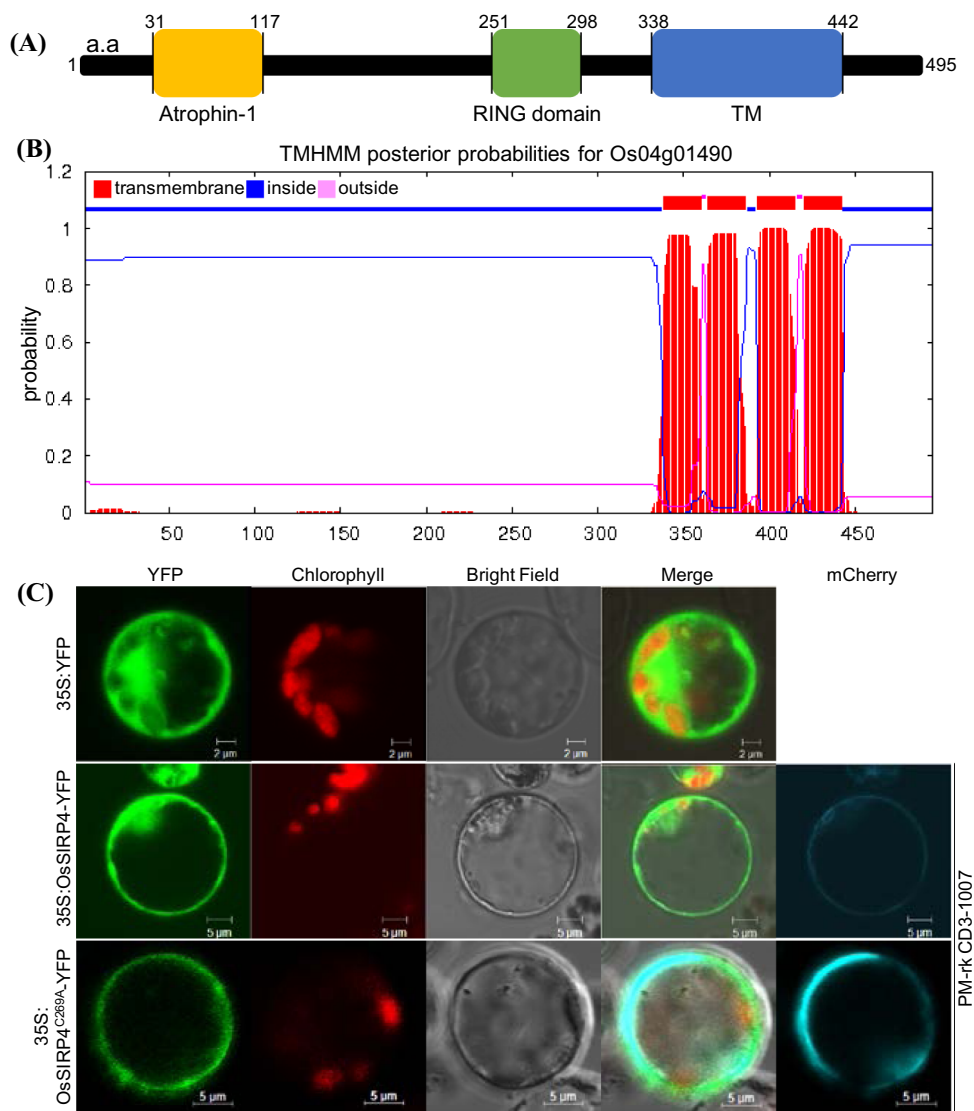
OsSIRP4 interacts with the peroxisomal biogenesis factor of OsPEX11-1

An E3 ligase can interact with specific proteins by recognizing substrate(s) and it is degraded via the ubiquitin/26S proteasome system (Deshaies and Joazeiro 2009; Saunders and Iconomou 2016). To investigate the OsSIRP4 interacting proteins, fused-Gal4 DNA binding domain (BD) OsSIRP4 was analyzed by screening in the Y2H system using prey cDNA library. As a result of Y2H screening, a total of six interacting partners were found (Fig. S3). One rice protein, a peroxisomal biogenesis factor 11-1 (OsPEX11-1) with strong activity, was selected. We first established a Y2H

assay to detect the OsSIRP4 interaction. The full-length OsPEX11-1 was fused to the Gal4 activation domain (AD) and BD-OsSIRP4 and co-transformed into the Gold yeast cells. Co-transformed cells resulted in the growth of yeast cells in SD medium lacking leucine and tryptophan (DDO, Fig. 3a, left) or leucine, tryptophan, histidine, and adenine (QDO, Fig. 3a, right). As expected, only cells containing both the BD-OsSIRP4/AD-OsPEX11-1 and the BD-p53/AD-T (positive control), but not the BD-OsSIRP4/AD-T (negative control) (Fig. 3a). To detect interaction between OsSIRP4 and OsPEX11-1, we attempted an in vitro pull-down assay using *E. coli*-expressed purified proteins. We found that the MBP-OsPEX11-1 protein was detected in the sample containing His-OsSIRP4 protein, but not the Trx-His-empty vector (Fig. 3b and Fig. S4).

To identify the subcellular localization of OsPEX11-1, the full-length OsPEX11-1 was cloned into the YFP-tagged vector. The yellow fluorescence was found as punctate signals (Fig. 3c, top). To verify these punctate signals as peroxisomes, we co-transfected the OsPEX11-1 with several organelle markers—ER, Golgi, mitochondria, and peroxisome—into the rice protoplasts (Nelson et al. 2007). Our results indicate that the fluorescent organelle markers were co-localized with the peroxisome (Px-rk CD3-983) (Fig. 3c and Fig. S5). Protein–protein interactions, such

the pull-down assay, Y2H assay, and BiFC assay are essential for understanding the molecular mechanism (Berggard et al., 2007; Hakes et al. 2008; Liu et al. 2017; Wang et al. 2016). For the BiFC assay, the OsSIRP4 and OsPEX11-1 full-length were fused with the split YFP N-terminal and C-terminal of the pSPYCE(M) (YFPc) and pSPYNE(R)173 (YFPn) vectors, respectively. After co-transfection using the PEG-mediated method, we detected yellow fluorescence signals on both the plasma membrane and the cytosol of the transfected rice protoplasts (Fig. 3c, bottom). To identify plasma membrane fluorescence, we co-expressed OsSIRP4-YFPc/OsPEX11-YFPn with the mCherry-fused plasma membrane marker in rice protoplasts, and the fluorescence signals were perfectly merged. These results indicate that OsSIRP4 can interact with OsPEX11-1 on both the plasma membrane and the cytosol in rice. Subsequently, the expression pattern of the *OsPEX11-1* with 150 mM NaCl was analyzed at different time intervals (0, 1, 12, 24, and 48 h).

Under salt stress, the expression level of the *OsPEX11-1* was significantly decreased except after 12 h of salt treatment (Fig. 3d, top), whereas the expression pattern of *OsSalT* was highly upregulated at all time intervals (Fig. 3d, bottom).

OsSIRP4 ligase regulates OsPEX11-1 via the 26S proteasome system

To verify the ubiquitination of the identified substrate proteins by OsSIRP4, we performed in vitro ubiquitination assay of OsPEX11-1 protein with OsSIRP4 protein. The purified MBP-tagged OsSIRP4 and His-Trx-tagged OsPEX11-1 were incubated with human E1, *Arabidopsis* E2 (AtUBC), and ubiquitin at 30 °C for 2 h and were subjected to immunoblot analysis with anti-Trx and anti-ub antibodies (Fig. 4a). Poly-Ub chains were detected in the presence of E1, E2, Ub, E3, and substrate protein, whereas
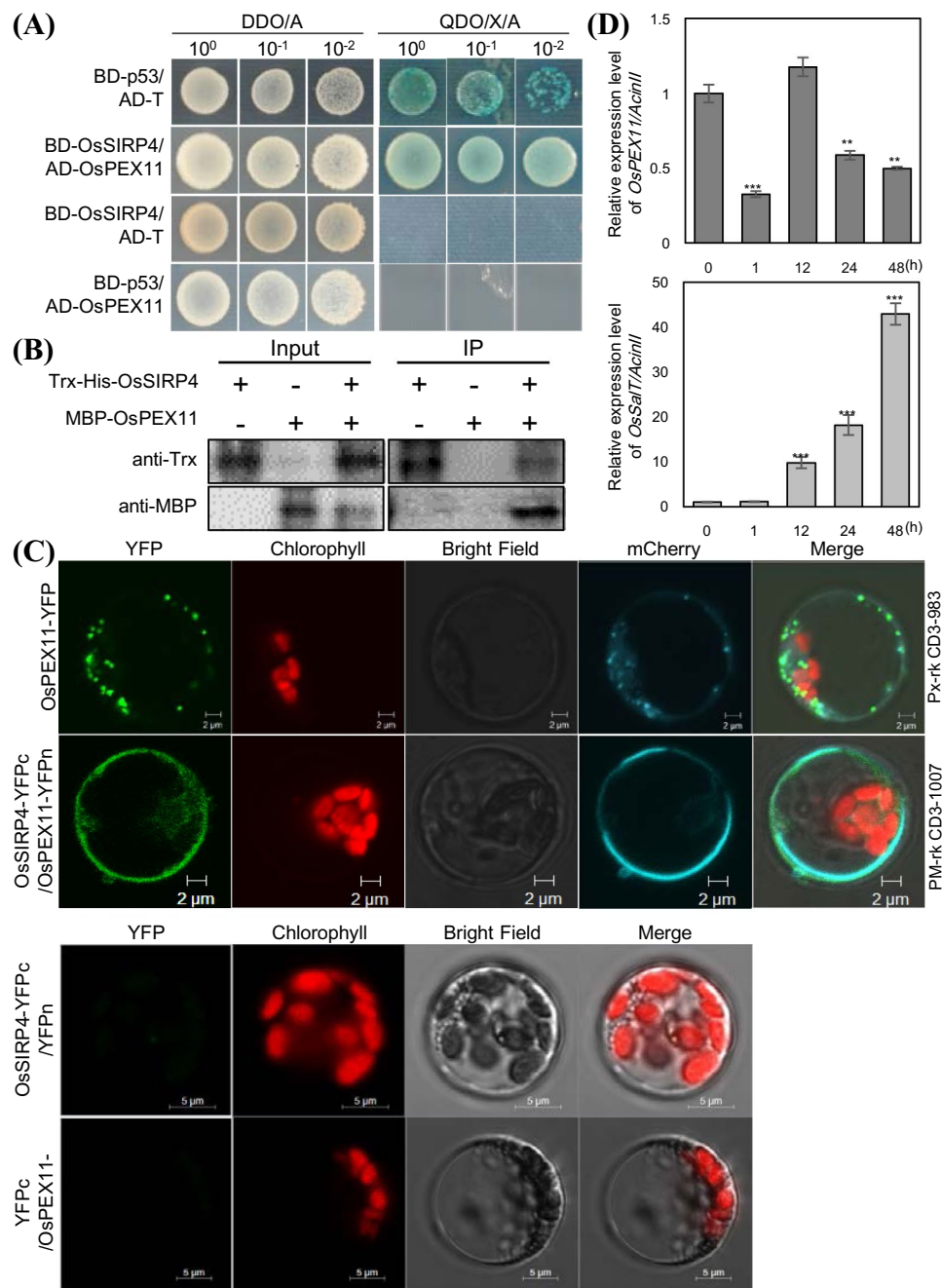
Fig. 3 OsSIRP4 interacts with OsPEX11-1. **a** Interaction between OsSIRP4 and OsPEX11-1 in a yeast two hybrid assay. The OsSIRP4 protein was fused with the GAL4 binding domain to generate BD-OsSIRP4 and OsPEX11-1 with the GAL4 activation domain to form AD-OsPEX11-1. Co-transformed clones grew in SD/-Trp-Leu (DDO) medium and SD/-Trp-Leu-His-Ade (QDO) medium indicating protein interaction in yeast cells. BD-OsSIRP4/AD-T, negative control; BD-p53/AD-T, positive control. **b** Pull-down assay showed the interaction between OsSIRP4 and OsPEX11-1. The MBP-OsPEX11-1 was pulled by the Trx-His-OsSIRP4 immobilized on resin and analyzed by immunoblotting. The input was immunoblotted using anti-MBP or -Trx antibodies. **c** Subcellular localization of the OsPEX11-1-YFP in rice protoplasts and BiFC analysis. OsSIRP4-PEX11-1 was colocalized with a mCherry-labeled peroxisome marker (Px-rk, CD3-983). In vivo interaction between OsSIRP4 and OsPEX11-1, determined using BiFC analysis. N- and C-terminal fragments of YFP (YFPn and YFPc) were fused to the C-terminus of OsSIRP4 and N-terminus of OsPEX11-1, respectively. OsSIRP4/OsPEX11-1 complexes were colocalized with a mCherry-labeled plasma membrane marker (PM-rk, CD3-1007). **d** Expression level of the *OsPEX11-1* gene under salt stress (150 mM NaCl) in the root. The *OsSalT* gene was used as marker gene. The mRNA expression levels were measured at 1, 12, 24, and 48 h. Data are presented as the mean $\pm$ standard deviation (SD), n = 9. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ compared with control (0 h) as 1. *ActinII* was used as the internal standard

Fig. 4 OsSIRP4 E3 ligase mediates the ubiquitination and degradation of OsPEX11-1. **a** In vitro ubiquitination of OsPEX11-1. The His-Trx-OsPEX11 fusion protein was assayed in the presence of E1 (human), E2 (*Arabidopsis*, UBC10), E3 (WT; Full-OsSIRP4, mutation; C269A-OsSIRP4), and ubiquitin. Ubiquitinated proteins were detected by immunoblotting with anti-Trx antibody and anti-Ub antibody. **b–c** OsSIRP4 promotes OsPEX11-1 protein degradation. In vitro protein degradation assay of OsPEX11-1 with OsSIRP4 E3 ligase. The MG132 was used as inhibitor of the 26S proteasome pathway. Anti-MBP or -His antibody was used for immunoblotting analyses. The Ponceau S staining of the Rubisco protein was used as a loading control
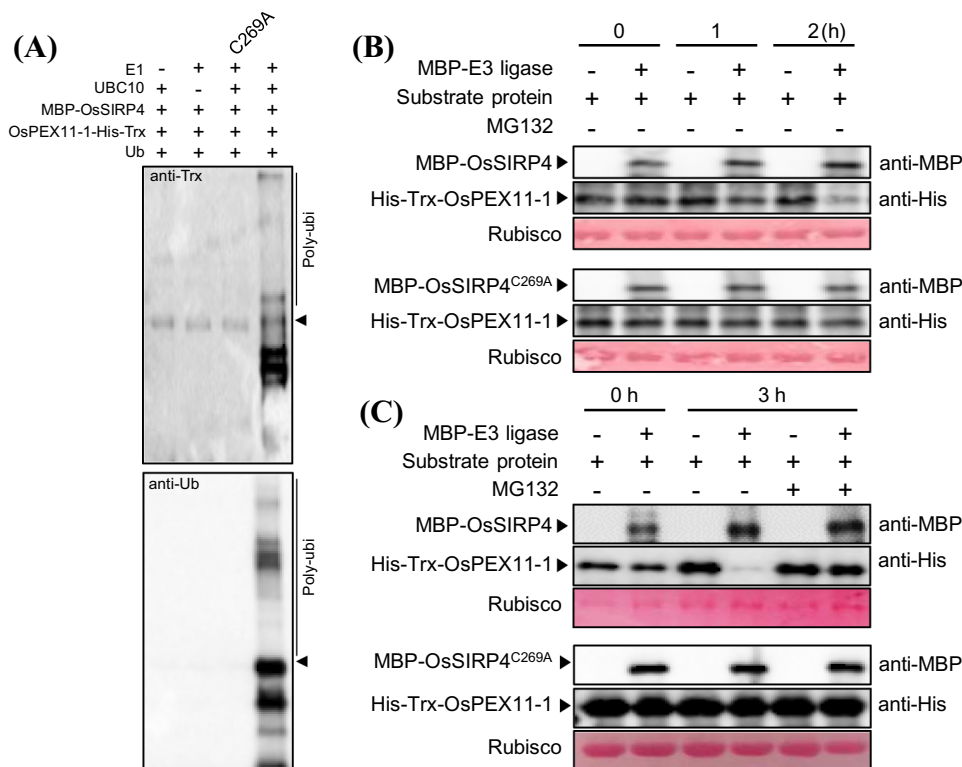
no polyubiquitinated products were detected in mutated OsSIRP4 (C269A) or in the absence of E1 or E2 (Fig. 4a, lanes 1–4).

To reveal whether OsPEX11-1 can be targeted directly for degradation by OsSIRP4, the purified MBP-OsSIRP4 and His-Trx-OsPEX11-1 were incubated to perform in vitro cell-free degradation assays. Results showed that OsSIRP4 promoted degradation of the OsPEX11-1 protein levels (Fig. 4b, top). Furthermore, we found no significant difference in OsPEX11-1 protein levels by the mutated OsSIRP4 (Fig. 4b, bottom). MG132 treatment (used as the 26S proteasome inhibitor) prevented the degradation of the OsPEX11-1 protein by the OsSIRP4 E3 ligase at 3 h of incubation. These results indicate that OsSIRP4 is responsible for OsPEX11-1 ubiquitination and facilitates its degradation by the 26S proteasome pathway.

OsSIRP4 has a function in response to salt stress

As the expression pattern of *OsSIRP4* was upregulated by salt stress, the functions of OsSIRP4 in plant responses to salt stress were analyzed. Three independent T_3 lines overexpressing OsSIRP4 or mutated overexpressing OsSIRP4 were obtained to confirm the physiological function of OsSIRP4 under salt stress. RT-PCR results showed that the transgenic *Arabidopsis* successfully expressed the exogenous OsSIRP4 (35S:OsSIRP4 #1, #2, and #3) and mutated OsSIRP4 (35S:OsSIRP4^C269A #1, #2, and #3) (Fig. S6). Under normal

conditions (non-treated), OE-OsSIRP4 and mutated OE-OsSIRP4 plants showed no substantial distinction in growth phenotype (Fig. 5a, b) when compared with the WT plants (*35S:YFP*). To test response related to salt stress, 3-day-old seedlings of OE-OsSIRP4, mutated OE-OsSIRP4, and WT plants in medium containing 150 mM and 200 mM NaCl, respectively, were treated with salt stress for 10 d. The OE-OsSIRP4 plants showed salt-sensitive root elongation and cotyledon greening under salt stress when compared with the WT plants (Fig. 5a, b, left). Meanwhile, the mutated OE-OsSIRP4 plants showed no significant difference in root elongation between the mutant and WT plants (Fig. 5a, b, right). Moreover, OE-OsSIRP4 treated with 150 mM and 200 mM NaCl exhibited a significantly lower green cotyledon rate than did the WT or mutated OE-OsSIRP4 from the 10th day after salt stress treatment (Fig. 5c).

In addition, to identify plant phenotypic effects under salt stress in soil, 2-week-old OE-OsSIRP4, mutated OE-OsSIRP4, and WT plants were exposed to salt stress with 150 mM NaCl for 24 d. More OE-OsSIRP4 leaves from all three lines were bleached when compared with WT plants (Fig. 5d). In contrast, the mutated OE-OsSIRP4 showed no significant difference in comparison with WT plants (Fig. 5e). The survival rate of the three transgenic lines in 150 mM NaCl was significantly low relative to that of the mutated OE-OsSIRP4 and WT plants (Fig. 5f). In the presence of 150 mM NaCl, growth of OE-OsSIRP4 was more inhibited and approximately 50% of plants were
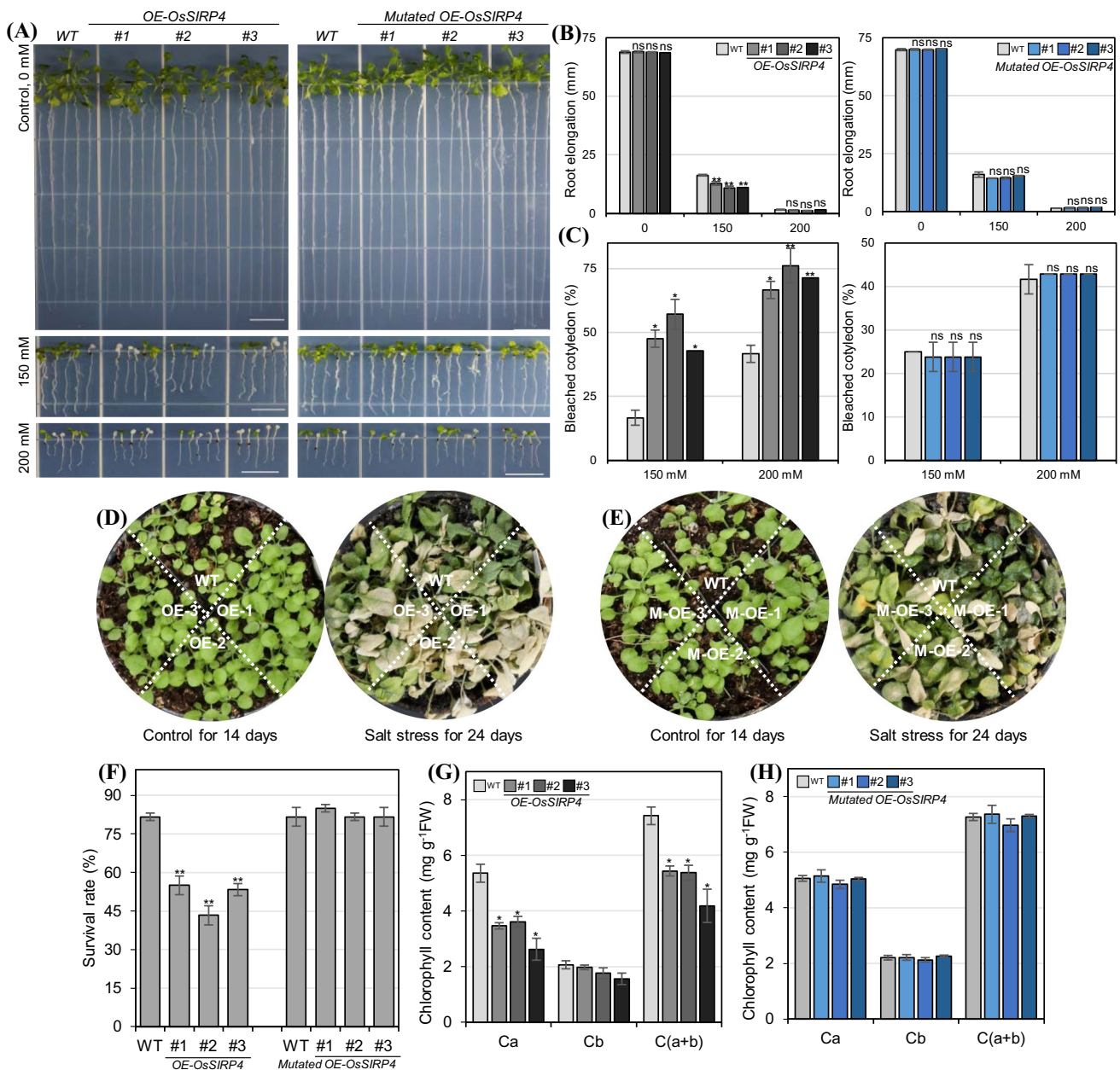
Fig. 5 Hypersensitivity to salt stress of the *35S:OsSIRP4* transgenic *Arabidopsis*. **a** Salt treatment of WT and transgenic plants. Three-day-old seedlings grown on half MS agar plates were transferred to half MS solid medium containing various concentrations of NaCl (150 and 200 mM). Data represent means ± SD from three biological replications (*n* = 10). Root lengths were measured at 10 d after treatment. **b** Root elongation of WT and transgenic plants on different salt media. **c** Quantification of seedling bleaching on plates such as those in A. **d**–**e** After 2 weeks of growth in soil, the seedlings were treated with 150 mM NaCl for 24 d and photographed. **f** Survival rates were calculated from the results of three independent experiments (n = 20). **g**–**h** Chlorophyll a or b, and total chlorophyll content in *Arabidopsis* plants. Three independent experiments were performed. The asterisks * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.001$, respectively, according to a two-tailed Student's *t*-test

alive at 24 d after treatment in soil. After 150 mM NaCl treatment, both WT and mutated OE-OsSIRP4 kept growing at 81% and 82%, respectively (Fig. 5f). The chlorophyll content showed that OE-OsSIRP4 exhibited significantly lower chlorophyll a and total chlorophyll when compared with WT and mutated OE-OsSIRP4 after 24 d

of salt treatment (Fig. 5g, h). Taken together, these results suggest that OsSIRP4 plays negative regulatory roles in the regulation of salt stress responses.

OsSIRP4 affects ROS and antioxidant enzyme activity

In a previous study, an *Oryza sativa* peroxisomal biogenesis factor 11 (OsPEX11) was shown to improve salt tolerance in response to high salinity (Cui et al. 2016). We measured the activities of ROS-scavenging anti-oxidative enzymes, including SOD, CAT, and POD on salt-induced oxidative stress. Figure 6 shows that salt stress modulated the accumulation of H_2O_2 and activities of SOD, CAT, and POD enzymes, and proline content and soluble sugar content in each of the *Arabidopsis* transgenic plants. H_2O_2 content in *Arabidopsis* transgenic plants was not significantly different between WT and OE-OsSIRP4 plants with no treatment, whereas H_2O_2 content was significantly higher in OE-OsSIRP4 plants than in WT and mutated OE-OsSIRP4 plants under salinity condition (150 mM NaCl) (Fig. 6a). Accordingly, the antioxidant activities, including activities
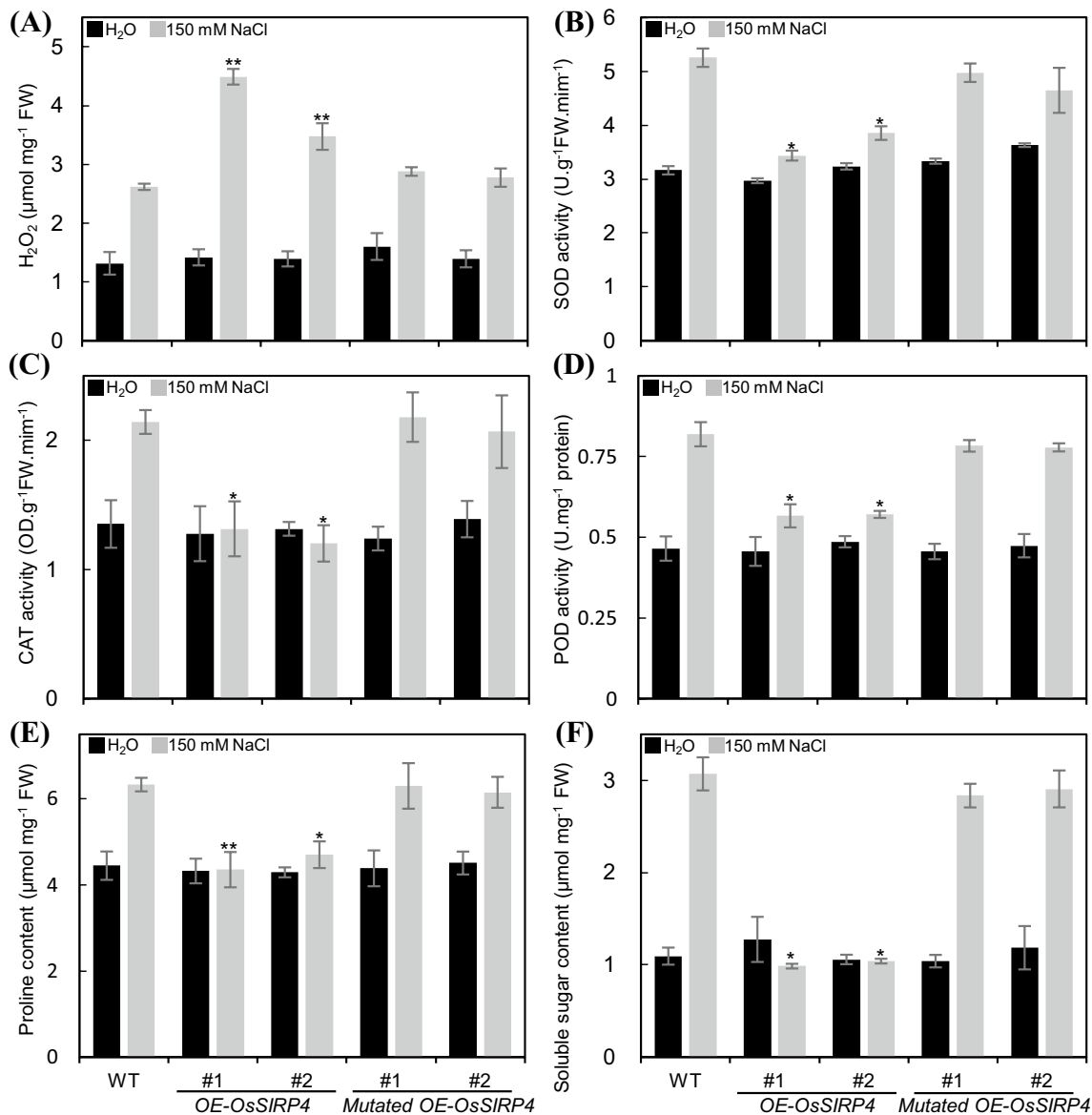

Fig. 6 Comparison of ROS-scavenging in 14-day-old WT and overexpressing-OsSIRP4 plants under control and salt stress. The *Arabidopsis* seedlings were treated with 150 mM NaCl for 24 h. The H_2O_2 content and activities of antioxidant enzymes (SOD, CAT, POD), proline content, and soluble sugar content were assayed (**a–f**). Values are means of three biological replicates. The asterisks * and ** indicate statistically significant differences at $P < 0.05$ and $P < 0.001$, respectively, according to a two-tailed Student's *t*-test

of SOD, CAT, and POD, were significantly reduced in OE-OsSIRP4 plants when compared with those in WT and mutated OE-OsSIRP4 plants under salt stress (Fig. 6b, c, d). Similarly, we found that both proline content and soluble sugar content were lowered in OE-OsSIRP4 plants under salt stress treatment (Fig. 6e, f). These results suggest that OsSIRP4 might have a negative role against salt-induced excessive ROS by inducing deficiency in antioxidant enzymes, proline, and soluble sugar accumulation.

OsSIRP4 regulates the expression of genes involved in the salt stress response

To better understand molecular regulation of OsSIRP4 to salt stress, we tested the expression levels of several salt-induced genes. Gene expression showed no significant difference in four genes, *AtSOS1* (K$^+$ uptake and transport; Wang et al. 2014), *AtAKT1* (K$^+$ uptake; Rubio et al. 2008), *AtNHX1* (Na$^+$/H$^+$ antiporter; Sottosanto et al. 2007), and *AtHKT1;1* (K$^+$ uptake and transport; Wang et al. 2014), in the OsSIRP4-overexpressing plants when compared with the mutant plants and WT under control conditions (Fig. 7). In contrast, the expression of these genes was significantly lower in OE-OsSIRP4 plants than in WT and mutated OE-OsSIRP4 after salt treatment for 24 h. These results suggest

that overexpression of OsSIRP4 in plants might down-regulate the expression levels of these salt-responsive genes under salt stress.

Discussion

Rice is one of the important cereal crops and is widely consumed globally. Rice grown under flooded and irrigated conditions is highly adaptable, but is adversely affected particularly by salt stress (Shrivastava and Kumar 2015; Singh et al. 2015). Several reports show that excess accumulation of salinity affects cellular metabolisms such as protein synthesis and enzyme activities, and hence, impairs plant growth, including leaf rolling, root growth, seed germination, spikelet sterility, and gain yield (Horie et al. 2012; Rahman et al. 2017). Therefore, much research is required to understand the molecular mechanism in response to salt stress in rice. To defend against environmental stress, plants have developed a self-resistance mechanism. In particular, the ubiquitin proteasome system (UPS) was documented several decades ago. E3 ubiquitin ligase in the UPS plays a crucial role in the regulatory mechanism of protein turnover. E3 ubiquitin ligase recognizes the target protein and degrades it via the 26S proteasome. Many studies have shown that the
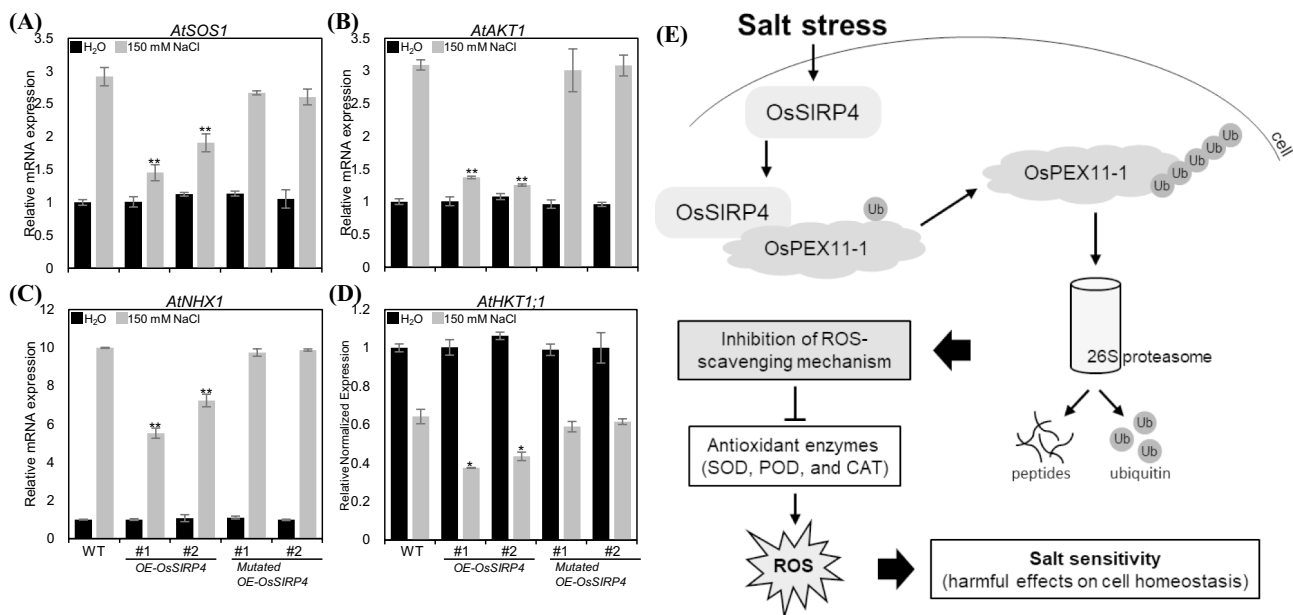

Fig. 7 Relative expression of the genes encoding Na$^+$ and K$^+$ homeostasis proteins under salt stress. **a** *AtSOS1,* AT2g01980; **b** *AtAKT1,* AT2G26650; **c** *AtNHX1,* AT5G27150; **d** *AtHKT1;1,* AT4G10310 in the *35S:OsSIRP4* and *35S:OsSIRP4^{C269A} Arabidopsis* seedlings treated with 150 mM of NaCl for 24 h. Data represent means ± SD from three biological replications, n = 9. *$P < 0.05$ and **$P < 0.01$ compared with control (non-treated WT) as 1. *ActinII* was used as the

internal standard. **e** Representative diagram of the findings of the present study. The OsSIRP4 gene was strongly induced under salt stress. The OsSIRP4 protein targeted OsPEX11-1 as a substrate protein, which in turn was degraded via the 26S proteasome pathway. These processes lead to the inhibition of ROS-scavenging mechanisms, and the increased ROS levels led to harmful effects, such as decreased stress tolerance, in cells

UPS is significantly involved in stress response pathways. In *Arabidopsis thaliana*, RING-DUF1117 E3 ubiquitin ligases (AtRDUF1)—products of a gene that is upregulated by salt—is involved in plant responses to salt stress. Overexpression of *AtRDUF1* leads to insensitivity to salt and osmotic stresses during germination and seedling growth (Li et al. 2013). Tomato (*Solanum pimpinellifolium*) RING Finger E3 Ligase (SpRing) is upregulated by salt, drought, and osmotic stresses, and overexpression of *SpRing* in *Arabidopsis* showed enhanced salt tolerance during seed germination and early seedling development, whereas silencing of *SpRing* led to increased sensitivity to salt stress in tomato (Qi et al. 2016). *Oryza sativa* drought-, heat-, and salt-induced RING finger protein 1 (OsDHSRP1) was highly expressed under abiotic stresses, including NaCl, drought, and heat and the phytohormone abscisic acid (ABA). The *Arabidopsis* plants overexpressing OsDHSRP1 showed hypersensitivity under drought, heat, and NaCl treatment (Kim et al. 2020). Similarly, *Oryza sativa* salt-, ABA- and drought-induced RING finger protein 1 (OsSADR1) resulted in sensitive phenotypes for salt- and mannitol-responsive seed germination and seedling growth in *Arabidopsis* (Park et al. 2018). In the present study, *OsSIRP4* was highly induced under salinity conditions in the rice shoot and root samples (Fig. 1a. In addition, OsSIRP4 as an E3 ligase encoding a 495 amino acid protein with a C_4HC_3-type RING domain, exhibited ubiquitination activity in vitro associated with the plasma membrane and cytosol (Fig. 1, Fig. 2 and S2). Our results demonstrated the molecular function of OsSIRP4 to regulate a peroxisome-localized protein (OsPEX11-1). For example, the OsSIRP4/OsPEX11-1 complex was observed in the plasma membrane and cytoplasm (Fig. 3c, bottom) and E3 ligase of OsSIRP4 led to degradation of OsPEX11-1 protein via the ubiquitin–proteasome system (Fig. 4). Finally, overexpression of *OsSIRP4* showed a significant low survival rate under salt stress (Fig. 5). Several lines of evidence support that OsSIRP4 plays an important role in response to salt responses in rice. Collectively, several E3 ligases, including OsSIRP4, might co-regulate salt responses in rice. Construction of the co-regulation network including a variety of RING E3 ligases is warranted in further work.

In most eukaryotic cells, including plants, peroxisome might be one of the major sites for intracellular H_2O_2 production because of their essentially oxidative metabolism. Peroxisomes, which possess diverse oxidative reactions, play pivotal roles in metabolic processes such as ROS detoxification, abiotic stress responses, and signaling (del Río et al. 2003; Nito et al. 2007; Hossain and Dietz 2016; Kao et al. 2018). A *PEX11* gene family is known to be involved in peroxisome biogenesis but remains largely unknown in rice. Nayidu et al. (2008) reported that five putative members of the OsPEX11 family (OsPEX11-1 to -5) had differential expression patterns under stresses, including ABA, drought,

H_2O_2, salt, low nitrogen, and low phosphorous. Overexpression of OsPEX11-3 (Os03g19010) showed tolerance and high activities of antioxidant enzymes, such as SOD, POD, and CAT, in response to salt stress when compared with the WT and OsPEX11-RNAi plants (Cui et al. 2016). Mutations of peroxisome biogenesis proteins showed severe developmental disabilities and stress-susceptibilities (Aung and Hu 2011; Burkhart et al. 2013; Cassin-Ross and Hu 2014). We found the degradation of OsPEX11-1 by OsSIRP4 E3 ligase, whose gene was down-regulated under salt stress (Fig. 3d). These results suggest the attractive hypothesis that inhibition of peroxisome biogenesis by OsPEX11-1 degradation via overexpression of OsSIRP4 might induce a highly sensitive response to salt stress. However, much work is required to rule out this hypothesis. Recent studies have shown that enhanced activities of antioxidant enzymes (SOD, POD, and CAT) trigger the ROS-scavenging mechanism. In contrast, overaccumulation of ROS might lead to metabolic disorders, cellular damage, and premature senescence or necrosis (Møller et al. 2007; Jaleel et al. 2009; Miller et al. 2010; Habib et al. 2016). Overexpression of genes involved in ROS detoxification leads to lower cellular damage and improvement in root growth under salt stress (Roy et al. 2014; Munns and Gilliham 2015). In addition, several lines of evidence indicate that the accumulation of proline and soluble sugars as osmoprotectants plays a key role in maintaining ROS balance in response to unfavorable environmental conditions in plants (Couée et al. 2006; Hayat et al. 2012; Rejeb et al. 2014; Rakshit and Singh 2018). Our results showed that overexpression of OsSIRP4 reduced the growth and survival rate under high salt stress when compared with both WT and mutated OsSIRP4 (Fig. 5). Under salinity conditions, compared with WT and mutated OsSIRP4, the overexpressing OsSIRP4 *Arabidopsis* plants showed increased H_2O_2 content and reduced antioxidant enzyme activities, including those of SOD, CAT, and POD (Fig. 6a, b, c, d). Similarly, lower proline and soluble sugar content were observed in the OsSIRP4-overexpressing plants than in the WT and mutated OsSIRP4 plants under salt stress conditions (Fig. 6e, f). The increased proline plays an important role in ROS scavenging and leads to a decrease in Na^+ toxicity in overexpression plants (Cui et al. 2016; Islam et al. 2016; Islam et al., 2015). Soluble sugar is known to be related to the maintenance of the ROS balance and increases in soluble sugars led to positive responses enhancing plant tolerance against abiotic stresses (Rathinasabapathi 2000; Rakshit and Singh 2018). Under non-stress conditions, all plants showed similar ROS-scavenging abilities and comparable proline and soluble sugar contents, whereas OsSIRP4-overexpressing plants under salt stress exhibited high ROS levels, low enzyme activity, and reduced proline and soluble sugar contents. These findings suggest that the OsPEX11-1 substrate protein can be degraded by the OsSIRP4 protein

as an E3 ligase, which leads to decreased stress tolerance (Fig. 7e). However, heterogenous overexpression leads to a question of how OsSIRP4 regulates salt sensitive response in *Arabidopsis*. An attractive hypothesis is that the *Arabidopsis* ortholog of OsPEX11-1 might be regulated by OsSIRP4. To examine this hypothesis, putative *Arabidopsis* ortholog of OsPEX11-1 (AT1G01820) with 76% similarity was tested (Fig. S7). The Y2H system showed strong interaction of AtPEX11-1 with OsSIRP4 (Fig. S8A). In addition, an in vitro pull-down assay showed that AtPEX11-1 was pulled down by Trx-His-OsSIRP4 protein (Fig. S8B). These results support the hypothesis that OsSIRP4 can regulate the protein levels of AtPEX11-1 *Arabidopsis* ortholog in transgenic plants and exhibits negative phenotypes due to oxidative damage under salt stress conditions.

Reducing Na^+ accumulation and maintaining K^+ stability is an important mechanism for enhancing salt tolerance or adaptation in plants (Apel and Hirt 2004; Foyer and and-Noctor 2005; Munns and Tester 2008; Noreen et al. 2010). The OsSIRP4 overexpressing plants showed a salt sensitivity phenotype (Fig. 5). To understand the salt-sensitive mechanism in OsSIRP4 overexpressing plants, the transcript levels of genes related to Na^+ and K^+ homeostasis were examined under control and salt stress conditions (Fig. 7). *AtHKT1;1* and *AtSOS1* are known to maintain Na^+ and K^+ homeostasis in *Arabidopsis* as a salt stress response (Wang et al. 2014). Under salt stress, *AtHKT1;1* unloads Na^+ and *AtSOS1* leads to Na^+ exclusion, thereby keeping the levels of Na^+ low. The *AtAKT* genes encoding the inward rectifier K^+ channels function to maintain K^+ uptake (Rubio et al., 2008). Rubio et al. (2008) reported that *AtAKT1* and *AtHAK5* have been shown to be major contributors to K^+ uptake in the roots (Hirsch et al. 1998; Gierth et al. 2005). A vacuolar Na^+/H^+ antiporter in *Arabidopsis*, *AtNHX1*, is ubiquitous and mediates the transport of Na^+ and K^+ into the vacuole, which influences plant development and confers salt tolerance (Sottosanto et al. 2007). T-DNA insertional mutant *nhx1 Arabidopsis* plants exhibited more salt sensitivity than did control plants (Apse et al. 2003). In this study, four salt-inducible genes, *AtSOS1*, *AtAKT1*, *AtNHX1*, and *ATHKT1;1*, were downregulated under saline stress condition in OsSIRP4 overexpressing plants when compared with the WT and mutated OsSIRP4 plants (Fig. 7). These results suggest that high Na^+ accumulation leading to increased Na^+ toxicity via down-regulation of genes is related to Na^+ and K^+ homeostasis in OsSIRP4-overexpressing plants that exhibit salt hypersensitive responses.

In summary, we examined the role of a rice E3 ligase, OsSIRP4, in response to saline stress. The expression of *OsSIRP4* was highly increased; however, that of an interacting gene, OsPEX11-1, was significantly decreased under salt treatment. The OsSIPR4 E3 ligase might regulates the degradation of ubiquitinated OsPEX11-1 protein via the 26S proteasomal system. The OsSIRP4-overexpressing plants showed a hypersensitive phenotype, such as inhibited root length, reduced cotyledon greening ratio, and decreased survival ratio and chlorophyll content under salt stress condition. In addition, OsSIRP4-overexpressing plants showed a high accumulation of H_2O_2; reduced activities of SOD, CAT, and POD; and reduced accumulation of proline and soluble sugar when compared with the WT and mutated OsSIRP4 plants, likely resulting in severe cell damage under salt stress. Here, we aimed to provide several lines of evidence to show that OsSIRP4 acts as a negative regulator of salt stress responses in rice. However, further work is required to fully elucidate the nature and mechanism of the OsSIRP4-mediated salt response via interaction with OsPEX11-1. Therefore, further studies on transgenic rice plants or mutant lines are required for a better understanding of the OsSIRP4-mediated salt responsive mechanism.

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