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Overexpression of rice F-box phloem protein gene *OsPP12-A13* confers salinity tolerance in *Arabidopsis*

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Abstract: Salinity is a serious challenge for agriculture production by limiting the arable land. Rice is a major staple food crop but very sensitive to salt stress. In this study, we used *Arabidopsis* for the functional characterization of a rice F-box gene *LOC_Os04g48270* (*OsPP12-A13*) under salinity stress. *OsPP12-A13* is a nuclear-localized protein that is strongly up-regulated under salinity stress in rice and showed the highest expression in the stem, followed by roots and leaves. Two types of transgenic lines for *OsPP12-A13* were generated, including constitutive tissue over-expression using the CaMV35S promoter and phloem specific over-expression using the pSUC2 promoter. Both types of transgenic plants showed salinity tolerance at the seedling stage through higher germination percentage and longer root length, as compared to control plants under salt stress in MS medium. Both the transgenic plants also exhibited salt tolerance at the reproductive stage through higher survival rate, plant dry biomass, and seed yield per plant as compared to control plants. Determination of Na^+ concentration in leaves, stem and roots of salt-stressed transgenic plants showed that Na^+ concentration was less in leaf and stem as compared to roots. The opposite was observed in wild type stressed plants, suggesting that *OsPP12-A13* may be involved in Na^+ transport from root to leaf. Transgenic plants also displayed less ROS levels and higher activities of peroxidase and glutathione S-transferase along with upregulation of their corresponding genes as compared to control plants which further indicated a role of *OsPP12-A13* in maintaining ROS homeostasis under salt stress. Further, the non-significant difference between the transgenic lines obtained from the two vectors highlighted that *OsPP12-A13* principally works in the phloem. Taken together, this study showed that *OsPP12-A13* improves salt tolerance in rice, possibly by affecting Na^+ transport and ROS homeostasis.

Abbreviations

CAT:	catalase
GFP:	green fluorescent protein
GST:	glutathione S-transferase
MDA:	malondialdehyde
POD:	peroxidase
ROS:	reactive oxygen species
SOD:	superoxide dismutase

Introduction

Salinity is among the major abiotic stresses that hamper plant growth and productivity (Munns and Tester, 2008; Zafar *et al.*, 2020c). Industrial development and excessive use of fertilizers are continuously increasing the land areas under salt stress (Alzubaidi *et al.*, 1990; Han *et al.*, 2015; Shrivastava and Kumar, 2015). In saline soils, the uptake of salts (mainly Na^+ ions) by roots increases manyfold, and salts are transported to the aerial parts of the plant, mainly leaves and shoot (James *et al.*, 2011; Byrt *et al.*, 2014). Since leaves are major photosynthetic organs, the accumulation of Na^+ ions seriously affects the rate of photosynthesis and leads to cell death in most cases (Chaves *et al.*, 2009; Kumar *et al.*, 2017). Thus, identification of the salt-tolerant varieties, understanding the mechanisms of salinity tolerance and identification of genes responsible for salt tolerance, will provide the most durable and eco-friendly solutions to cope with this major issue (Huang *et al.*, 2008; Chaves *et al.*, 2009; Rahnama *et al.*, 2011; Zafar *et al.*, 2015; Zafar *et al.*, 2020c).

Crops differ in their ability to tolerate salt stress, and rice being a major staple crop is highly sensitive to salt stress (Martínez-Atienza *et al.*, 2007; Huang *et al.*, 2008; Liu *et al.*, 2013). Salt stress usually causes the accumulation of Na^+ ions in leaf cells, which affects various metabolic processes such as protein synthesis and activation of key metabolic enzymes (Munns *et al.*, 2006; Munns and Tester, 2008). Plants manage

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to exclude the excessive Na^+ ions from the leaf and shoot to the root cells to protect from cellular damage (Byrt *et al.*, 2007; Han *et al.*, 2018). Thus, Na^+ exclusion or recirculation from leaves has been regarded as an important mechanism of salinity tolerance in plants (Munns *et al.*, 2006; Byrt *et al.*, 2007; Munns and Tester, 2008; Han *et al.*, 2018). Several genes have been identified from the model plant *Arabidopsis thaliana* and the major crop wheat that regulate salinity tolerance via Na^+ exclusion or recirculation from leaves (Huang *et al.*, 2008; James *et al.*, 2011). Among these, Salt Overly Sensitive (SOS) pathway genes, such as *SOS1* and *SOS2*, play key roles in maintaining the ion homeostasis in cells and contribute significantly to salt tolerance (Martínez-Atienza *et al.*, 2007; Cheng *et al.*, 2019). In addition, Na^+/H^+ antiporter genes have shown a potential role in salinity tolerance in different crops, including *Arabidopsis* (Sottosanto *et al.*, 2007), kiwifruit (Tian *et al.*, 2011), and mungbean (Kumar *et al.*, 2017). Thus, all these gene families play important roles in salinity tolerance mainly by regulating Na^+ transport and exclusion in leaves.

In addition to the Na^+ accumulation, salt stress also causes oxidative damage to plants through the overaccumulation of ROS in cells (Abogadallah, 2010; Abdelgawad *et al.*, 2016; Kumar *et al.*, 2017). This oxidative damage induces membrane lipid peroxidation and thus leads to cell death in different tissues (Abdelgawad *et al.*, 2016; Zafar *et al.*, 2020b). However, plants have a huge genetic variation to cope with this stress under harsh climates, which depends on their antioxidant defense system (Abogadallah, 2010; Zafar *et al.*, 2020a). The antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), and glutathione S-transferase (GST) have the ability to scavenge or detoxify the ROS molecules in order to protect plants from oxidative damage induced by environmental stresses (Abogadallah, 2010; Abdelgawad *et al.*, 2016; Zafar *et al.*, 2020a).

Fox box domain proteins are a large family with around 700 members in *Arabidopsis* and rice (Xu *et al.*, 2009). Several F-box genes have been shown involved in salinity tolerance (Jain *et al.*, 2007; Gonzalez *et al.*, 2017; An *et al.*, 2019). In rice, a member of the F-box protein family, known as *MAIF1* (*miRNAs regulated and abiotic stress induced*), has been shown to negatively regulate salt tolerance by affecting root growth (Yan *et al.*, 2011). In *Arabidopsis*, another F-box protein, EST1, also negatively affects salinity tolerance by regulating plasma membrane Na^+/H^+ antiport activity (Liu *et al.*, 2020). Overexpression of another F-box gene, *OsMsr9*, enhanced salinity tolerance in *Arabidopsis* and rice by increased root and shoot growth, higher production of proline, and less malondialdehyde (MDA) contents (Xu *et al.*, 2014). Similarly, overexpression of a wheat F-box gene *TaFBA1* in tobacco enhanced drought and salinity tolerance by regulating antioxidant, reactive oxygen species (ROS) production, as well as Na^+ and K^+ levels in cells (Zhou *et al.*, 2014; Zhao *et al.*, 2017b). A novel F-box gene, *CaF-box*, in pepper has also been reported to play a role in multiple abiotic stress tolerance, including salinity (Chen *et al.*, 2014). A genome-wide analysis of F-box proteins in *Medicago truncatula* identified several other functional domains in the C-terminal region, such as LRR, Kelch, FBA, and PP2, in addition to the conserved domains (Song *et al.*, 2015). These F-box genes are speculated to play a role in salt and heavy metal stresses (Song *et al.*, 2015). These studies indicated a potential role of F-box genes in salinity tolerance, and thus identification of new F-box genes in rice would play important role in breeding salt-tolerant cultivars.

In this study, we have reported the role of a rice F-box domain-containing protein *OsPP2-A13* in salinity tolerance. *OsPP2-A13* was identified as a hub gene predicted to play a major role in salt tolerance in rice (Zhu *et al.*, 2019). We showed that overexpression of *OsPP2-A13* in *Arabidopsis* ecotype Columbia-0 displayed enhanced salinity tolerance at seedling and reproductive stages, probably by modifying Na^+ transport from root to leaves. *OsPP2-A13* affects the expression of antioxidant-associated genes, which probably caused higher antioxidant activities under salt stress and ROS levels under normal range in transgenic lines.

Materials and Methods

Plant materials, growth conditions and stress treatments

Seeds of japonica rice cultivar Nipponbare were sown in Petri plates under high moisture conditions at 37°C in the dark for good germination (3–4 days). Uniformly germinated seeds were transferred to Yoshida solution (Yoshida *et al.*, 1971) and grown for 4 weeks at 28°C with 70% relative humidity. Leaf, stem, and root tissues were collected at this stage for tissue-specific relative gene expression analysis. Then seedlings were shifted to a new Yoshida solution having 150 mM NaCl (Quan *et al.*, 2018), and the samples were harvested at 0, 3, 9, 24, and 48 h of salt treatment for RNA isolation.

For the salt treatment of *Arabidopsis thaliana* at the seedling stage, seeds were sown on half-strength Murashige and Skoog (MS) medium and laid on 4°C for 3 days and then shifted to a growth chamber at 22°C with a light intensity of 120–150 μmol/m^2.s and relative humidity of 50%. After 10 days, seedlings were shifted to a new MS medium with 200 mM NaCl. Root length was observed after 7 days of salt treatment, and data for root length were recorded. For the estimation of germination percentage, seeds were plated initially on MS medium with 200 mM NaCl and germination rate was recorded after 5 days.

For salt stress at the reproductive stage, *Arabidopsis* plants were grown in a growth chamber at the above-mentioned conditions, and 250 mM NaCl solution was applied to four-weeks old plants every three days interval. Leaf samples were collected at this stage for various physiological assays including Na^+ concentration. The number of survived plants, plant dry biomass, and seed yield per plant (mg) were recorded at the time of complete maturity.

RNA isolation and real time PCR

RNA was isolated using the RNAprep Pure Kit (for Plants; Tiangen) and quantified in NanoDrop. 1 μg total RNA was reverse transcribed into cDNA using a first-strand cDNA synthesis kit (Takara). Quantitative real-time PCR was performed using SYBR green master mix on an ABI7500 sequence detection system (Zhao *et al.*, 2017a). *OsActin1* gene was used as an internal control for rice (Zafar *et al.*, 2020a), and *AtActin2* was used for *Arabidopsis* (Zhao *et al.*, 2017a). The primer sequences for all genes are listed in Suppl. Tab. 1.

Phylogenetic analysis and gene structure

Protein sequence for rice *OsPP12-A13* gene was retrieved from Rice genome annotation project (http://rice.plantbiology.msu.edu/) under locus name *LOC_Os04g48270*. The protein sequences for ortholog genes of different species were retrieved from NCBI via BLAST search against *OsPP12-A13*. The amino acid sequences were aligned using CLUSTALX software (Wang *et al*., 2018), and a phylogenetic tree was constructed using MEGA 7 with 1000 bootstrap replicates (Zafar *et al*., 2020b). The gene structure of the *OsPP12-A13* gene was constructed using a gene structure display server (Hu *et al*., 2014).

Vector construction and transgenics development

To construct a binary vector for gene overexpression in transgenic plants, the coding sequence of *OsPP12-A13* cDNA was amplified using a forward primer (5'-ATCGTCTAGAATGGGGGCGGGGG-3', X*ba*I site is underlined) and a reverse primer (5'-ATCGGGTACC TTACTTGCAGATTGTGC-3', K*pn*I site is underlined). The PCR product was confirmed by sequencing. Then, the gene fragment was digested with X*ba*I and K*pn*I and cloned into the plant binary vector ProkII under the control of the CaMV 35S and pSUC promoters to generate the 35S::OsPP12-A13 and pSUC::OsPP12-A13 constructs, respectively. For pSUC::OsPP12-A13, the pSUC promoter was first constructed into the ProkII vector. These constructs were introduced into *Agrobacterium tumefaciens* strain LBA4404 after sequencing and then transformed into *Arabidopsis* ecotype Col-0 by the floral dipping method (Zhang *et al*., 2006). Empty vectors were also introduced in *Arabidopsis* as controls.

Subcellular localization

Localization of OsPP12-A13 protein was first predicted using the WoLF PSORT database (www.genscript.com/tools/wolf-psort). For experimental validation, the coding sequence of OsPP12-A13 cDNA was fused with GFP and cloned into vector pBWA(V)HS-GLosgfp. This construct was co-transformed along with the nucleus marker vector pBWA(V)HS-Nucleus-mKate into *Arabidopsis* protoplasts and observed under a confocal microscope. For the negative control, an empty vector pBWA(V)HS-GLosgfp containing only the GFP gene was transformed into *Arabidopsis* protoplasts.

Determination of Na^+ concentration

Na^+ concentration from leaf, stem, and root tissues was determined by the freeze-thawed method as described earlier (Wu *et al*., 2019). Plant samples were harvested and frozen (−80°C) immediately for 60 min and then thawed again, followed by squeezing to release cell sap. The cell sap was centrifuged at 5000 x *g* for 10 min, and the supernatant was collected. The 10 µL of supernatant was diluted to 25 mL and used for Na^+ determination with a flame photometer.

Measurement of H_2O_2, MDA and antioxidant activities

Quantitative measurement of H_2O_2 was performed using a spectrophotometric method as described earlier (Zafar *et al*., 2020a). Briefly, 0.1 g fresh leaves were harvested from *Arabidopsis* plants and extracted with 1 mL of 50 mM sodium phosphate (pH 7.4) buffer and kept on ice for 20 min. The mixture was centrifuged at 12000 × *g* for 15 min and quantified using a spectrophotometer. Measurement of MDA, SOD, POD, CAT, and GST activities was performed using the kits provided by Nanjing Jiancheng bioengineering Institute, China (Zafar *et al*., 2020b). One unit of SOD activity was defined as 1 g tissue, among which the inhibition rate of SOD is 50%. One unit of CAT activity was estimated as the amount of enzyme that decomposes 1 µmol H_2O_2 per second in 1 g tissue. One unit of POD activity was defined as an absorbance change of 0.01 per minute.

Electrolyte leakage assay

The relative electrolyte leakage was determined using the following method (Bajji *et al*., 2002). Leaf segments of 1 cm were harvested from selected plants and washed with deionized water to clean out the solutes from the leaf surface. The cut segments were then put into test tubes containing 20 mL deionized water, and electrical conductivity was measured with an electrical conductivity meter.

Statistical Analyses

All the data were analyzed with the R software (www.r-project.org). One-way analysis of variance was performed by comparing each transgenic line to the vector control plants. This was followed by the Tukey HSD test for mean comparison. The error bars were calculated with data from a single experiment.

Results

OsPP12-A13 is a salt responsive gene

F box proteins have been shown to play a role in diverse processes, including response to environmental stresses (Jain *et al*., 2007; Gonzalez *et al*., 2017; An *et al*., 2019). However, its role under salinity stress has not been studied in most crop species, including rice. Here, we studied one of rice F box proteins, PHLOEM PROTEIN 2-LIKE A13 (OsPP2-A13), which is predicted to work through phloem tissue and may be involved in the transportation of Na^+ ions for salinity tolerance (Zhu *et al*., 2019). We first investigated whether *OsPP2-A13* is responsive to salt stress. We applied salt stress of 150 mM NaCl to rice seedlings for different time durations from zero to 48 h and detected mRNA abundance of *OsPP2-A13*. The quantitative real-time PCR (qRT-PCR) analysis showed that *OsPP2-A13* expression was strikingly induced under 150 mM NaCl, and the expression increased proportionally with the duration of salt stress (Fig. 1a). We further tested the tissue-specific expression of *OsPP2-A13* in different tissues of rice using qRT-PCR. This showed the highest relative expression in the stem, followed by root and leaf, which have almost similar expression levels (Fig. 1b). The lowest expression was observed in seed tissues. These results indicate that *OsPP2-A13* is a salt responsive gene and may have a role in salinity tolerance.

Evolutionary study and subcellular localization of OsPP12-A13

The protein sequence of rice OsPP12-A13 was obtained from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/)

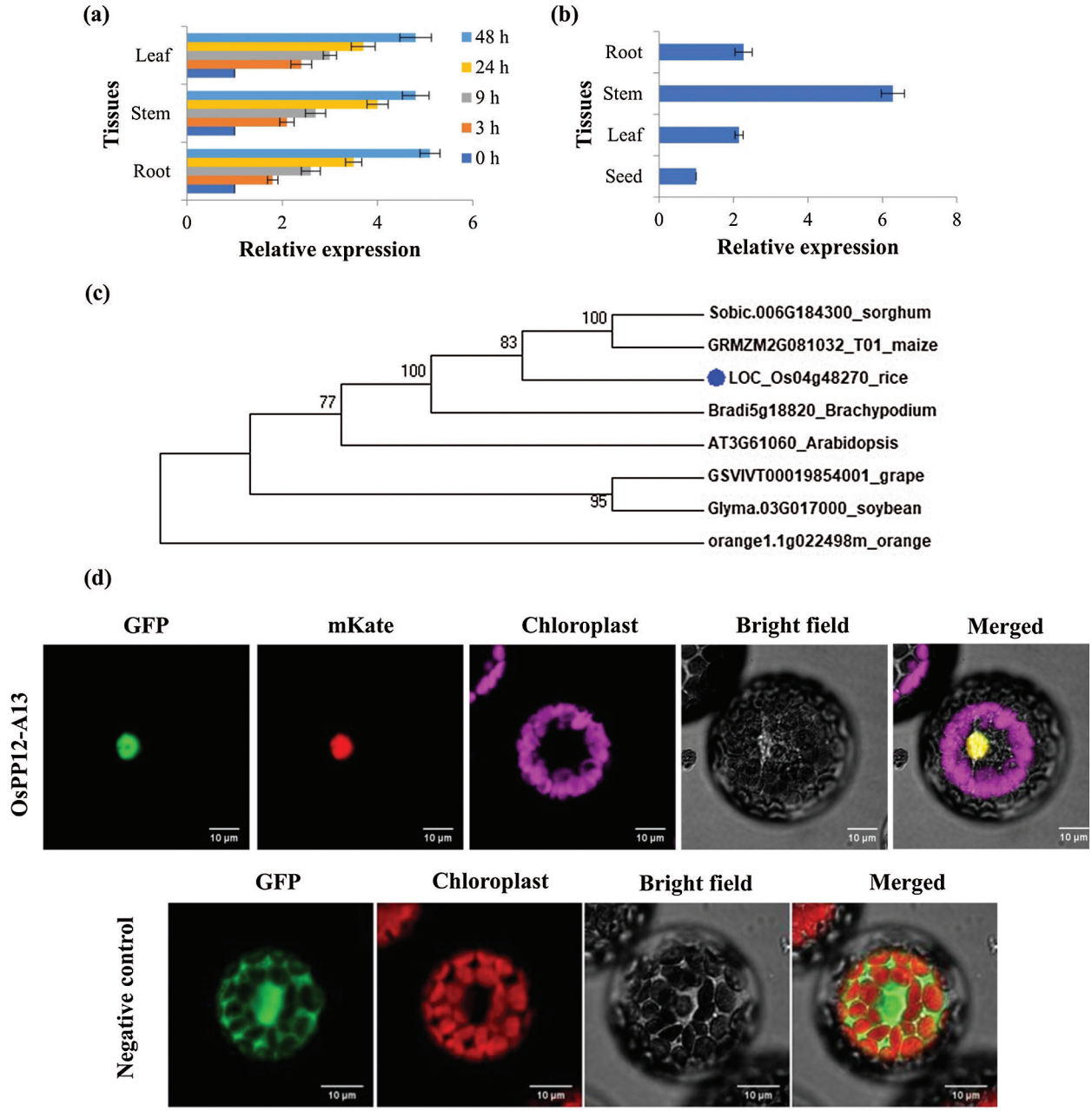

FIGURE 1. Expression pattern and subcellular localization of *OsPP2-A13*.
(a) Relative expression of *OsPP2-A13* under different time courses salt treatment in leaf, stem, and roots of rice. (b) Relative expression of *OsPP2-A13* in different tissues of rice. (c) Phylogenetic tree from amino acid sequences of *OsPP2-A13* and its orthologs in different species (*Sorghum bicolor* (sorghum), *Zea mays* (maize), *Brachypodium distachyon* (Brachypodium), *Arabidopsis thaliana* (*Arabidopsis*), *Vitis vinifera* (grape), *Glycine max* (soybean) and *Citrus sinensis* (orange)); gene codes are from NCBI. (d) Confocal microscopy images for subcellular localization of OsPP2-A13 protein. *Arabidopsis* protoplasts were co-transformed with constructs containing *OsPP2-A13*-GFP and mKate (upper row) or GFP alone (lower row).

under gene locus *LOC_Os04g48270*. The *OsPP12-A13* gene is located on chromosome 4 of rice with a nucleotide length of 3362-bp and CDS of 915 bp. The gene structure analysis showed that it has 3 exons and 2 introns (Suppl. Fig. S1a). The *OsPP12-A13* encodes a protein of 305 amino acids having an F-box domain at the N terminal and a large phloem protein 2 (PP2) domain near the C-terminal (Suppl. Fig. S1b). The molecular weight of the protein is 33.7 kDa, and the predicted isoelectric point (pI) is 7.18. To study the evolutionary relationship of OsPP12-A13 with its orthologs from other species, we retrieved the amino acid sequences of its orthologs from different species, including monocots and dicots. Phylogenetic analysis of protein sequences indicated that all orthologs are evolutionarily related to each other, and rice OsPP12-A13 was closer to monocot members (maize, sorghum, and brachypodium) as expected (Fig. 1c). The dicots were also closer to each other, with grape and soybean clustered into one branch while the orange F-box ortholog was in a clearly separate clade, which implies a high sequence variation in orange (Fig. 1c).

To study the subcellular localization of OsPP12-A13 in rice, we first predicted its location using available bioinformatics tools. WoLF PSORT server (www.genscript.com/tools/wolf-psort) predicted that OsPP12-A13 is

localized in the nucleus similar to its ortholog *AT3G61060* in *Arabidopsis thaliana*. For the experimental validation of this result, we fused the *OsPP12-A13* gene with green fluorescent protein (GFP) and transformed it into *Arabidopsis* protoplasts. We also transformed a vector containing mKate protein into these protoplasts. Co-localization of GFP and mKate signals under the confocal laser microscope confirmed that OsPP12-A13 is a nuclear-localized protein (Fig. 1d). For the negative control, we transformed an empty vector into *Arabidopsis* protoplasts, which showed GFP signals not only in the nucleus but also in the cytoplasm and other organelles. These results confirm that OsPP12-A13 is a nuclear-localized protein.

OsPP12-A13 increases germination percentage and root length of seedlings in transgenic Arabidopsis plants under salt stress

Germination percentage and root length are the key traits related to salinity tolerance in plants (Zafar *et al*., 2015). To see if *OsPP12-A13* can improve the germination percentage and root length, we fused *OsPP12-A13* under CamV35S promoter and developed tissue constitutive overexpression lines for model plant *Arabidopsis thaliana* (Suppl. Fig. S2a). For the negative control, we transformed a vector containing only the CamV35S promoter without the *OsPP12-A13* gene. We obtained nine positive homozygous F3 lines for CamV35S–*OsPP12-A13 plants* and 15 lines for CamV35S plants. Four positive overexpressing lines were selected after qRT-PCR analysis for stress treatment and other physio-molecular assays (Suppl. Fig. S3). Results demonstrate that germination percentage was significantly reduced to 40% under salinity stress of 200 mM NaCl in MS medium for control plants (35s_VC, plants transformed with a vector containing the CamV35S promoter but not the *OsPP12-A13* gene). However, constitutive overexpression of *OsPP12-A13* increased germination percentage up to 90% under salt stress in transgenic *Arabidopsis* seedlings (Fig. 2a). *OsPP12-A13* has been described as a phloem protein. To see if *OsPP12-A13* works mainly in phloem tissues, we further developed phloem specific over-expression plants using the pSUC2 promoter (Truernit and Sauer, 1995; Wippel and Sauer, 2012) fused with *OsPP12-A13* (Suppl. Fig. S2b). We obtained 17 positive homozygous F3 overexpressing lines for pSUC2–*OsPP12-A13* plants and 21 lines for *pSUC2* plants. Four positive overexpressing lines from both vectors were selected after qRT-PCR analysis for further experimental evaluation (Suppl. Fig. S3). The transgenic overexpressed plants (pSUC2–*OsPP12-A13)* also improved the germination percentage up to 90% compared to control plants (pSUC2_VC) having only pSUC2 promoter but not *OsPP12-A13* (Fig. 2a).

To observe the effects of these overexpression constructs on root length, we measured root length of 10 days old seedlings grown in MS medium with and without 200 mM NaCl, respectively. *OsPP12-A13* overexpressing plants from positive lines (CamV35S-*OsPP12-A13* and pSUC2-*OsPP12-A13)* significantly increased root length up to 2 folds under salt stress compared with controls lines (CamV35S_VC and pSUC2_VC) (Figs. 2b, 2c). These results demonstrate that *OsPP12-A13* improves salt tolerance via regulating root length and improved germination percentage.

OsPP12-A13 increases transgenic plants survival and seed yield under salt stress

To observe the role of *OsPP12-A13* in salt tolerance at later growth stages, we evaluated plant survival, plant dry biomass, and seed yield per plant by growing transgenic overexpression plants in soil under control and salt stress (250 mM NaCl) at the reproductive stage. Under salt stress, only less than 20% of plants survived for either of the control plants (CamV35S_VC or pSUC2_VC) (Fig. 3a). However, both the CamV35S-*OsPP12-A13* and pSUC2-*OsPP12-A13* significantly increased the rate of plant survival up to 90% under salt stress (Figs. 3a and 3d). Plant dry biomass is also a good indicator of salt tolerance. The plant dry biomass was significantly reduced under salt stress in control plants (CamV35S_VC and pSUC2_VC) (Fig. 3b). But both the constitutive and phloem specific overexpression of *OsPP12-A13* significantly increased plant dry biomass under salt stress compared with controls (Fig. 3b). Seed yield is the ultimate objective and the most important trait for grain crops, including rice (Chun *et al*., 2020). We observed a significant increase in seed yield per plant under salt stress in transgenic *Arabidopsis* plants overexpressing *OsPP12-A13* compared to their vector controls CamV35S_VC and pSUC2_VC (Fig. 3c). Based on these results, we conclude that *OsPP12-A13* positively regulates plant survival, plant biomass, and seed yield under salt stress. Furthermore, the similar responses of constitutive and phloem specific overexpressing plants further support our conclusions that *OsPP12-A13* works mainly in phloem tissues.

OsPP12-A13 regulates salinity tolerance by affecting Na^+ transport

Plants absorb salts mainly Na^+ ions from roots and translocate them into aerial parts mainly leaf and stem. Under conditions of high salinity in the soil, these salts are accumulated in higher concentrations in leaf cells, which are toxic for cells and leads to cell death (Munns and Tester, 2008). Thus, protecting Na^+ accumulation in leaf cells is a mechanism of salinity tolerance by plants (Munns and Tester, 2008; Roy *et al*., 2013; Byrt *et al*., 2014). To see if *OsPP12-A13* may be involved in regulating salinity tolerance by affecting Na^+ transport, we measured the Na^+ concentration of leaf, stem, and root tissues under control and salt stress conditions. Our results showed that Na^+ concentration was significantly higher in leaf and stem tissues in control vectors under salt stress; however, Na^+ concentration in these tissues was significantly less in *OsPP12-A13* overexpressing plants as compared to control vectors CamV35S_VC and pSUC2_VC (Figs. 4a, 4b). We then measured Na^+ concentration in root tissues, which indicated a significantly higher Na^+ concentration in *OsPP12-A13* overexpressing plants as compared to vector control plants CamV35S_VC and pSUC2_VC under salt stress (Fig. 4c). These results demonstrate that *OsPP12-A13* regulates salinity tolerance in *Arabidopsis*, probably by limiting Na^+ transport from root to leaf cells.

Electrolyte leakage is an indicator of cell membrane stability, which is used as an important trait for stress tolerance (Elbasyoni *et al*., 2017; Zafar *et al*., 2020a). To find the role of *OsPP12-A13* in maintaining cell membrane stability, we measured relative electrolyte leakage from leaf tissues under salt stress. We observed a strikingly higher

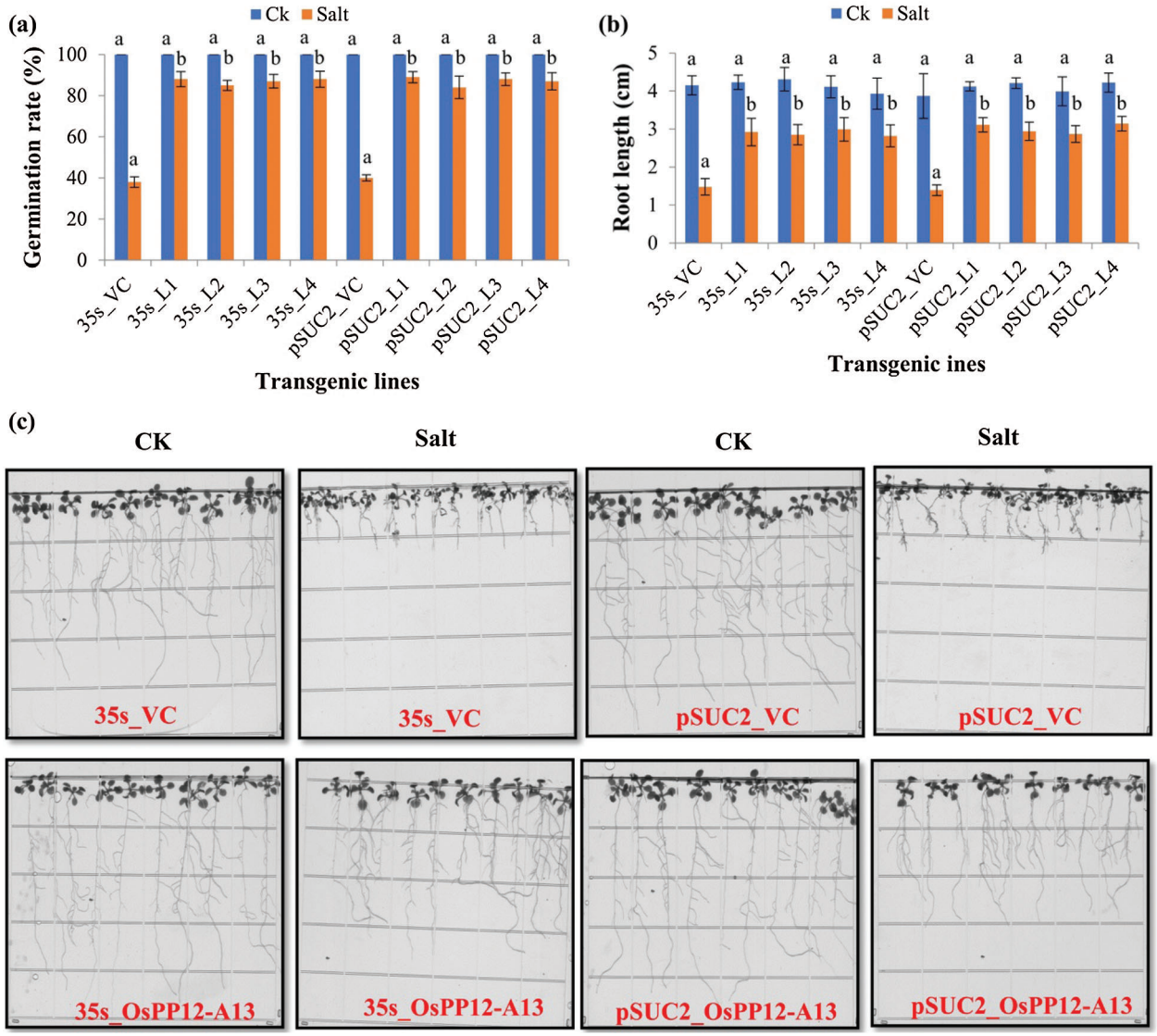

FIGURE 2. Overexpression of *OsPP2-A13* improves germination and root length in transgenic *Arabidopsis* plants under salt stress. (a) Statistical analysis for the quantitative data of germination percentage in transgenic *Arabidopsis* overexpression plants and vector control plants. N = 50 seeds for each line. (b) Statistical analysis for the quantitative data of root length in transgenic *Arabidopsis* overexpression plants and vector control plants. N = 30 plants for each line. Statistically significant values ($p < 0.05$) are shown using different letters (a and b) in bar diagrams with one-way ANOVA following Tukey's test. Error bars represent standard error. (c) Phenotypic analysis of root length of transgenic *Arabidopsis* seedlings grown on MS medium with (Salt) and without (CK) 200 mM NaCl.

electrolyte leakage under salt stress in the control plants CamV35S_VC and pSUC2_VC, showing higher damage to cell membrane under stress (Fig. 4d). Nevertheless, the electrolyte leakage was significantly less in transgenic plants overexpressing *OsPP12-A13* under both the constitutive and phloem specific promoters, suggesting higher cell membrane stability under salt stress to avoid tissue damage (Fig. 4d).

OsPP12-A13 maintains ROS homeostasis under salt stress

Abiotic stresses often lead to oxidative stress caused by the overaccumulation of ROS (Zafar *et al.*, 2018b; Zafar *et al.*, 2020a). To examine whether *OsPP12-A13* affects the ROS levels, we measured cellular H_2O_2 (hydrogen peroxide, the most stable ROS species). Our results demonstrate that H_2O_2 level was significantly increased under salt stress in the control plants (CamV35S_VC and pSUC2_VC); however, it was considerably low in transgenic plants overexpressing *OsPP12-A13* under both CamV35S and pSUC2 promoters (Fig. 5a). ROS usually leads to membrane lipid peroxidation, which is measured in terms of malondialdehyde (MDA) (Zafar *et al.*, 2020b). Measurement of MDA contents showed that MDA level was significantly higher under salt stress in the control plants (CamV35S_VC and pSUC2_VC) (Fig. 5b). In contrast, we observed significantly less MDA contents in transgenic plants overexpressing *OsPP12-A13* under CamV35S as well as pSUC2 promoters (Fig. 5b), suggesting that *OsPP12-A13* protects plants from oxidative damage by keeping lower ROS levels.

Enzymatic antioxidants such as CAT, SOD, POD, and GST serve as important ROS scavengers in plant and animal tissues (Di Meo *et al.*, 2019; Zafar *et al.*, 2020a). We thus estimated the activities of these antioxidant enzymes to understand the mechanism of ROS scavenging and balance in transgenic plants. According to our results, activities of CAT and SOD were significantly enhanced under salt stress in both the control vectors as well as transgenic plants

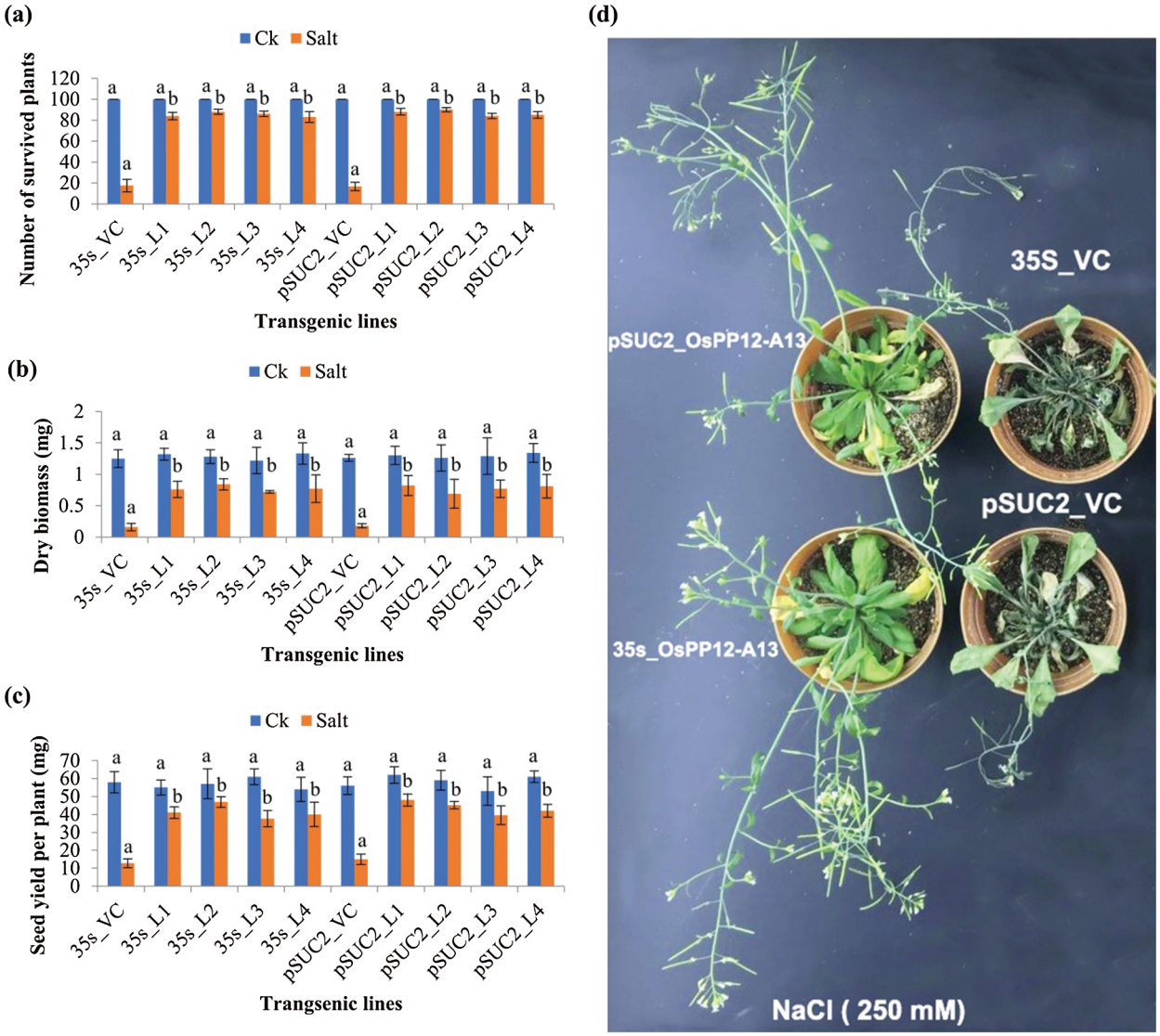

FIGURE 3. Assay for survival rate and seed yield in transgenic *Arabidopsis* plants under salt stress.
(a–c) Statistical analysis for the quantitative data of the number of survived plants, n = 100 plants for each line (a), plant dry biomass, n = 15 plants for each line (b) and seed yield per plant, n = 15 plants for each line (c) from transgenic *Arabidopsis* overexpression plants and vector control plants grown in soil with and without NaCl stress of 250 mM. Statistically significant values ($P < 0.05$) are shown using different letters (a and b) in bar diagrams with one-way ANOVA following Tukey's test. Error bars represent standard error. (d) Phenotypic observation for plant survival of transgenic *Arabidopsis* seedlings grown in soil with NaCl stress of 250 mM.

overexpressing *OsPP12-A13* (Figs. 5c, 5d). Since the activities were increased irrespective of the *OsPP12-A13* gene, this suggests that *OsPP12-A13* has no role in the increased activities of CAT and SOD, perhaps it could be due to compensatory response by the plant under stress. On the other hand, activities of POD and GST were significantly increased in the transgenic plants overexpressing *OsPP12-A13* under CamV35S as well as pSUC2 promoters, but not significantly high in the control plants (Figs. 5e, 5f). These results suggest that *OsPP12-A13* maintains ROS homeostasis in the plant under salt stress by regulating activities of POD and GST specifically and protect tissues from oxidative damage.

OsPP12-A13 regulates ROS homeostasis by modulating the expression of salt and stress responsive genes

To get insight into the underlying molecular mechanism of *OsPP12-A13* mediated salt stress tolerance, we investigated the expression of stress-responsive and antioxidant-related genes in *Arabidopsis* leaf (Martínez-Atienza *et al.*, 2007; Cheng *et al.*, 2019; Zafar *et al.*, 2020a). The Salt Overly Sensitive (SOS) pathway plays a key role in maintaining ion homeostasis in cells and contributes significantly to salt tolerance (Cheng *et al.*, 2019). Since SOS genes positively regulate salt tolerance, we studied their expression in response to salt stress in transgenic plants overexpressing *OsPP12-A13* compared to control plants. We observed around 1.5–2.0- fold upregulation in the expression of *AtSOS1* and *AtSOS2* genes compared to control plants under salt stress (Fig. 6). *AtNHX1* and *AtHNX2* genes play important roles in vacuolar compartmentalization of Na^+, which is an important mechanism of salt tolerance (Yokoi *et al.*, 2002). We observed 2.5–3.6-fold upregulation in the expression of *AtNHX1* and *AtHNX2* genes compared to control plants under salt stress (Fig. 6). *Arabidopsis thaliana high-affinity potassium transporter 1* (*AtHKT1*) regulates salinity tolerance by limiting the transport of Na^+ from

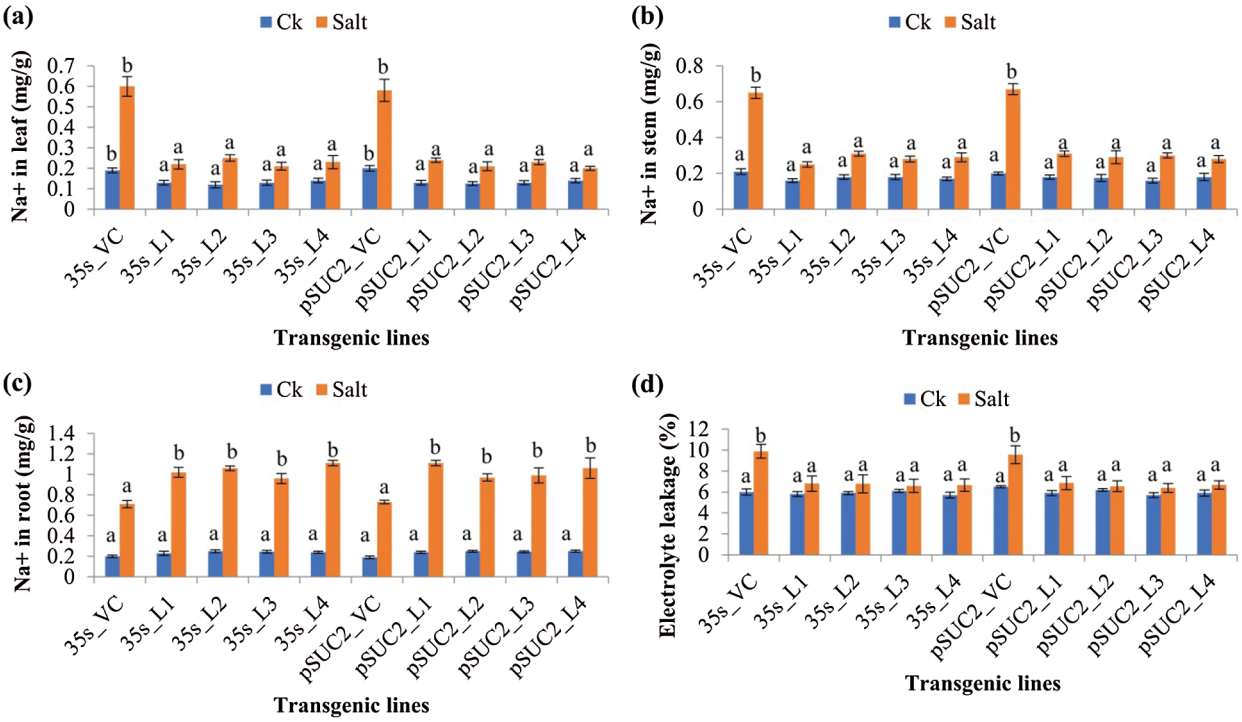

FIGURE 4. *OsPP2-A13* affects Na^+ transport in transgenic *Arabidopsis* plants under salt stress.
Statistical analysis for the quantitative data of Na^+ concentration in leaves (a) stem (b) and roots (c), and electrolyte leakage (d) from transgenic *Arabidopsis* overexpression plants and vector control plants grown in soil with and without NaCl stress of 250 mM. Statistically significant values ($p < 0.05$) are shown using different letters (a and b) in bar diagrams with one-way ANOVA following Tukey's test. Error bars represent standard error.

roots to shoots (Rus *et al.*, 2001; An *et al.*, 2017). We observed more than 3 folds upregulation in the expression of *AtHKT1* in transgenic plants overexpressing *OsPP12-A13* compared to control plants under salt stress (Fig. 6). This suggests that *OsPP12-A13* affects the expression of *AtHKT1* and thereby maintain low leaf Na^+ concentration in transgenic plants.

Next, we studied the expression levels of antioxidant-related genes. We observed a slight upregulation in the expression levels of *AtSOD1*, *AtSOD2*, *AtCAT1*, and *AtCAT3* genes in transgenic plants overexpressing *OsPP12-A13* compared to control plants under salt stress (Fig. 6). Unlikely, we observed a moderate upregulation in the expression of *AtPOD1* and *AtPOD2* in overexpressing plants compared to control plants under salt stress (Fig. 6). Notably, we observed a significant upregulation of up to 5 folds in the expression of *ATGST1* and *ATGST2* genes under salt stress in the transgenic plants overexpressing *OsPP12-A13* compared to control plants (Fig. 6). These gene expression profiles were almost consistent with the recorded antioxidant activities (Fig. 5) and suggest that *OsPP12-A13* regulate the expression of *AtGST1* and *AtGST2* genes and maintain redox balance in transgenic plants under salt stress.

Discussion

OsPP2-A13 is salt responsive nuclear protein

Environmental stresses such as drought, heat, and salinity pose a serious threat to global crop production (Zafar *et al.*, 2020c). The most sustainable and eco-friendly approach to tackle this challenge is the development of climate-smart varieties (Munns and Tester, 2008; Zafar *et al.*, 2018b). Salinity has been a key challenge for sustainable crop production as it seriously affects crop yield, especially in rice (Shrivastava and Kumar, 2015; Zafar *et al.*, 2015; Abdelgawad *et al.*, 2016). Thus, identification of novel genes regulating salinity tolerance from either wild species or natural genetic variation will provide a useful genetic resource to breed salinity tolerant cultivars in rice (Huang *et al.*, 2008; Rahnama *et al.*, 2011; Quan *et al.*, 2018). F-box genes are a large family with several hundred members in *Arabidopsis* and rice (Xu *et al.*, 2009). Overexpression of an F-box gene *OsMsr9* enhanced salinity tolerance in *Arabidopsis* and rice by increased root and shoot growth, higher production of proline, and less malondialdehyde (MDA) contents (Xu *et al.*, 2014). Similarly, overexpression of another F-box gene, *TaFBA1*, in tobacco enhanced salinity tolerance by regulating antioxidant, reactive oxygen species (ROS) production and Na^+ and K^+ levels in cells (Zhao *et al.*, 2017b). These studies indicated a potential role of F-box genes in salinity tolerance, and thus identification of new F-box genes for their role in salt tolerance would play important role in breeding salt-tolerant rice cultivars. In this study, we have characterized the role of a rice F-box *phloem protein 2-LIKE A13* (*OsPP2-A13*) in salinity tolerance. We first detected that *OsPP2-A13* is highly responsive to salt stress, and its expression was highest in the stem, followed by roots and leaves (Fig. 1). Transformation of *OsPP2-A13* with GFP tag into *Arabidopsis* protoplasts revealed that *OsPP2-A13* is localized to the nucleus, similar to its *Arabidopsis* ortholog *AT3G61060*. Since transcription factors are known to regulate the expression of stress-responsive

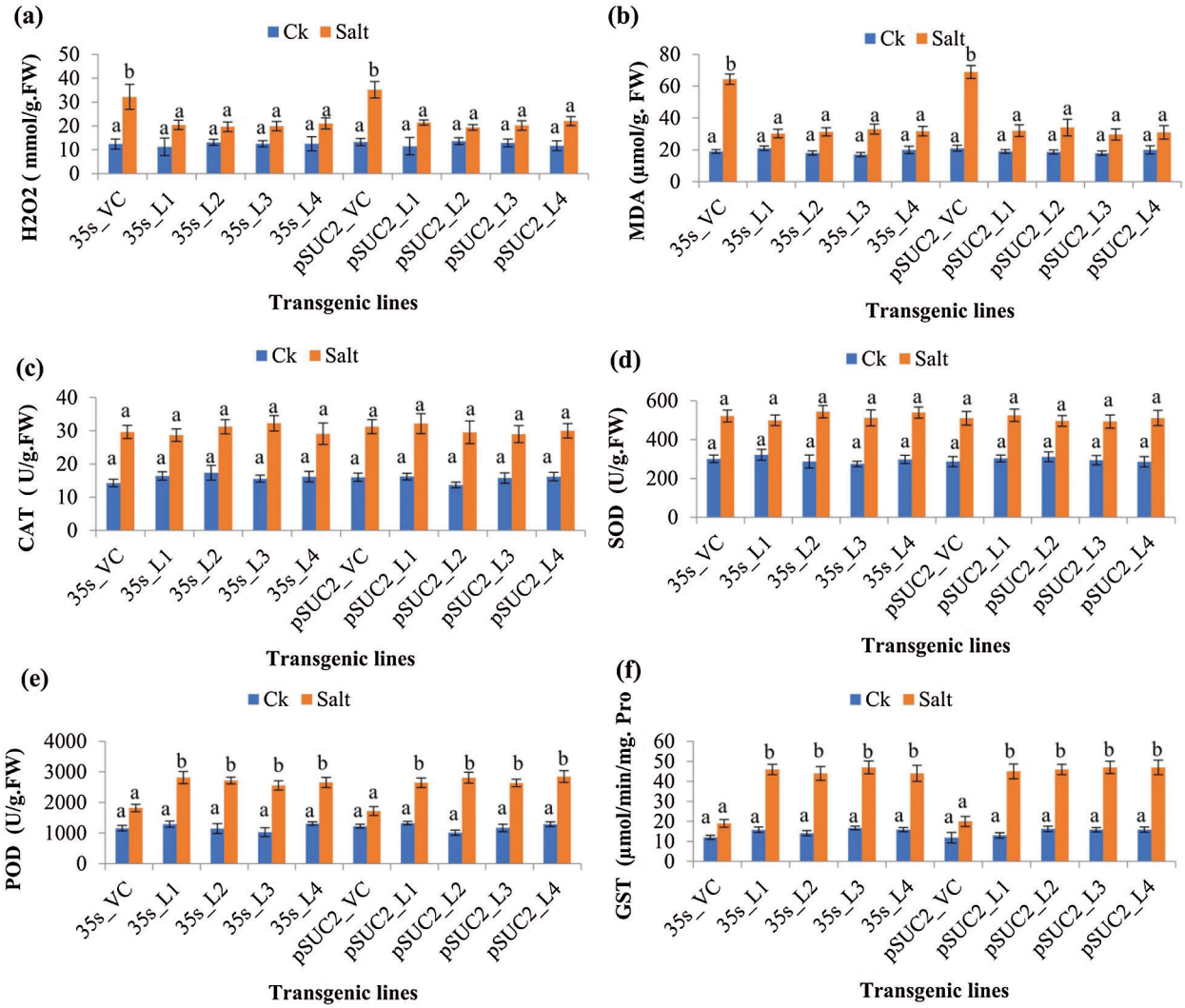

FIGURE 5. Analysis of ROS and antioxidants in transgenic *Arabidopsis* plants under salt stress.
Statistical analysis for the quantitative data of H_2O_2 concentration (a), MDA (b), CAT (c), SOD (d), POD (e), and GST (f) from leaves of transgenic *Arabidopsis* overexpression plants and vector control plants grown in soil with and without NaCl stress of 250 mM. Statistically significant values ($p < 0.105$) are shown using different letters (a and b) in bar diagrams with one-way ANOVA following Tukey's test. Error bars represent standard error.

	CamV35S-OsPP2-A13/35s_VC			pSUC2-OsPP2-A13/pSUC2_VC		
	L1	L2	L3	L1	L2	L3
ATSOS1	2.3	1.9	1.89	2.67	1.86	2.24
ATSOS2	1.4	2.1	1.5	1.67	1.94	2.59
ATNHX1	3.6	2.5	2.9	2.24	1.56	2.35
ATNHX2	2.7	2.8	2.5	2.33	1.77	1.64
ATHKT1	3.4	2.9	3.1	3.25	2.01	2.16
ATSOD1	1.5	1.3	1.02	1.18	0.95	0.77
ATSOD2	1.2	1.4	0.89	1.16	0.74	0.85
ATPOD1	2.33	2.1	1.63	1.465	1.87	1.52
ATPOD2	1.95	2.4	1.75	1.95	1.51	1.76
ATCAT1	0.98	1.23	0.92	0.72	0.89	0.81
ATCAT3	1.23	1.11	0.85	0.83	0.95	1.05
ATGST1	4.5	3.7	3.8	4.79	4.38	4.01
ATGST2	5.1	4.4	4.6	3.95	4.18	3.85

FIGURE 6. Expression fold-change of various Na^+ transport and antioxidant-related genes under salt stress in *Arabidopsis* leaf.
qRT-PCR based expression fold change (transgenic overexpression lines/vector control lines) of key genes involved in Na^+ transport and antioxidant activities in salt stress response. Values indicate the mean of three biological repeats.

genes and are located in the nucleus (Zhang *et al.*, 2013; Li *et al.*, 2017; Ali *et al.*, 2018; Dai, 2019; Zhu *et al.*, 2020), this suggests that *OsPP2-A13* may interact with some key transcription factors and regulate salt-responsive genes to impart salinity tolerance.

OsPP2-A13 regulates salinity tolerance probably via phloem tissues

We showed with a number of evidences that overexpression of *OsPP2-A13* in *Arabidopsis* imparts salt tolerance at both seedling and reproductive stages. We constitutively expressed *OsPP2-A13* under CamV35S promoter in *Arabidopsis* and observed a significantly higher germination percentage and root length in *Arabidopsis* seedlings grown in MS medium with 200 mM NaCl (Fig. 2). Alongside, we observed a significantly higher survival rate, dry biomass, and seed yield per plant in transgenic CamV35S-*OsPP2-A13* plants grown in soil with 250 mM NaCl (Fig. 2). Since root length, survival rate, dry biomass and seed yield are the key traits associated with salinity tolerance (Liu *et al.*, 2013; Farooq *et al.*, 2015; Zafar *et al.*, 2015), we conclude that *OsPP2-A13* improves salinity tolerance in *Arabidopsis*.

Phloem serves as an important medium for salinity tolerance by translocating Na^+ salts from leaves and shoot

(place of higher Na^+ concentration) to the root (place of low Na^+ concentration) (Berthomieu *et al.*, 2003; Kong *et al.*, 2012; Wu, 2018). Phloem played a key role in salinity tolerance in several species, including maize, clover, and sweet pepper (Tester and Davenport, 2003). Since *OsPP2-A13* is a phloem protein gene, we tested if *OsPP2-A13* works mainly via the phloem to improve salinity tolerance. We expressed *OsPP2-A13* under pSUC2 promoter (phloem specific promoter) (Truernit and Sauer, 1995; Wippel and Sauer, 2012) in *Arabidopsis* and evaluated salinity tolerance. We found that pSUC2-*OsPP2-A13* plants showed almost similar results for the recorded traits, which proved that *OsPP2-A13* works mainly via the phloem to regulate salinity tolerance. This suggests that *OsPP2-A13* restricts Na^+ transport from roots to leaves, and phloem plays a central role.

Na^+ transport is an important mechanism of salinity tolerance in plants (Wu, 2018). We therefore investigated if *OsPP2-A13* affects Na^+ transport from roots to leaves. Our results showed that both the CamV35S-*OsPP2-A13* and pSUC2-*OsPP2-A13* plants had significantly reduced Na^+ concentration in leaf and stem tissues under salt stress compared with control plants (Fig. 4). However, Na^+ concentration was considerably higher in roots under salt stress. Since a high concentration of Na^+ in roots is not detrimental to the plant as compared to the leaf and stem (Berthomieu *et al.*, 2003; Kong *et al.*, 2012; Wu, 2018), we speculate that *OsPP2-A13* regulates salinity tolerance mainly by downregulating Na^+ transport from root to leaves. Thus, *OsPP2-A13* could serve as an important functional gene to modulate salinity tolerance in breeding programs.

OsPP2-A13 protects from oxidative damage under salt stress

One of the most common and frequent responses to abiotic stresses is the excessive production of ROS which often leads to detrimental effects. The accumulation of ROS beyond certain levels causes membrane lipid peroxidation, cell death, reduced fertility, and poor seed setting (Abogadallah, 2010; Abdelgawad *et al.*, 2016; Zafar *et al.*, 2020a; Zafar *et al.*, 2020b). Plants protect themselves from oxidative damage by activating stress-responsive genes and antioxidant defense mechanisms (Abogadallah, 2010; Abdelgawad *et al.*, 2016). In this study, although the ROS level was increased under salt stress, ROS level was significantly less in transgenic plants overexpressing *OsPP2-A13* as compared to control plants with normal *OsPP2-A13* expression (Fig. 5). A similar trend was observed for MDA (Fig. 5), which is used as an oxidative stress marker (Zafar *et al.*, 2020a). This indicates that *OsPP2-A13* helps to keep the ROS levels under normal ranges. Plants adapt various defense mechanisms against oxidative stresses (Larson, 1995). Among these, the activation of antioxidant machinery is one of the major defense strategies (Abogadallah, 2010; Gill and Tuteja, 2010). Antioxidant enzymes such as SOD, POD, CAT, and GST are key enzymes that detoxify ROS molecules and protect cellular organelles from ROS damage (Jiang and Yang, 2009; Zafar *et al.*, 2018a). We found a dramatic increase in the activities of POD and GST under salt stress in the transgenic plants expressing *OsPP2-A13* as compared to control plants without *OsPP2-A13* (Fig. 5). This proves that low ROS levels in the transgenic plants expressing *OsPP2-A13* could be attributed to the higher POD and GST activities, which protected the plants from oxidative damage under salt stress (Abogadallah, 2010; Hussain *et al.*, 2019; Zafar *et al.*, 2020a). Since *TaFBA1* (an F-box gene) also controls salinity tolerance by regulating antioxidant and ROS production and Na^+ and K^+ levels in cells, this suggests some common evolution and mechanism of action of these F-box genes (Zhao *et al.*, 2017b).

OsPP2-A13 regulates the expression of Na^+ transport and antioxidant related genes

Our results suggest that *OsPP2-A13* improves salinity tolerance probably by affecting Na^+ transport and antioxidant defense response. To understand the molecular basis of this response, we studied the expression levels of various Na^+ transport and antioxidant-related genes under salt stress. We observed a slight to moderate upregulation of *AtSOS1*, *AtSOS2 (Salt overlay sensitive pathway genes)* *AtNHX1*, *AtHNX2* (Na^+/H^+ antiporter genes) and *AtHKT1 (high-affinity potassium transporter gene)* (Fig. 6). The SOS pathway plays a key role in maintaining ion homeostasis in cells and contributes significantly to salt tolerance (Cheng *et al.*, 2019). Na^+/H^+ antiporter genes play an important role in vacuolar compartmentalization of Na^+, which is an

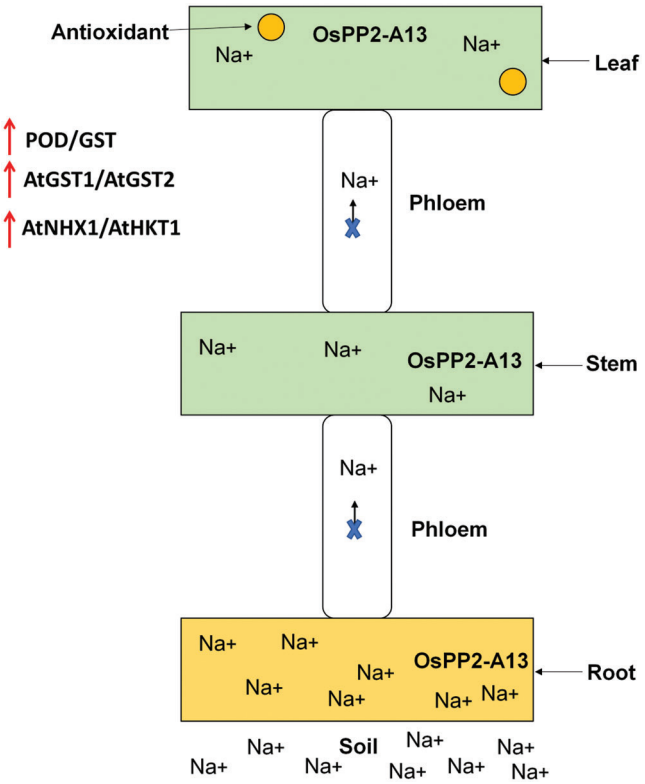

FIGURE 7. Working model of *OsPP2-A13* for Na^+ transport to regulate salinity tolerance.

Under salt stress, plants absorb Na^+ ions from roots and transport them to aerial parts mainly stem and leaves. Overexpression of *OsPP2-A13* limits Na^+ ions transport from root to leaves via phloem tissues. In addition, transgenic plants also induce activities of peroxidase and glutathione S-transferase and expression of antioxidant (*AtGST1* and *AtGST2*) and Na^+ transport (*AtNXH1* and *AtHKT1*) related genes to maintain low ROS level and Na^+ concentration in leaves.

important mechanism of salt tolerance (Yokoi *et al*., 2002). Similarly, *AtHKT1* regulates salinity tolerance by limiting the transport of Na^+ from roots to shoots (Rus *et al*., 2001; An *et al*., 2017). Thus, the increased expression of these genes in *OsPP2-A13* overexpressing plants may be correlated with the Na^+ transport regulation mechanism imparting salt tolerance. Notably, the increased expression of *AtHKT1* further supports the hypothesis that *OsPP2-A13* enhances salinity tolerance by downregulating Na^+ transport from roots to upper plant parts and that *AtHKT1* and *OsPP2-A13* may work in the same pathway of Na^+ transport.

The enzymatic activities are often positively correlated with the expression level of their corresponding genes (Yin *et al*., 2017). To see if increased activities of antioxidant enzymes are correlated with increased transcripts levels of corresponding genes (Das *et al*., 2019; Zafar *et al*., 2020a), we measured the relative mRNA abundance of CAT, SOD, POD, and GST related genes in *Arabidopsis*. We found that the expression of *AtSOD1*, *AtSOD2*, *AtCAT1*, and *AtCAT3* increased slightly, *AtPOD1* and *AtPOD2* increased moderately; however, *ATGST1* and *ATGST2* showed a 5-fold increase in the expression under salt stress in transgenic plants expressing *OsPP2-A13*. The relative transcript levels were consistent with the observed antioxidant activities, as observed previously under salt and other abiotic stresses in rice (Das *et al*., 2019; Zafar *et al*., 2020a), suggesting that the enzymatic activities have a moderate positive correlation with the expression levels of their corresponding genes. These results suggest that *AtNHX1, AtHKT1, ATGST1*, and *ATGST2* could be downstream target genes of *OsPP2-A13*, which could be further validated using yeast two-hybrid, pull-down, or split luciferase assays. Taken together, our study describes an important role of a rice F-box gene in enhancing salt tolerance in *Arabidopsis via* modulating multiple traits.

Conclusions

In summary, this study demonstrated the important role of rice F-box phloem protein *OsPP2-A13* in regulating salinity tolerance in transgenic *Arabidopsis* plants (Fig. 7). We showed that OsPP2-A13 is a nuclear-localized protein that functions mainly via the phloem, probably by affecting Na^+ transport from root to leaves. Lastly, we showed that *OsPP2-A13* protect plants from oxidative stress by maintaining higher antioxidant activities and regulating the expression of *AtGST1* and *AtGST2* genes. *OsPP12-A13* knock-out or RNAi experiments are needed directly in rice in order to have a complete understanding of the role of this gene in salt tolerance. Further analysis to study allelic variation and development of molecular markers linked to this gene may facilitate rapid screening of salt-tolerant rice germplasm in breeding programs.

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Availability of Data and Materials: Data supporting the results described in this manuscript are available in the text and its supplementary files.

Authors' Contributions: Chunkun Fan: Design, experimental execution, paper writing; Yongpeng Zhang, Chunbao Yang, Yawei Tang, Ji Qu, Bu Jie, Deji Quzhen: Experimental execution, validation, and paper revision; Liyun Gao: Design, supervision, funding acquisition, paper revision. All authors have read and approved the final version of the manuscript.

Ethics Approval: Not applicable.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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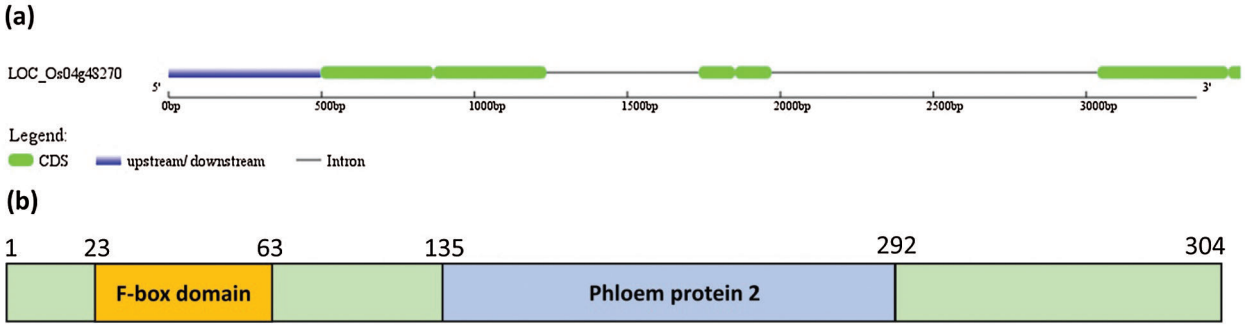
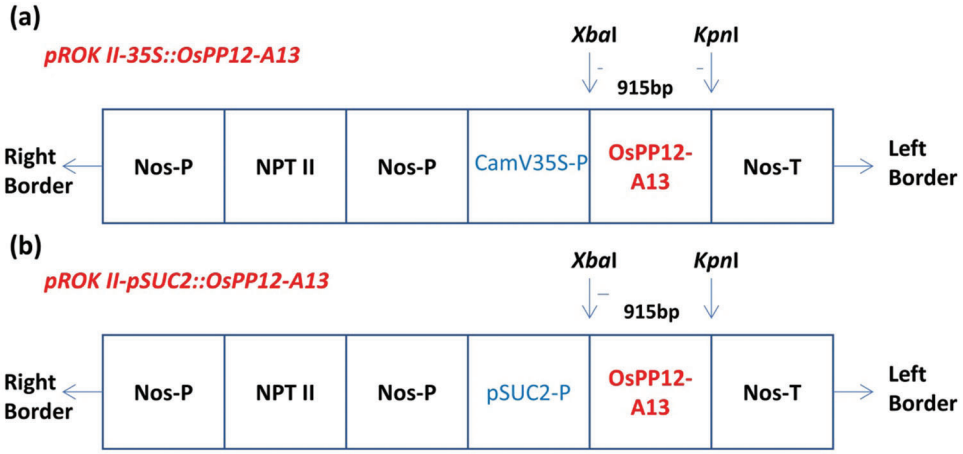
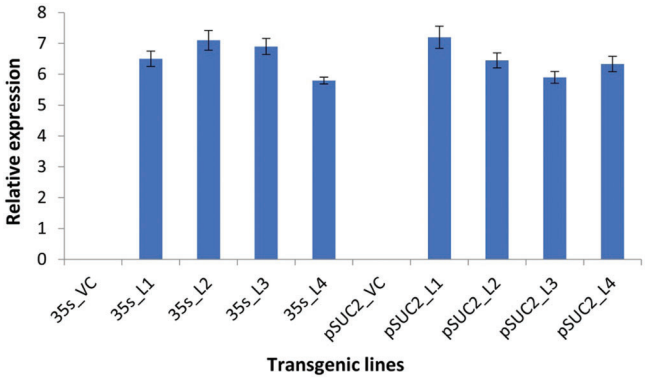


SUPPLEMENTARY FIGURE S1. Domain analysis and gene structure of *OsPP2-A13*.



SUPPLEMENTARY FIGURE S2. Map of vectors used for *OsPP2-A13* overexpression.



SUPPLEMENTARY FIGURE S3. qRT-PCR analysis of overexpression in transgenic *OsPP2-A13* lines.

SUPPLEMENTARY TABLE 1

List of primer sequences used for qRT-PCR analysis

Gene	GenBank accession number	Forward primers (5'-3')	Reverse primers (5'-3')
OsACTIN	LOC4333919	CTTCATAGGAATGGAA	CGACCACCTTGATCTT
OsPP12-A13	LOC_Os04g48270	AGCAGGGAAGCAGAAA	GAGAAGAACAAGGGTGGT
ATACTIN2	AT3G18780	TCCTGCTCGTAGTCAA	CTCCCGCTATGTATGT
ATSOS1	AT2G01980	CTTGGATCTCTCGAATATG	GGAAACGTGACCTTCACAAG
ATSOS2	AT5G35410	GATAGAATTGTTCATAAAGGG	GGAGTTCCACATGTGGTACGC
ATNHX1	AT5G27150	CTTTAGTGAAGATCTTTTC	GAATCTGTTGCAGCAAATATG
ATNHX2	AT3G05030	GAGGGAAAAACTCACATCTC	CGCCCAAGTCAAAGGTCC
ATHKT1	AT4G10310	ATCTGGCTCCTAATCCCTCAA	CCGTCACTCCAAGAAGAACAC
ATSOD1	AT1G08830	GATGGTAAAACACACGGTGC	GCCAGGCTGAGTTCATGGCCTC
ATSOD2	AT1G12520	GTCACCCGGAACCCACAGC	CCGAATAAAAGGCCTCTCC
ATPOD1	AT1G24110	TCTGACCGTTCAAGAAATGG	TGGAGCAACCCGTAACCGTG
ATPOD2	AT2G18140	TCCGGGAGCCACACCATTGG	TGGTCGGAATTCAACAGTC
ATCAT1	AT1G20630	ACGGACGAAGAATACA	CCAGTGCTAAGGGTTT
ATCAT3	AT1G20620	GAAACGGACAATAACC	CTCTTCCCTCACCATC
ATGST1	AT5G41210	GCTCGGGTTCGGTAAA	AAAAGCAACAATGCCTCA
ATGST2	AT2G29480	GCCCGAGCAAGACAAA	TCTCAAACTTAAAGGCGTAC