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Overexpression of a rice phospholipase C gene (OsPLC1) in Arabidopsis thaliana improves salinity stress tolerance

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Abstract

Phospholipase C (PLC) in plants is divided into two families, phosphatidylinositol-specific phospholipase C (PI-PLC) and non-specific PLC (NPC), according to their substrate usage. The importance of PI-PLCin the plant's response to various environmental stresses has been documentedin several studies. In the present study, we generated transgenic Arabidopsis plants with constitutive expression of OsPLC1, a member of the PI-PLCs, which has been reported to play a key role in the response to salt stress of rice. Two independent transgenic Arabidopsis lines, OE-5 and OE-6, showed tolerance to salt stress compared to wild-type (WT) plants. Overexpression of *OsPLC1*led to reduced NaCl sensitivity during seed germination and early seedling development of Arabidopsis, as compared to WT plants. Expressions of a subset of salt stress-related genes were altered in OE lines. The expressions and the enzyme activities of SOD (superoxide dismutase), CAT (catalase), andAPX (ascorbate peroxidase) were significantly increased in both two OE lines. Besides,the overexpression lines exhibited higher proline, soluble sugars, and soluble proteins, leading to lower reactive oxygen species (ROS) levels under salt stress compared with those of WT plants. Additionally, we found that OsPLC1 improved the water retention ability of leaves in Arabidopsis under dehydration conditions. Taken together, our results indicated that OsPLC1 can enhance tolerance to salt stress in Arabidopsis by regulating ROS signaling cascades.

Key message

Overexpression of rice enhances salt tolerance in transgenic Arabidopsis by modulating ROS signaling, elevating antioxidant enzyme activities, and accumulating osmotic protectants, thereby reducing oxidative damage and improving water retention.

Introduction

Plants frequently encounter adverse environmental conditions, such as drought, flooding, extreme temperatures, and soil salinity, which can severely limit plant growth and development, and greatly reduce the quality and quality of crops (Gong et al. 2020; Hirayama and Shinozaki 2010; Nadarajah 2020). Among them, soil salinity is a major global problem limiting land usage and crop production. There is an estimated one-fifth of the irrigated lands in the world are affected by salinity (Morton et al. 2019). To alleviate the potentially harmful damage of such stresses, plants have evolved various tiers of physiological, biochemical, molecular, and cellular processes that represent avoidance or adaptive mechanisms.

Homeostasis of ROS, such as hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-), in the cell, is achieved through a balance between its production and scavenging in unstressed conditions (Gill and Tuteja 2010; Halliwell 2006; Hasanuzzaman et al. 2021). When subjected to a stressful environment, excessive production and accumulation of ROS in plant cells would lead to oxidative damage (Pucciariello and Perata 2016; Yu et al. 2021; Gechev and Petrov 2020). For example, the intracellular macromolecules (such as DNA and proteins) would be destroyed, and the lipid peroxidation reaction of

cytomembrane activated, causing damage to membrane functions and photosynthesis inhibition, leading to plant growth inhibition and even death (Das and Roychoudhury 2014; Gill and Tuteja 2010). Numerous studies have reported that manipulating antioxidant abilities, such as the elevation of ROS scavenging enzyme (SOD, CAT, and APX, etc.) activities and antioxidative substances (proline, soluble proteins and sugars, etc.) levels, is a valuable way to achieving stress tolerance in plants (Mittler 2002; Miller et al. 2008; Nadarajah 2020; Mittler et al. 2022).

Phospholipids are not only important components of cellular bio-membranes but also act as signaling compounds in salt stress response (van Zelm et al. 2020; Han and Yang 2020). The function of phospholipids such as phosphatidic acid (PA) and phosphoinositides (PIs) in the salt stress response mechanism has been studied extensively, mainly by analyzing the regulatory function of proteins involved in salt stress signal transduction (Han and Yang 2020). For example, PA and phosphatidylinositol 4,5-bisphosphate (PIP_2) levels rapidly increased within 30 min of salt treatment using the ^{32}P labeling technique (Darwish et al. 2009). Similarly, a recent study revealed that PA accumulates rapidly in the Arabidopsis root within 10 min of salt treatment by PA-specific biosensor, indicating the regulatory role of PA in the related signaling (Li et al. 2019). PI can bind the C-terminus of PM (plasma membrane) H^+-ATPase and inhibit its activity under normal conditions. However, salt stress does induce the turnover of PI to PI_4P (phosphatidylinositol 4-phosphate), which in turn stimulates SOS1 (Salt Overly Sensitive 1) activity to exclude excess Na^+ from the cell (Yang et al. 2021).

PI-PLC hydrolyses PIs to generate two potent cellular messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG), whereby IP_3 increases free cytoplasmic Ca^{2+} in cytoplasm DAG that interacts with and activates protein kinase C (PKC) and other proteins (Wang et al. 2001; Nakamura et al. 2005; Yu et al. 2021). In plants, increasing studies indicate that PI-PLC members have participated in multifarious cellular processes such as cell proliferation, differentiation, migration, and fate decisions. Nine PI-PLC family members (named AtPLC1-9) have been predicted in the Arabidopsis genome (Mueller-Roeber and Pical 2002; Tasma et al. 2008). Among them, only *AtPLC1-5* and *AtPLC9* have been investigated to encode functional PI-PLC enzymes and play important biological functions in previous studies. For example, AtPLC1 is needed for the secondary responses to ABA, and the increasing activity of AtPLC1 is essential for maximal gene induction by ABA (Sanchez et al. 2001). The loss function of AtPLC2 causes infertility and disruption of root development (Li et al. 2015; Chen et al. 2019). When compared to WT plants, the lateral root densities were significantly reduced in *AtPLC3* knock-down mutants (Zhang et al. 2018). AtPLC4 negatively regulates the salt tolerance of Arabidopsis seedlings by Ca^{2+} signaling pathway (Xia et al. 2017). Overexpression of *AtPLC5* triggers senescence in Arabidopsis leaves (Zhang et al. 2015). AtPLC9 is involved in thermotolerance and HS (heat shock) signal transduction in Arabidopsis (Zheng et al. 2012). Four members of PI-PLCs have been identified in the rice genome (Singh et al. 2013), and the roles of *OsPLC1*, *OsPLC3*, and *OsPLC4* in the rice responding to osmotic stresses have been demonstrated in previous studies (Li et al. 2017; Yu et al. 2021; Deng et al. 2019). For instance, after NaCl treatment, OsPLC1 and OsPLC4 can enhance salt tolerance by activating the IP_3 (inositol triphosphate)-Ca^{2+}-mediated signaling (Li et al. 2017; Deng et al. 2019). However, the

overaccumulation of ROS in *OsPLC3*-OE plants leads to high sensitivity to osmotic stress (Yu et al. 2021).

In this study, we aimed to determine similarities in the transduction of salt stress signals through PLC in rice and Arabidopsis, and to determine whether OsPLC1 can enhance the salinity stress tolerance in dicotyledonous Arabidopsis plants. Our results suggest that OsPLC1 may enhance salt tolerance in Arabidopsis via modulating ROS homeostasis. OsPLC1 conferred salt resistance both in monocots and dicots and these results implied that important elements of the salt response are conserved between monocots and dicots. Our findings suggest that OsPLC1 is an important and valuable resource for improving salt resistance in molecular breeding programs. Furthermore, this study highlights the potential for heterologous transformation to assist in molecular breeding.

Materials and methods

Plant materials and growth conditions

Rice seeds (*Oryza sativa* L. japonica cv. nipponbare (Nip)) were surface sterilized by 5% sodium hypochlorite and germinated on half-strength Murashige and Skoog (1/2 MS) agar medium containing 3% sucrose (w/v) at 28 ◦C.

All Arabidopsis plants used in this study were in the Columbia-0 (Col-0) accession. Seeds were surface sterilized in 20% NaClO (v/v) for 3 min, then washed three times in sterile water before being sown on 1/2 MS plates containing 1% sucrose (w/v) and 0.7% agar (w/v) at pH 5.8. For stratification, the plates were placed in the dark for 2 days at 4 ◦C. All seedlings were grown under the same conditions (16 h light/8 h dark at 22 ◦C).

Analysis of gene expressions

The expression levels of genes were examined in the experiments of qRT-PCR, by following the protocol reported previously (Yu et al. 2021; Yu et al. 2022). Concisely, total RNA was isolated with TRIzol reagent (Invitrogen, USA). RNA samples were amplified with the CFX connect real-time PCR detection system 185–5201 (Bio-Rad, USA), and the relative gene expression levels were then analyzed using the CFX manager software (Bio-Rad, USA). Three biological replicates with two technical replicates of each sample were used for qRT-PCR analysis and the transcript levels relative to *OsACTIN*2 (*Os10g0510000*) in rice or *AtActin2* (*At3g18780*) in Arabidopsis. The primers used for qRT-PCR analysis are listed in Supplementary Table 1.

Generation of transgenic Arabidopsis plants overexpressing OsPLC1

To generate the *OsPLC1*-overexpressing transgenic lines, the plasmid pBA002-OsPLC1 was constructed, by following the method reported in our previous studies (Dong et al. 2021; Yu et al. 2021). The full-

length coding sequence (CDS) of OsPLC1 (1797 bp) was amplified from cDNA of Nip plants using OsPLC1-F and OsPLC1-R primers (Supplementary Table S1), and was then cloned into the binary vector pBA002 using EcoRI/XhoI restriction sites.

For plant transformation, plasmid was first introduced into Agrobacterium (*Agrobacterium tumefaciens*) strain GV3101 and then transformed into three weeks Col-0 plants using the floral dip method (Clough and Bent 1998). Basta-resistant T0 seedlings of Arabidopsis expressing the OsPLC1 were selected. Transcript analysis was performed for the T2 transgenic Arabidopsis lines by semi-quantitative PCR and qRT-PCR. Two independent transgenic lines (OE-5 and OE-6) based on transcript analysis were selected for further studies.

Stress tolerance assays

All seeds of Arabidopsis were harvested at the same period and stored in the same condition. To break seed dormancy, seeds were sterilized and incubated at 4°C for 2 days. For assessing seed germination, sterilized seeds were planted on 1/2 MS agar medium containing 3% sucrose supplemented with or without NaCl (150 mM). Then they were placed in a growth chamber with a photoperiod of 16 h light/8 h dark, at 22 ◦C. The germination rate was expressed as a percentage of the total number of seeds plated. A seed was considered as germinated once the radical protruded through the seed coat. The germination rate was scored daily for 7 days.

For root elongation analysis, five-day-old seedlings were grown vertically on 1/2 MS plates before being transferred to 1/2 MS plates containing NaCl (0 and 200 mM) and returned to the growth chamber (16 h light/8 h dark, at 22 ◦C). The root length, relative fresh weight, and chlorophyll content were measured at seven more days (12-day-old seedlings) after vertical growth.

4-week-old plants were used for observing the effects of salt stress in nutrient soil. For salt stress, the culture solution was changed from distilled water to NaCl solution (0 and 200 mM), and the survival rates of each line were then calculated after 7- and 14-day treatment, respectively. After treatment, the contents of chlorophyll and ROS in the leaves of plants were measured.

Histochemical NBT and DAB staining

In situ detection of H_2O_2 by 3′, 3′-diaminobenzidine (DAB) staining and O_2^- Nitroblue Tetrazolium (NBT) staining was performed according to methods previously described (Dong et al. 2021; Jia et al. 2021). The study sampled five fresh leaves per plant from 5 to 7 independent plants. Excised leaves were immersed in 1 mg/L DAB and 0.5 mg/L NBT in the dark at 22 ◦C for about 3 h and 2 h, respectively. The samples were then cleared to remove chlorophyll at 4 ◦C in 80% (v/v) ethanol for 2 h and immersed in 10% (v/v) glycerol for observations.

Determination of physiological parameters

For measuring the relative water content (RWC) and water loss rate (WLR) of plants, fully expanded leaves were detached from plants and weighed for the fresh weight (FW), turgid leaf weight (TW), and dry weight (DW). The WLR and RWC were calculated by following the equations: WLR (%) = (FW-TW)/(FW-DW) × 100% and RWC (%) = (FW-DW)/(TW-DW) × 100% (Yuan et al. 2019; Yu et al. 2021).

For measuring electrolyte leakage, the detached leaves were immediately immersed in the deionized water and vacuumed for 30 min. After incubation with gentle agitation for 2.5 h, the initial conductivity was then measured with a conductivity meter (DDBJ-350, Shanghai, China). Next, the above samples were boiled for 15 min, and the total conductivity was measured after cooling. The relative electrolyte leakage was calculated by following the equations: Relative electrolyte leakage (%) = (initial conductivity) / (total conductivity) × 100% (Wang et al. 2014).

For the ROS quantification assay, the H_2O_2 content was detected following a previously reported protocol described by Noreen and Ashraf (2009), and the O_2^- content was determined according to the method of hydroxylamine (Elstner and Heupel 1976). Total chlorophyll content in leaves was measured via the method described previously (Xu et al. 2018; Yu et al. 2021). The content of the soluble protein was detected by the method using Coomassie brilliant blue G-250 (Kruger, 1994). Contents of proline and soluble sugar were determined using the proline assay kit (Solarbio, China) and the plant soluble sugar assay kit (Solarbio, China), respectively. For the antioxidant enzyme activity assay, the activities of antioxidant enzymes, SOD, CAT, and APX, were assessed by following the instructions of the assay kits (Solarbio, China), respectively.

Statistical analysis

In each independent experiment, it contained at least three biological replicates and all experiments were repeated for three times. Data represented as the mean ± standard deviation (SD). The statistical analysis was performed using SPSS 22.0 software (IBM, Armonk, New York, USA)), and results were illustrated using GraphPad Prism 7.0 or Microsoft Excel 2021 software.

Results

Generation and confirmation of transgenic Arabidopsis overexpressing OsPLC1

In our previous study, we demonstrated that predominant *OsPLC1* expression was detectable in tissues of various stages of rice, especially during the young stage and tilling stage, and OsPLC1 plays important roles in the control of flowering time and grain quality (Yu et al. 2022). The role of OsPLC1 in the response to salt stress of rice has also been demonstrated (Li et al. 2017). However, whether OsPLC1 can enhance the salinity stress tolerance in dicotyledonous Arabidopsis plants, is elusive. Firstly, we analyzed the behaviors of *OsPLC1* transcripts in 7-day-old Nip seedlings that were treated with NaCl by semi-quantitative RT-PCR and qRT-PCR. Both results showed that the expression of *OsPLC1* was

markedly induced under NaCl (Fig. S1). This result was consistent with that shown in the study reported by Li et al (2017).

To assess the salt tolerance potential of OsPLC1 in Arabidopsis, Agrobacterium-mediated transformation of Arabidopsis was conducted. After Basta selection and PCR analysis, we obtained six independent T0 transgenic lines (Fig. S2A). To determine the transcript expression level of *OsPLC1* in transgenic Arabidopsis, 3-week-old T3 generation leaves were used for semi-quantitative RT-PCR (Fig. S2B). Finally, we have selected two *OsPLC1* transgenic Arabidopsis plants for further analysis, named OE-5 and OE-6, and grown in the same Petri dish or pot with different stress conditions.

To investigate the effects of OsPLC1 on the normal growth (without stress) of Arabidopsis, surface sterilized seeds of Col and *OsPLC1* overexpressing Arabidopsis were sown on 1/2 MS liquid medium for 3 days, and the seed germination were then compared. As a result, no detectable morphological differences in seed germination of all lines tested were found (Fig. S3A). To track the effects of OsPLC1 on the subsequent growth of Arabidopsis, the seedling growth, morphological traits of floral organs, and siliques of Col and *OsPLC1* overexpressing Arabidopsis were then analyzed. No detectable morphological differences in plant growth and development of all lines tested were found (Fig. S3B, C, D).

Decreased germination sensitivity of OsPLC1-overexpressing transgenic Arabidopsis to salt stress

Maturation and germination of seeds are two crucial processes in alternating generation for flowering plants (Xiong et al. 2018; Yin et al. 2021; Yu et al. 2022). The dry and dormant seeds will rapidly germinate in a suitable environment. Given that overexpression of *OsPLC1* can significantly improve salt resistance in rice (Li et al. 2017), we examined and compared seed-germination phenotypes in transgenic Arabidopsis and WT in response to NaCl (Fig. 1A). In the absence of NaCl (Control), the two OE lines exhibited no significant differences in seed germination phenotype compared to the wild type (Fig. S3A, Fig. 1A and B). Both OE lines and WT seeds showed 80% germination after sowing one day, and their germination rate was nearly 100% at 3 days (Fig. 1B). In the presence of 150 mM NaCl, germinations of seeds were inhibited both in OE lines and WT seeds. However, higher germination rates were observed in the OE lines than for WT seeds (Fig. 1B), indicating that the *OsPLC1* overexpression transgenic seeds were less sensitive to salt.

OsPLC1 overexpressing transgenic Arabidopsis seedlings exhibit resistance to salt stress

To further verify the influence of NaCl treatment on seedling growth, we also analyzed the expression of *OsPLC1* in seedlings at the post-germination stage in the presence or absence of NaCl. 5-day-old seedlings of OE and WT lines grown on 1/2 MS medium were transferred to 1/2 MS medium with or without NaCl (200 mM) for more than 7 days. As a result, the primary root lengths and relative water contents of the *OsPLC1* transgenic lines and WT plants did not differ significantly without exogenous NaCl (Fig. 2). When NaCl was present in the medium, the root lengths and relative water contents of both the transgenic lines and the WT plants were significantly reduced. As expected, we observed that the

roots of the transgenic lines were significantly shorter than those of WT plants (Fig. 2). Moreover, the leaves of the WT plants turned yellow, and exterior quality reduced after NaCl treatment for 7 days, and leaves of the OE lines were still green (Fig. 2). And the contents of chlorophyll in OE plants were relatively remarkably higher than WT plants under salt stress (Fig. 2). Thus, our results indicated that resistant to salt is conferred at the germination and post-germination stages by the overexpression of *OsPLC1*.

Overexpression of OsPLC1 activates expressions of salt stress-responsive genes in Arabidopsis

Previous study demonstrated that the elevated salt tolerance of *OsPLC1* overexpressing plants in rice was accompanied by the upregulation of a subset of stress-responsive genes (Li et al. 2017). Based on the foregoing phenotypical and physiological analyses, it has become important to further investigate the participation of *OsPLC1* overexpression in the adaptive responses of plants under salt stress. For this purpose, qRT-PCR analysis was performed to compare the expression profiles of several salt responsive genes, including *RD22* (*responsive dehydration 22*), *RAB18* (*responsive to ABA 18*), *DREB2A* (*dehydration-responsive element binding protein 2A*), *DREB2B*, *RD29A* (), *RD29B*, *MYB20* (*myeloblastosis 20*), *MYB44*, *NCED3* (*9-cis-epoxycarotenoid dioxygenase 3*), *SOS1*, *SOS2* and *SOS3*, in the *OsPLC1* overexpressing and WT plants after NaCl treatment. As shown in Fig. 3, under normal conditions, there was no significant difference in expression levels of the above genes between OE lines and WT plants, while not coincidentally, all of them were substantially increased by NaCl treatment in both genotypes (Fig. 3). Nevertheless, notably higher expression levels of *RD22*, *RAB18*, *DREB2B*, *RD29A*, *RD29B*, *MYB20*, *MYB44*, *SOS1*, *SOS2* and *SOS3* were observed in both OE lines than in the WT under NaCl treatment, particularly in *DREB2B*, *RD29A* and *MYB44*. Yet the increment of *DREB2A* and *NCED3* expressions had no significant difference between OE and WT plants after salt treatment (Fig. 3). To sum up, these results revealed that *OsPLC1* was involved in regulating several major stress-responsive genes in transgenic Arabidopsis plants, thereby enhancing plant tolerance to salt stress.

OsPLC1 overexpression lines accumulate less ROS under salt stress

In plants, substantial accumulation of ROS is mainly triggered in the adverse environment, and can cause irreversible oxidative damage, ultimately leading to cell injury and even death (Waszczak et al. 2018; Halliwell 2006; Gill and Tuteja 2010). We then used DAB and NBT as chromogenic substrates to monitor the accumulation of two key ROS species ROS, H_2O_2 and O_2^-, respectively. The results showed that both OE and WT plants accumulated exceptionally low ROS, and did not differ much between these lines under normal conditions (Fig. 4A and B). However, the NaCl stress-induced ROS has been considerably reduced in *OsPLC1* overexpression plants in comparison to WT (Fig. 4A and B). We also measured the contents of the two key ROS species in leaves of OE and WT plants grown under salt stress with biochemical methods. The results indicated that contents of H_2O_2 and O_2^- were greatly decreased in transgenic Arabidopsis plants compared to those in WT plants under salt stress treatment (Fig. 4C and D), which corresponded to the histochemical experiment results. Collectively, the results suggested that overexpression of *OsPLC1* accumulates less ROS contents in transgenic Arabidopsis plants.

OsPLC1 overexpression lines show enhanced antioxidant activities under salt stress

Antioxidant levels in plants are key parameters to decrease and alleviate the damage by stress conditions, and the efficient antioxidant system (including antioxidant enzymes (SOD, CAT, APX, etc.) and osmosis-regulating substances (proline, soluble protein, soluble sugar, etc.)) helps plants in ROS scavenging under stress conditions (Yang and Guo 2018; Hasanuzzaman et al. 2020; Choudhury et al. 2017). In order to investigate the effects of salt stress on the antioxidant system of WT and OE lines, the related physiological parameters of WT and OE plants under salt stress for 6 h and normal conditions were measured.

We first analyzed expressions of a set of genes encoding antioxidant enzymes, including SODs (Cu/ZnSOD1, Cu/ZnSOD2, MnSOD, FeSOD1, FeSOD2), CATs (CAT1, CAT2, and CAT3) and APXs (APX1, APX3, APX5, and APX6), in the OE and WT lines quantitatively. qRT-PCR analysis using 10-day-old seedlings treated with 200 mM NaCl revealed that, there was no significant difference in the expression levels of all genes detected between *OsPLC1* overexpression lines and WT plants under normal growth conditions (Fig. S4). In the presence of NaCl, expressions of *SODs*, *CATs* and *APXs* in all plants were significantly induced. Nevertheless, the relative expression levels of *Cu/ZnSOD2*, *MnSOD*, *CAT2*, *APX1*, and *APX3* in *OsPLC1* overexpression plants were differentially higher than those in the WT plants under NaCl treatment (Fig. S4). However, transcript levels of *Cu/ZnSOD1*, *FeSOD1*, *CAT1*, *CAT3* and *APX6* in WT and *OsPLC1*-overexpressing transgenic plants were similar under both normal and salt conditions (Fig. S4).

Next, we examined the effects of *OsPLC1* overexpression on antioxidant enzyme activities of SOD, CAT, and APX under normal and stress conditions with biochemical methods (Fig. 5A). Similarly, there was no statistically significant in the above enzyme activities between the transgenic and WT plants in control groups (Fig. 5A). When exposed to salt stress, considerable increases in the SOD, CAT, and APX activities in both the WT and *OsPLC1* overexpressing plants, while the increases were more pronounced in the OE plants (Fig. 5A). SOD and CAT activities of OE-5 and OE-6 upon salt stress were markedly enhanced in comparison to WT, and the APX activity in overexpression lines was also slightly higher in OE plants than that in WT plants following salt stress (Fig. 5A).

Osmosis-regulating substances are important biomarkers to abiotic stressed plants; hence we analyzed contents of proline, soluble protein and soluble sugar in overexpression lines as well as WT before and after salt stress treatments. Our results showed no significant difference in proline, soluble protein, and soluble sugar contents at normal conditions (without salt stress) between the overexpression lines and WT plants (Fig. 5B). By contrast, the proline content of the *OsPLC1* transgenic lines was significantly increased by ~ 7- to 8-fold at 200 mM NaCl from the control condition, respectively. Whereas the rise in WT was about 4-fold significantly lower than that of transgenic plants (Fig. 5B). Also, the findings of the soluble protein and soluble sugar assays showed a similar pattern as proline (Fig. 5B). Hence, these results indicate that overexpressing *OsPLC1* in Arabidopsis leads to phenotypes with induced ROS scavenging and, as a consequence, induced increased resistance to salt stress.

Overexpressing of OsPLC1 enhanced salt tolerance of mature transgenic plants

Given that *OsPLC1* overexpression can improve the salt resistance of Arabidopsis in the stages of seed germination and seedling growth (Fig. 1 and Fig. 2), we decided to explore whether overexpression of *OsPLC1* improves the salt resistance of Arabidopsis during following-up development, such as the transition from vegetative stage to reproductive stage. To this end, 4-week-old plants of OE and WT lines grown in nutrient soil were watered containing 200 mM NaCl every second day for more than 14 days. The results showed no significant differential phenotypes between *OsPLC1* overexpression lines and WT plants under normal conditions (Fig. 6A). Under salt stress, most leaves of the WT plants exhibited more severe stress symptoms (wilting and chlorosis) along with growth retardation, and even death (Fig. 6A). In contrast, those of the *OsPLC1* overexpression transgenic plants remained green or presented only mild etiolation under the same treatments, and could even blossom normally (Fig. 6A). The survival rates and the chlorophyll contents of the transgenic lines were significantly higher than that of WT after NaCl treatment (Fig. 6B). Similarly, we measured the relative water content and Chlorophyll content in plants of all tested lines under NaCl stress. As the results, both the relative water content and Chlorophyll content in *OsPLC1* overexpression lines were significantly higher than that in the WT plants after NaCl treatment (Fig. 6C). Moreover, the change of relative electrolyte leakage in leaves of OE and WT plants was also compared. Under salt stress, two OE lines had significantly lower relative electrolyte leakage when compared with the WT plants (Fig. 6C). Together, the data indicate that overexpression of *OsPLC1* enhances plant sensitivity to salt stress.

Overexpressing OsPLC1 improves the water-retaining capacity of plants

High salinity and drought are two major abiotic stresses in the natural environment, that influence plant development by the alternation of the osmotic pressure of cells (Munns and Tester 2008; Hasanuzzaman et al. 2021). Since overexpression of *OsPLC1* could enhance the salt resistance of Arabidopsis plants, we wonder to investigate whether *OsPLC1* affects the drought resistance of plants. Hence, the growth and development of 4-week-old plants of *OsPLC1* overexpression transgenic lines and WT lines grown in the same condition following the drought treatment for two weeks were then investigated. As shown in Fig. S5, growths of all WT and *OsPLC1* overexpression transgenic plants were inhibited, and all plants withered and exhibited severe water loss-related symptoms. After drought for two weeks, almost all the WT and *OsPLC1* overexpression transgenic plants had died (about 9% survival rate, Fig. S5). Thus, we speculated that *OsPLC*1 did not enhance the drought resistance of Arabidopsis, which further demonstrated that there are some differences in the signaling pathways and in the regulation mechanism that affect the plant growth by salt stress and drought stress.

In addition, detached leaves from 3-week-old plants of *OsPLC1* overexpression transgenic lines and WT lines grown in nutrient soil were washed, and placed on the clean filter paper for dehydration treatment. The water loss rates of leaves were then calculated according to the fresh weights at different time periods. The results showed that, after 2 h dehydration, the water loss rates of OE-5 and OE-6 leaves were 23% and 25% respectively, while the water loss rate of WT leaves was up to 31% (Fig. 7). The water

loss rates gradually increased with the dehydration time both in leaves of *OsPLC1* overexpression transgenic lines and WT plants. However, the water loss rates of overexpression transgenic leaves were persistently lower than that of WT leaves (Fig. 7), suggesting that *OsPLC1* enhances the water-holding capacity of Arabidopsis leaves under dehydration conditions.

Discussion

Phospholipids are key components of cell membranes and also rich sources for generating cellular mediators (Wang et al. 2014). The content and composition of phospholipids in different membranes of a cell are not identical, and the phospholipid composition of a membrane is also diverse during cell development and in response to stresses (Wang 2001; Wang et al. 2012).

PI-PLC catalyzes the substrate PIP_2 to generate two key second messengers in cells, inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG), which promotes the release of Ca^{2+} from intracellular stores and activates some members of the protein kinase C (PKC) family, respectively (Li et al. 2015; Li et al. 2017; Wang et al. 2012; Yu et al. 2021). Increasing studies indicate that PI-PLCs play vital roles in plant growth, development, and response to abiotic and biotic stresses. Plants contain multiple gene families, with the Arabidopsis genome encoding 9 PLC genes, rice 4, and wheat 8 (Tasma et al. 2008; Sigh et al. 2013; Wang et al. 2020). Expressions of different family members are distinct in various cells and tissues, during different developmental stages of plants, and in response to various stresses. In Arabidopsis, researchers demonstrated that AtPLC1, AtPLC3, AtPLC4, AtPLC5, and AtPLC9 are involved in responses of ABA (abscisic acid), salt, drought, and high-temperature stresses, respectively (Sanchez and Chua 2001; Zhang et al. 2018; Xia et al. 2017; Zhang et al. 2015; Zheng et al. 2012). AtPLC2 is required for gametophyte development and early embryogenesis by regulating the mitotic process (Li et al. 2015; Chen et al. 2019). In rice, both OsPLC1 and OsPLC3 are involved in responses to salt stress, and OsPLC4 modulates rice response to salt and drought (Li et al. 2017; Deng et al. 2019), qRT-PCR analyses also revealed that the mRNA accumulation of *OsPLC1* was significantly promoted by salinity (Fig. S1). This study investigated the involvement of OsPLC1 salt stress response in Arabidopsis during seed germination, young seedling growth, and mature stage.

Salinized soil is a major environmental factor that affects seed germination, plant growth, and even crop production (Jin et al. 2021). According to statistic, about 40% soil in the world is influenced on excessive salt (Munns and Tester 2008; Han and Yang 2021). Excessive soluble salts in the soil will reduce the water absorption of plants, resulting in a water shortage of plants, and eventually causing plant death (Jin et al. 2021; Hasanuzzaman et al. 2021). In the present study, we found that overexpression of *OsPLC1* in Arabidopsis plants significantly enhanced the salt resistance as compared to WT plants.

Increasing evidence has demonstrated that the primary effect of salt stress led to osmotic stress and was conveyed in ABA-dependent and ABA-independent manners (Manuka et al. 2018). The expression analysis of all the ABA-dependent salt-stress-responsive genes, such as *RD22*, *RAB18*, *DREB2B*, *RD29A* and *RD29B* induced under salt treatment and showed a statistically higher expression in the *OsPLC1*

overexpression plants than WT plants (Fig. 3). In addition, several MYB proteins involved in the ABA response also participate in regulating salt tolerance by regulating some gene expressions in ABA signaling (Wang et al. 2021). For example, the expression of *MYB20* can be increased by NaCl treatment, and overexpression of *MYB20* significantly enhances salt stress tolerance (Cui et al. 2013). MYB44 also takes part in the salt response through the regulation of PP2Cs (the type 2C protein phosphatases), the key negative regulators of ABA signaling, by directly binding to their promoters, thus leading to salt tolerance in plants (Persak and Pitzschke 2014). Here, the expressions of *MYB20* and *MYB44* were up-regulated both in *OsPLC1* overexpressed lines and WT plants, while raised higher in OE plants than in WT (Fig. 3). Thus, it is likely that OsPLC1 might be a positive regulator for salt response by influencing the expression of stress-related genes through ABA-dependent pathways.

In the past two decades, a crucial progress in the understanding of plant salt tolerance is the identification of the *SOS* genes (Zhu, 2001). Among these *SOS* genes, *SOS1*, *SOS2*, and *SOS3* encode proteins that constitute the SOS signaling pathway, and any mutation of these genes is hypersensitive to NaCl (Gong et al. 2020). *SOS1* encodes a plasma membrane Na^+/H^+ antiporter, which can reduce the concentration of Na^+ in the cytoplasm (Shi et al. 2002; Shi et al. 2003). SOS2 is an autoinhibitory kinase under normal conditions but its autoinhibition is released by binding with SOS3 (a Ca^{2+}-binding protein with three EF-hands) when plants are exposed to hypersaline conditions (Guo et al. 2001). In the SOS signaling pathway, SOS3 senses the increasing Ca^{2+} triggered by salt stress, and the Ca^{2+}-bound SOS3 rapidly interacts with SOS2. The SOS2-SOS3 complex is recruited to the plasma membrane, and SOS1 is phosphorylated and activated by SOS2. The intracellular Na^+ concentration is eventually reduced by Na^+ efflux transport through SOS1 (Gong et al. 2020). We therefore analyzed expression levels of the above *SOS* genes, and found that the transcription levels of all three *SOS* genes significantly up-regulated in the *OsPLC1* overexpressed lines than in WT plants following their exposure to salt stress (Fig. 3). So, we hypothesize that the *OsPLC1*-OE lines can enhance salt tolerance by positively regulating *SOS* genes to maintain a low cytosolic concentration of Na^+.

In unstressed conditions, ROS is generated within the cell at low levels (Mittler et al. 2022; Choudhury et al. 2017). Under high saline conditions, salt imposes an oxidative stress caused by the production of ROS, and then results in a cascade of damage in plant cells, including mitochondrial dysfunction, lipid peroxidation, and protein loss (Hasanuzzaman et al. 2021). Increased resistance in *OsPLC1* overexpressing lines was associated with lower accumulation of ROS under salinity stress (Fig. 4).

RWC status was comparatively better in transgenic plants under salt stress (Fig. 6). The imbalance in water status of plants due to salt treatment which in turn lowered cell water potential together with turgor pressure, causing brown and wilting in the stem and leaves of WT plants, ultimately leads to plant death (Fig. 6). Chlorophyll is one of the fundamental components in plant photosynthesis which is susceptible to salt stress (Manuka et al. 2018). Degradation of Chlorophyll is one of the significant markers that signify stress intensity and damage (Alavilli et al. 2016). In our study, no remarkable differences were detected between transgenic lines and WT plants under normal conditions. However,

the *OsPLC1* transgenic lines depicted significantly higher leaf chlorophyll content after salt treatment, which may lead to enhanced fresh weight of the transgenic line and presented better tolerance compared with that of WT plants (Fig. 2C).

Diverse stresses such as drought and salt stress lead to the accumulation of ROS in cells, which brings a potential security threat to the normal growth and metabolism of plants, ultimately affecting the productivity of crops (Mittler 2002; Miller et al. 2008; Pucciariello and Perata 2016; Nadarajah 2020). To cope with these stresses, plants have evolved many adaptive mechanisms, that consist of enzymatic and non-enzymatic components (Hossain et al. 2015). Antioxidant enzymes such as SOD, CAT, and APX play significant roles in scavenging ROS in animal or plant cells (Gechev and Petrov 2020; Hasanuzzaman et al. 2021; Mittler et al. 2022). Numerous studies have demonstrated that the activities of these antioxidant enzymes are upregulated to defend protein systems. As the first-line enzyme in the oxygen-scavenging enzyme system, SOD is primarily required for converting O_2^- to H_2O_2, and the H_2O_2 was then converted into H_2O and O_2 by CAT (Hu et al. 2012; Choudhury et al. 2017; Hasanuzzaman et al. 2020; Waszczak et al. 2018). The balance between SOD and CAT activities in cells is important for determining the content of O_2^- and H_2O_2 (Miller et al. 2008; Mittler 2002). The APX is recognized as a hydrogen peroxide-scavenging enzyme that is unique to plants (Nadarajah 2020). It plays an essential role in protecting the structural integrity of cellular organelles from H_2O_2 and/or other toxic hydroxyl radicals (Halliwell 2006; Gill and Tuteja 2010; Pucciariello and Perata 2016). In the present work, the enzyme activities of SOD, CAT, and APX in *OsPLC1* overexpression plants were significantly higher than in WT plants after subjection to salt (Fig. 5A), indicating that the overexpression lines effectively maintained a relatively lower level of ROS either by limited ROS production or increased the activities of ROS scavenging related enzymes, ultimately enhanced the salt tolerance.

Reports have also implicated that proline, soluble protein, and soluble sugar in cells are necessary for the growth and development of plants, which protect cells from damage by acting as radical scavengers and osmotic agents (Liang et al. 2013; Ma et al. 2021). Enrichment of proline and soluble sugar may be a defensive response that prevents various oxidative stresses as well as protein stabilization properties by scavenging ROS (Ma et al. 2021). Under drought stress, the accumulation of proline in *VvNAC08* overexpressing plants markedly enhanced the drought resistance of Arabidopsis (Ju et al. 2020). Our recent study revealed that overexpressing *OsPLC3* in rice reduces the contents of soluble protein and soluble sugar during osmotic stress, leading to a decline in ROS scavenging ability (Yu et al. 2021). In this study, under salt stress, the overexpression *OsPLC1* lines accumulated higher proline, soluble protein, and soluble sugar compared to WT (Fig. 5), which could explain the higher tolerance of the plants to salt stress conditions.

Declarations

Conflict of interest The authors declare no conflict of interest.

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Author Contribution

Min Yu conceived and designed research. Min Yu and Dong Huang conducted experiments and analyzed data. Min Yu, Xiaoming Yin and Jiaojiao Bai wrote the manuscript. Baolu Cui improvised the language of manuscript. All authors read and approved the manuscript.

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