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Research article

Aquaporin *PIP2;1* affects water transport and root growth in rice (*Oryza sativa* L.)

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ABSTRACT

Aquaporins are key proteins in regulating water transport, plant growth and development. In this study, we investigated the function of plasma membrane intrinsic proteins (PIPs) in both yeast (*Saccharomyces cerevisiae*) and rice (*Oryza sativa* cv. Nipponbare). Three *OsPIP1s* (*OsPIP1;1*, *OsPIP1;2* and *OsPIP1;3*) and four *OsPIP2s* (*OsPIP2;1*, *OsPIP2;3*, *OsPIP2;4* and *OsPIP2;5*) were successfully amplified and expressed in yeast. Overexpression of *OsPIP2s*, especially *OsPIP2;1*, increased yeast membrane water permeability (P_f). Root hydraulic conductivity (Lpr) was decreased by approximately four-fold in OsPIP2; 1 RNAi knock-down plants, resulting in a decrease in *OsPIP2;1* expression levels of 70% and 50% in line 3 and line 4, respectively, compared to the wild type (WT) plants. No significant differences in the photosynthetic rate, transpiration rate, mesophyll conductance and chloroplast CO_2 concentration were observed between WT and OsPIP2; 1 RNAi plants. Higher stomatal conductance and intercellular CO_2 concentrations were observed in line 3 plants than in WT plants. In addition, lower root total length, surface area, root volume and fewer root tips were found in the RNAi plants than in the WT plants. Finally, the RNAi plants were more sensitive to drought stress. The results indicate that PIP2; 1 plays an important role in the regulation of water transport and plant growth.

1. Introduction

Aquaporin in plants is a large protein family, consisting of five subfamilies, i.e., plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), nodulin-26 like intrinsic proteins (NIPs), small basic intrinsic proteins (SIPs) and X-intrinsic proteins (XIPs) (Fox et al., 2017; Danielson and Johanson, 2008). In rice (*Oryza sativa* L. cv. Nipponbare), 33 members were identified, including 11 PIPs, 10 TIPs, 10 NIPs and 2 SIPs (Sakurai et al., 2005).

Previous evidence indicated the involvement of aquaporin in regulating plant growth and water transportation (Chaumont and Tyerman, 2014, 2017; Maurel et al., 2015). Expression profiles in different organs and diurnal regulation were investigated in response to environmental stress (Yu et al., 2006; Sakurai et al., 2008; Yooyongwech et al., 2013). In many cases, changes in water transportation were correlated with variations in the expression of aquaporins in rice, especially those located in plasma membranes (Sakurai-Ishikawa et al., 2011; Ding et al., 2016a; Meng and Fricke, 2017). Similar to *Arabidopsis thaliana*, putative protein sequences from rice, showing higher similarity to aquaporins, were initially found to be located in the plasma membrane, namely, PIPs. Among the PIP genes in rice, *OsPIP1;1* and *OsPIP2;1* were found to be upregulated in both roots and leaves in response to abscisic acid or water depletion (Guo et al., 2006), indicating their important role in the regulation of water transport under abiotic stress. Heterogeneous *in vivo* expression systems were developed to characterize aquaporin functions, such as *Xenopus* oocytes and yeast. Assuming a comparable expression level, the abilities of PIP1; 1 and PIP1; 2 to facilitate membrane water diffusion were relatively low or absent, while those of PIP2; 4 and PIP2; 5 were high (Sakurai-Ishikawa et al., 2011).

Although the abovementioned studies provided evidence of an aquaporin function in water permeability, direct measurements of

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aquaporin functions in rice are still missing. As exemplified for model plants such as *Arabidopsis* or tobacco, a causal relationship can be obtained by comparison of plants with impaired aquaporin gene expression to control plants (Kaldenhoff et al., 1998, 2008; Siefritz et al., 2002a; Heckwolf et al., 2011). Phenotypes associated with physiological or morphological processes can be induced by the down regulation of specific aquaporins, indicating the specific function of a certain aquaporin. Particularly for PIP2 aquaporins, functional evidence from down-regulation studies for the participation of PIP2 aquaporins in plant water transport is rare, even though these aquaporins were found to induce high water diffusion rates in cellular assays. One example was given by the analysis of *pip2;2* knockout lines in *Arabidopsis* (Javot et al., 2003).

In this study, we systematically analyzed the water permeability of rice aquaporin PIPs using a yeast expression system via a stopped flow spectrophotometer. Furthermore, the water permeability of PIP2; 1 was verified via an RNA interference (RNAi) approach. The root hydraulic conductivity and plant growth were studied and compared between WT and PIP2; 1 RNAi plants. All of the above results provide a clear overview of the function of PIP family members and their role in regulating water permeability.

2. Materials and methods

2.1. Gene amplification and expression in yeast

The cDNA sequence of PIPs from rice plants (OsPIP1; 1 [AK061769], OsPIP1; 2 [AK098849], OsPIP1; 3 [AK102174], OsPIP2; 1 [AK072519], OsPIP2; 3 (Sakurai et al., 2005), OsPIP2; 4 [AK072632], and OsPIP2; 5 [AK107700]) was isolated from the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/), amplified with specific primers (Table S1), and inserted into the pYES-DEST52 yeast expression vector using the Gateway®cloning strategy (Invitrogen™, Karlsruhe, Germany) (Otto et al., 2010). Constructs were further verified via sequencing. Verified constructs were transformed into yeast strain *Saccharomyces cerevisiae* SY1 (Matα, ura3-52, leu 2-3112, his 4-619, sec6-4ts, GAL2). Single transformants containing the corresponding aquaporin were selected via ura3 complementation.

2.2. Water permeability measurement

The water permeability of intact yeast protoplasts was measured by stopped flow spectrophotometry, as described previously (Otto et al., 2010). In brief, the protoplasts were exposed to a low outwardly directed osmotic gradient to induce protoplast swelling. Volume change was followed by the decrease of scattered light intensity in a stopped flow spectrophotometer (SFM-300, Bio-Logic SAS, Claix, France). Quantification of water permeability was achieved by fitting a single exponential function to the initial 100 ms of the swelling kinetics using Biokine software (Bio-Logic SAS, Claix, France). The osmotic water permeability coefficients (P_f) were calculated as described by van Heeswijk and van Os (Van Heeswijk and Van Os, 1986) using the following equation: $P_f = (1/\tau) \times (V_0/(S_0.V_w.C_{out}))$, where τ is the time constant for the exponential decay, V_0 and S_o are the initial mean protoplast volume and surface area, respectively, V_w is the partial molar volume of water (18 $cm^3.mol^{-1}$), and C_{out} is the external osmolarity after the mixing procedure. The initial size of the protoplast was determined by light microscopy (Leica, Germany). Calculation of P_f values resided on at least five independent experiments of two independently transformed clones, with an average of 20 measurements each ($n \geq 100$).

2.3. Preparation of OsPIP2; 1 RNAi rice plants

To generate the RNAi construct, a 287-bp fragment of OsPIP2; 1 was amplified with primers that included restriction sites for *Spe*I and *Sac*I (F, GACACTAGTTACAAGCACCAGACGGACG and R, GACGAGCTCCCGTACCTGTTGAAGTAGG). The same reaction was run using a different set of primers that included restriction sites for *Kpn*I and *Bam*HI (F, GACGGTACCTACAAGCACCAGACGGACG and R, GACGGATCCCCGTACCTGTTGAAGTAGG). The products were inserted into pMD19-T vector (TaKaRa, Dalian, China) and then into pTCK303, which carries the cauliflower mosaic virus 35 S promoter (Wang et al., 2004). Transgenic rice plants (*Oryza sativa* L. ssp. *japonica* 'Nipponbare') were generated from *Agrobacterium tumefaciens*-mediated cocultivation (Ai et al., 2009). Transgenic plants were selected on a medium containing 50 $mg.L^{-1}$ hygromycin (Roche, Shanghai, China), and hygromycin-resistant rice plants (T0 generation) were transplanted to soil and grown to maturity for seeds in the field.

2.4. Plant growth conditions

T2 generation rice seeds were surface-sterilized with 70% (v/v) ethanol for 2 min and 4% (v/v) NaClO for 15 min, washed five times with sterilized water, and then transferred into sterilized 1/2 Murashige and Skoog culture media. Seeds were placed into a light incubator with a 14/10-h day/night period. One-week old uniform plants were selected and transferred into a 7-L bucket for hydroponic culture. The macronutrient concentrations (mM) in the solution were as follows: 2.86 N as a mixture of equimolar amounts of $(NH_4)_2SO_4$ and $Ca(NO_3)_2$; 0.32 P as KH_2PO_4; 1.02 K as K_2SO_4 and KH_2PO_4; and 1.65 Mg as $MgSO_4$. The micronutrients (μM) were as follows: 35.8 Fe as Fe–EDTA, 9.10 Mn as $MnCl_2{\cdot}4H_2O$, 0.52 Mo as $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 18.5 B as H_3BO_3, 0.15 Zn as $ZnSO_4{\cdot}7H_2O$, 0.16 Cu as $CuSO_4{\cdot}5H_2O$, and 100 Si as $Na_2SiO_3{\cdot}9H_2O$. A nitrification inhibitor (dicyandiamide [DCD]) was added to each nutrient solution to prevent the oxidation of NH_4^+. The nutrient solutions were changed every 3 days, and the pH was adjusted to 5.50 ± 0.05 every day with HCl or NaOH. For polyethylene glycol (PEG)-simulated drought stress treatment, 15% (w/v) PEG 6000 was added to the nutrient solution. The plants were grown in a greenhouse and phytotron. In the greenhouse, the temperature was 25 ± 5 °C during the daytime, and the maximum light intensity at midday was > 1500 $\mu mol\ m^{-2}.s^{-1}$ at the top leaf level. The phytotron had a 16-h light (30 °C) and 8-h (22 °C) dark photoperiod, and the relative humidity was maintained at approximately 60%. Light intensity was maintained at 200 $\mu mol\ m^{-2}.s^{-1}$ at the top leaf level.

2.5. Real-Time quantitative PCR (RT-qPCR)

After germination, plants were cultured for six weeks in hydroponic culture. Total RNA was extracted from the roots of wild type (WT) and PIP2; 1 RNAi plants using TRIzol reagent (Invitrogen, Carlsbad, CA,USA) according to the manufacturer's instructions. For RT-qPCR analysis, DNase I-treated total RNAs were used for reverse transcription using the PrimeScript™ RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China). The expression of three *PIP1s* (*PIP1;1, PIP1;2 and PIP1;3*) and six *PIP2s* (*PIP2;1, PIP2;2, PIP2;3, PIP2;4, PIP2;5* and *PIP2;6*) was analyzed by RT-qPCR using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, USA), and the products were labeled using SYBR Green Master Mix (SYBR Premix Ex *Taq*II, TaKaRa, Dalian, China) according to the manufacturer's instructions. The primers were used according to a previous study (Sakurai-Ishikawa et al., 2011; Ding et al., 2016a). Actin and 18s RNA genes were used as internal controls, and the $2^{-\Delta Ct}$ method was used to normalize the data.

2.6. Root hydraulic conductivity measurement

After germination, hydroponic culture was conducted in a greenhouse, and six-week-old rice plants were used for root hydraulic conductivity (Lpr) measurement. Lpr of the whole root system was measured using a high pressure flow meter (Tsuda and Tyree, 2000) (HPFM; Dynamax Inc., Houston, USA). Aboveground portions of rice plants were removed approximately 2 cm above the root/shoot interface, and the HPFM was attached to the detached root using a compression fitting and dental silicone. Positive pressure (Pi) was applied to force water from the base of the excised root to the root tip (opposite to the normal direction of flow during transpiration). The Pi at the base increased rapidly from 0 to 0.5 MPa at a constant rate of 3–7 kPa s^{-1} while the flow (F) and applied pressure (Pi) were measured every second. The slope (Kr, $kg.s^{-1}.MPa^{-1}$) of the relationship between F and Pi was taken as a transient measurement. After Kr was measured using transient methods, the root surface area (Sr) was measured using a root scanner system and analyzed with WinRHIZO 2008a software (Regent Instruments, Quebec, Canada). All Kr values were calibrated to the values at 25 °C. Lpr ($kg.s^{-1}.MPa^{-1}.m^{-2}$) was calculated using the following equation: Lpr = Kr/Sr.

2.7. Gas exchange measurement

Gas exchange measurements were performed using a Li-cor 6400 portable photosynthesis system with a 6400-40 Leaf Chamber Fluorometer (LICOR Biosciences, Lincoln, NE, USA) with six-week-old rice plants in a greenhouse. The newly expanded leaves were used for gas exchange measurement. Leaf temperature during the measurement was maintained at 28 °C, and photosynthetic photon flux density (PPFD) was 1500 μmol $m^{-2}.s^{-1}$. The CO_2 concentration in the cuvette was controlled at 400 μmol mol^{-1} with a CO_2 cartridge (LICOR Biosciences, Lincoln, NE, USA). After equilibration to a steady state, the fluorescence was recorded (Fs) and a 0.8 s saturating pulse of light (approx. 8000 μmol $m^{-2}.s^{-1}$) was applied to measure the maximum fluorescence (Fm'). Photosynthetic rate (A), stomatal conductance (gs), transpiration rate (Tr) and intercellular CO_2 concentration (Ci) were recorded simultaneously. The efficiency of photosystem II (Φ_{PSII}) was calculated as Φ_{PSII} = 1-Fs/Fm'. Then, the total electron transport rate (J_T) was calculated as $J_T = \Phi_{PSII} \times PPFD \times \alpha_{leaf} \times \beta$, in which α_{leaf} and β were the leaf absorption and the proportion of quanta absorbed by photosystem II, respectively. The value of $\alpha_{leaf} \times \beta$ was calculated from the slope of the relationship between Φ_{PSII} and the quantum efficiency of CO_2 uptake (Φ_{CO2}), obtained by varying light intensity under nonphotorespiratory conditions at < 2% O_2, through connecting the Li-cor with N_2 gas. In this study, the value of $\alpha_{leaf} \times \beta$ was 0.512 for WT and PIP2; 1 RNAi plants. The mesophyll conductance (g_m) was calculated by variable J_T methods (Harley et al., 1992) as follows:

$$g_m = A/\{Ci\text{-}\Gamma^*[\ J_T + 8(A + R_d)]/[\ J_T\text{-}4(A + R_d)]\}$$

Where A is the light-saturated photosynthetic rate, Ci is the intercellular CO_2 concentration, and Γ^* and R_d are the CO_2 compensation point and the rate of dark respiration, respectively, as measured according to Li et al. (2013). Briefly, under the dark conditions, the PPFD in the cuvette was controlled as 150, 300 and 600 μmol $m^{-2}.s^{-1}$. At each PPFD, the CO_2 concentration was adjusted to 25, 50, 80 and 100 μmol mol^{-1} with a CO_2 cartridge. A and Ci were recorded at each PPFD and CO_2 concentration. Then A/Ci response curves were plotted, and three linear curves crossed the same point. The A at this point represented -Rd, and Ci represented Γ^*. Before the measurement, stomatal opening was induced under a PPFD of 600 μmol $m^{-2}.s^{-1}$ and a CO_2 concentration of 100 μmol mol^{-1} for 30 min in the dark. The chloroplast CO_2 concentration (Cc) was calculated as follows: Cc = Ci-A/g_m

2.8. Statistical analysis

All statistical analyses were performed using JMP statistical software (SAS Institute, Cary, USA). Significant differences among treatments (indicated by different letters) were identified by an analysis of variance followed by Tukey's honest significant difference test ($P < 0.05$).

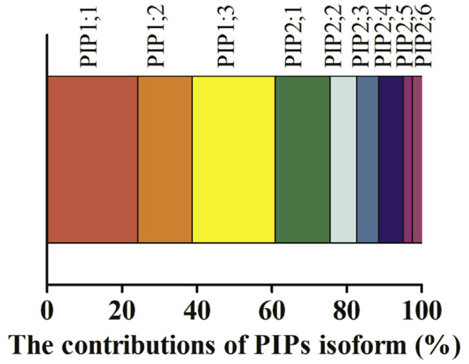

Fig. 1. The expression contribution of a single *PIP* to the nine *PIPs* in roots. The expression of *PIP1;1*, *PIP1;2*, *PIP1;3*, *PIP2;1*, *PIP2;2*, *PIP2;3*, *PIP2;4*, *PIP2;5* and *PIP2;6* was checked by RT-qPCR. After germination, the WT plants were cultured hydroponically for six weeks, and the root samples were collected for RNA extraction and RT-qPCR.

3. Results

3.1. The expression of PIPs in roots

Aquaporins from rice are composed of 33 members, including 11 PIPs (Sakurai et al., 2005). Among the 11 PIPs, we checked the expression of nine *PIPs* in roots by RT-qPCR, i.e., *PIP1;1, PIP1;2, PIP1;3; PIP2;1, PIP2;2, PIP2;3, PIP2;4, PIP2;5* and *PIP2;6* according to Sakurai-Ishikawa et al. (2011). *PIP2;7* and *PIP2;8* were omitted due to their low abundance in roots, while *PIP2;6* failed to give positive results during the experiments, and the expression data of *PIP2;6* were taken from *PIP2;5*, as they had similar expression levels in roots (Sakurai-Ishikawa et al., 2011). The three *PIP1s* were highly expressed in roots, contributing to more than 60% of the entire *PIP* expression. The expression of *PIP2;1* was the highest among the six *PIP2s*, with a relative expression among all the tested *PIPs* of 15% (Fig. 1).

3.2. Comparison of the water permeability of yeast cells expressing PIPs

Within the seven selected PIPs, PIP1; 1, PIP1; 2, and PIP1; 3, as well as PIP2; 1, PIP2; 3, PIP2; 4, and PIP2; 5 from 11 PIPs were successfully amplified and expressed in yeast. Selection criteria of the PIP2 family were based on previous studies by Sakurai-Ishikawa et al. (Sakurai et al., 2005; Sakurai-Ishikawa et al., 2011). PIP2; 7 and PIP2; 8, were not selected due to the low abundance in roots. Finally, four other PIP2s, PIP2; 1, PIP2; 3, PIP2; 4 and PIP2; 5 resulted in successful constructs for yeast expression while PIP2; 2 and PIP2; 6 failed during the amplification processes. Cell walls of yeasts were enzymatically degraded and the resulting spheroplasts were subjected to hypo-osmotic conditions in a stopped flow spectrophotometer. After a dead time of 10–12 ms, changes in light scattering were recorded. Arbitrary units from light scattering were normalized as indicated in Fig. 2 (PIP1s, Fig. 2a; PIP2s, Fig. 2b). The resulting curves were used for a nonlinear fit to obtain the water permeability P_f (see methods for the functions used in the calculation) (Fig. 2c). Compared to control cells, the expression of PIP1 in yeast displayed only a slight or barely detectable increase in P_f with a range similar to controls. For the cells expressing PIP2s, a significant increase in P_f was detected compared with the cells not expressing any PIPs. These were different and were reliant upon the expression of the particular aquaporin species. Curves obtained from experiments with cells expressing PIP2; 1 appear steeper than those obtained with cells expressing one of the remaining aquaporins. Accordingly, the highest P_f value was obtained with overexpression of PIP2; 1, which was six-fold and 10-fold greater than that with overexpression of PIP2; 5 and PIP1; 1, respectively.

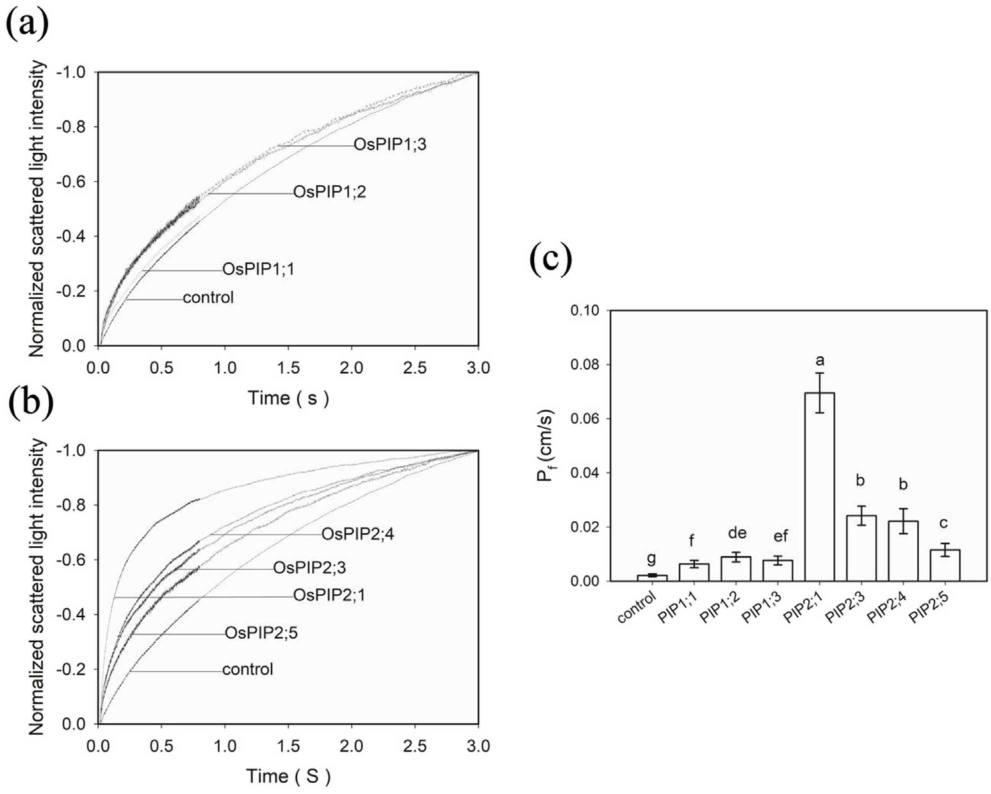

Fig. 2. Comparison of membrane water permeability in yeast expressing PIP1s and PIP2s. (a–b) Time course of normalized scattered light intensity in response to hypo-osmotic conditions. (c) Calculated osmotic water permeability coefficients (P_f) values of corresponding yeast plasma membranes given as the mean ± SD ($n > 10$). Yeast cells expressed PIP1s and PIP2s, respectively, noninduced cells were a control as indicated. Different letters above each column indicate significance groups according to Tukey's honest significant difference test ($P < 0.05$).

3.3. *The expression of PIP2;1 and the other PIPs in the PIP2; 1 RNAi and WT plants*

As indicated in 3.1 and 3.2, *PIP2;1* was highly expressed in roots and showed an effective water diffusion activity when expressed in yeast. We therefore considered PIP2; 1 to play a major role in water transportation. To further verify its physiological roles, two PIP2; 1 RNAi lines (line 3 and line 4) were generated via *Agrobacterium tumefaciens* transfection. The expression levels of *PIP2;1* and other *PIPs* were determined by RT-qPCR. As shown in Fig. 3a, the expression of *PIP2;1* was decreased by approximately 70% and 50% in line 3 and line 4, respectively, compared with the WT, while no large differences were observed in the expression of *PIP1;1*, *PIP1;2*, *PIP2;2*, *PIP2;3* and *PIP2;4* between WT and PIP2; 1 RNAi plants (Fig. 3b). A lower expression level of *PIP2;5* was observed in PIP2; 1 RNAi plants than in WT plants.

3.4. *Comparison of root hydraulic conductivity (Lpr) and plant growth between PIP2; 1 RNAi and WT plants*

In the two PIP2; 1 RNAi lines, Lpr was reduced by approximately four-fold in comparison with the WT (Fig. 4). This indicated that PIP2; 1 is a component of the water transport system in rice and is responsible for the reduction of water transport resistance.

At the reproductive growth stage, the RNAi rice plants showed a smaller shoot and yield reduction compared to WT plants (Fig. 5a, Fig. 5b). In the vegetative growth stage, the root appeared elongated and smaller in PIP2; 1 RNAi plants than in WT plants (Fig. 5c). For two-month-old rice plants, both root and shoot dry weights were significantly lower in the RNAi plants than in WT plants (Fig. 5d and e). In comparison to the WT, the dry weights of the root and shoot were decreased by 39% and 29%, respectively, in line 3.

Significant decreases in root total length, surface area, root volume and root tips were observed in the PIP2; 1 RNAi plants, especially in line 3, in comparison with the WT (Table 1). No difference in root diameter was detected between WT and PIP2; 1 RNAi plants.

3.5. *The comparison of gas exchange between the PIP2; 1 RNAi and WT plants*

No significant differences in A and Tr were found between WT and PIP2; 1 RNAi plants (Table 2). Interestingly, there was a higher g_s and Ci in line 3 than in the WT. Additionally, a slightly lower g_m was observed in line 3 than in the WT. Indeed, there was no difference in Cc between WT and PIP2; 1 RNAi plants (Table 2).

3.6. *Effect of drought stress on the growth of PIP2; 1 RNAi plants*

To determine the effect of drought stress on the growth of PIP2; 1 RNAi plants, hydroponic cultures were grown in a greenhouse and phytotron under control and PEG treatment. In the greenhouse, the phenotype of leaf rolling was detected in WT and PIP2; 1 RNAi plants after 15% PEG6000 treatment for one week. The results showed that the rolling of leaves was more serious in PIP2; 1 RNAi plants than in WT plants (Fig. 6a). In the phytotron, less difference in shoot growth, root and shoot dry weight was observed between WT and PIP2; 1 RNAi seedlings (two-week old) under the control water treatment (Fig. 6b and d). A smaller shoot was observed in the PIP2; 1 RNAi plants than in the WT plants under PEG treatment (Fig. 6c). Indeed, a significantly lower shoot dry weight was shown in line 3 in comparison with the WT under PEG treatment for one week (Fig. 6e). The root dry weight was also lower, but not significantly, in the PIP2; 1 RNAi plants than in WT plants (Fig. 6e).

4. Discussion

4.1. *PIP2; 1 facilitates water transport*

The expression of nine *PIPs* (*PIP1;1, PIP1;2; PIP1;3; PIP2;1, PIP2;2, PIP2;3, PIP2;4, PIP2;5* and *PIP2;6*) was tested in roots, and the three *PIP1s* contributed to more than 60% of the total expression of the nine *PIPs* (Fig. 1). Moreover, *PIP2;1* was the most expressed gene in the

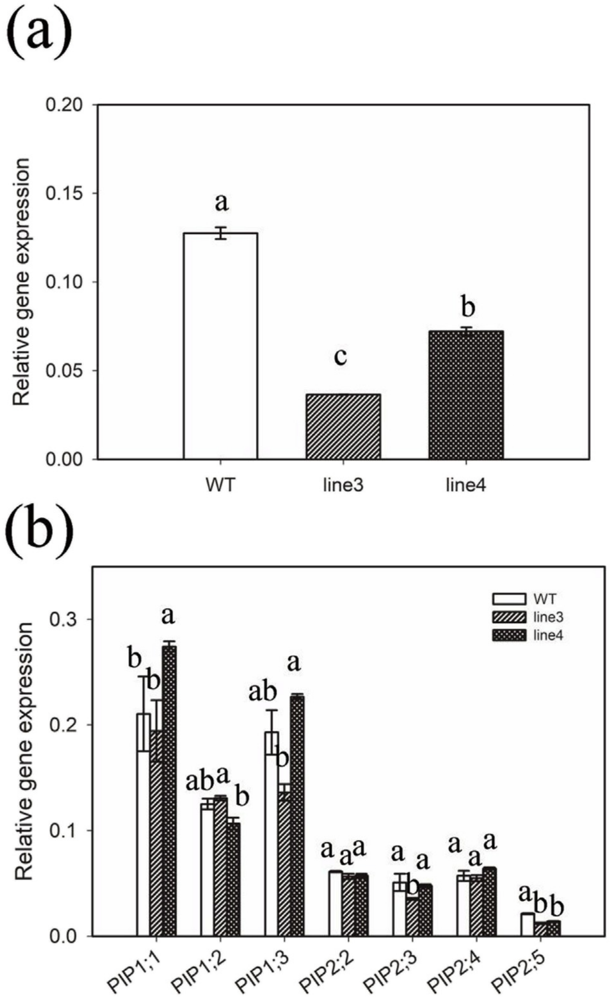

Fig. 3. Comparison of the expression of *PIP2;1* and other *PIPs* between WT and PIP2; 1 RNAi plants, line 3 and line 4. The expression of *PIPs* was assessed by RT-qPCR. The plants were cultured with hydroponics for six weeks and then root samples from three plants were collected for RNA extraction. The $2^{-\Delta Ct}$ method was used to normalize the data. Actin and 18sRNA were used as control genes. The bars indicate the means ± SD (n = 3). Different letters above each column indicate significance groups according to Tukey's honest significant difference test (P < 0.05).

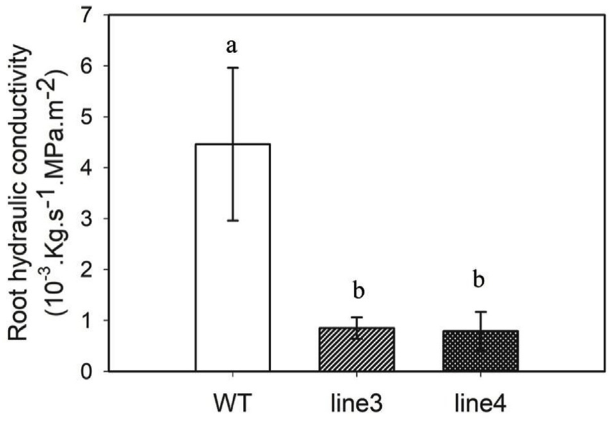

Fig. 4. Comparison of root hydraulic conductivity (Lpr) between WT and PIP2; 1 RNAi plants. Hydroponic culture was conducted in this experiment. Six-week-old rice plants were used to measure root hydraulic conductivity. The bars indicate means ± SD; n (WT) = 27, n (line 3) = 18, n (line 4) = 15. Different letters indicate different significance groups using Tukey's honest significant difference test (P < 0.05).

PIP2s, indicating its important role in the regulation of water transport in rice roots (Sakurai-Ishikawa et al., 2011; Ding et al., 2016a). Other studies showed that PIP2; 1 also played an important role in balancing water permeability during chilling recovery (Yu et al., 2006). Additional results showed that PIP2; 1 was mostly located at the cells of the endodermis, where water flow was blocked by apoplastic barriers (Sakurai et al., 2008; Sakurai-Ishikawa et al., 2011), indicating that PIP2; 1 may facilitate water passage through the barriers. Based on both previous reported results and our own data, we further tested the water permeability of PIPs, including three PIP1s (PIP1; 1, PIP1; 2 and PIP1; 3) and four PIP2s (PIP2; 1, PIP2; 3, PIP2; 4 and PIP2; 5) in yeast (*Saccharomyces cerevisiae*). Membrane water permeability was significantly increased by overexpressing PIP2s in yeast (Fig. 2). This confirmed our hypothesis that PIP2s play an important role in regulating water transport in roots. In contrast, highly abundant PIP1s in roots did not lead to an obvious increase in water permeability when PIP1s were overexpressed in yeast. This might indicate another mechanism of PIP1s in regulating water transport. As reported before, membrane water permeability was even higher when both PIP1s and PIP2s were coexpressed in comparison with expression of PIP2s alone in *Xenopus* oocytes (Fetter et al., 2004), which could be explained by the formation of heterotetramers between PIP1s and PIP2s (Bienert et al., 2018; Vajpai et al., 2018).

Knock down of *PIP2;1* was performed by generating RNAi plants, and the expression of *PIP2;1* was decreased by 70% and 50% in line 3 and line 4, respectively (Fig. 3); while no major difference was observed with regard to the expression of *PIP1;1, PIP1;2, PIP2;2, PIP2;3, PIP2;4* between WT and RNAi plants. Lpr was significantly lower in the RNAi plants than in the WT plants (Fig. 4), which was tightly related to the regulation of the morphology, anatomy, and especially aquaporin content (Bramley et al., 2009) of roots. Previous studies demonstrated that Lpr was changed by the genetic modification the expression of aquaporin. In *NtAQP1* antisense tobacco plants, Lpr was decreased by 42% (Siefritz et al., 2002a), and in *pip1;2* knockout Arabidopsis plants, Lpr was decreased by 20–30% (Postaire et al., 2010). Gambetta et al. (2017) indicated that the contribution of aquaporin to Lpr was highly variable across species, ranging from 0 to 90%, depending on the type of aquaporin inhibitor and the methods applied for measuring Lpr. In comparison to other herbaceous species, rice roots tend to have higher root hydraulic resistance per root surface area, resulting from outer apoplastic barriers, sclerenchymatous tissue (Miyamoto et al., 2001), and aerenchyma formation (Yang et al., 2012). Aquaporins may play an even more important role in the regulation of Lpr in rice plants. Indeed, the contribution of aquaporin to Lpr could be up to 79% under normal water conditions and 85% under drought stress in rice (Grondin et al., 2016). In this study, the results showed that Lpr decreased by approximately 80% in the PIP2; 1 RNAi plants. These results were consistent with previous reports that PIP2; 1 localized at the endodermis and central cylinder, where water flow was blocked by apoplastic barriers, in rice plants (Sakurai-Ishikawa et al., 2011), indicating that PIP2; 1 facilitates water passage through these specific cells.

PIP2;1 was the most highly expressed gene among all *PIP2s* in roots (Fig. 1) (Sakurai-Ishikawa et al., 2011), and showed the largest increase in membrane water permeability when overexpressed in yeast compared to other PIPs (Fig. 2). However, no direct functional data have been reported for PIP2; 1 in rice. Here, we provide evidence for the function of PIP2; 1 as a molecular component of plant water transport with transgenic knockdown as direct evidence. To date, causal evidence by knockdown or knockout assays for the function of PIP2 in plant water transport was provided solely by analysis of an *Arabidopsis* T-DNA insertion mutant (Javot et al., 2003). A PIP2; 2 dependent reduction of hydraulic conductivity of only 14% was detected in comparison to an approximately roughly 80% reduction in this study. It's possible that the difference between *Arabidopsis* and rice root architecture is the reason

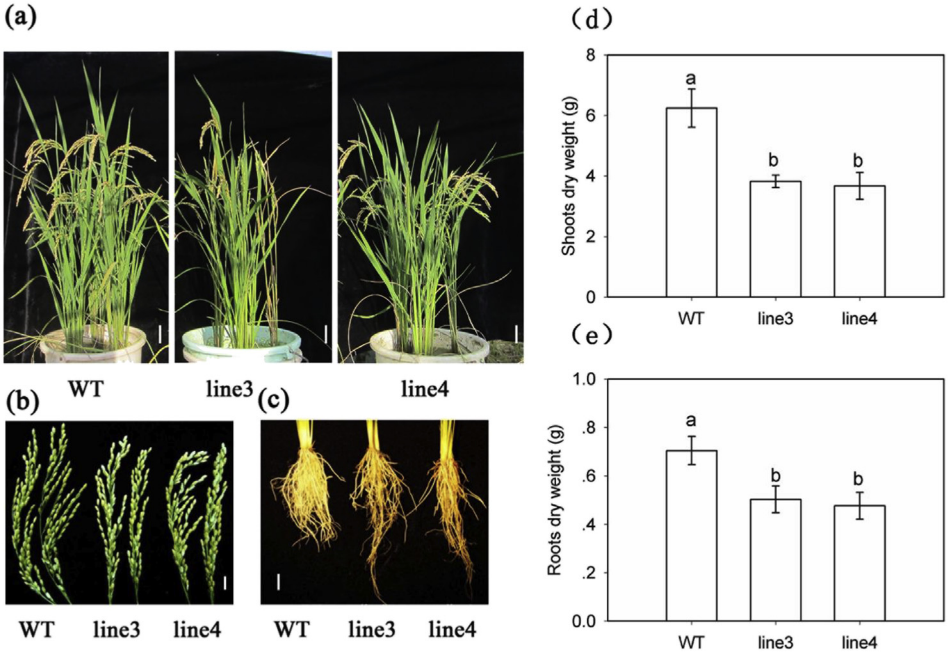

Fig. 5. Comparison of plant growth between WT and PIP2; 1 RNAi plants. (a) Visual comparison of characteristic WT and PIP2; 1 RNAi plants at the reproductive stage (bar = 5 cm) as well as (b) comparison of rice seeds (bar = 2 cm), (c) comparison of root development (bar = 2 cm), and (d, e) dry weights of shoots and roots. Soil pot experiments were conducted for (a) and (b). Hydroponic culture in a greenhouse was used for root development comparison, shoot and root dry weight measurements. After 5 weeks of culture, roots were imaged with a camera, and two-month-old rice plants were harvested for dry weight measurements. The values were from five replications, and the error bars indicate ± SD in (d) and (e). Different letters indicate different significance groups using Tukey's honest significant difference test ($P < 0.05$).

that the effect of PIP2;*2* absence is smaller in *Arabidopsis* and that of *PIP2;1* is more pronounced in rice.

4.2. PIP2; 1 affects plant growth under control and drought stress

In the PIP2; 1 RNAi plants, root growth and development were different compared to the WT (Fig. 5; Table 2). Analysis of root development showed that total root length, surface area, root volume and tip number decreased in PIP2; 1 RNAi plants, particularly in transgenic line 3 (Table 2), indicating that the emergence of new roots was inhibited by the down regulation of *PIP2;1*. It has been demonstrated that cell growth and development are tightly related to the regulation of hydraulic and turgor pressure, associated with aquaporin (Chaumont and Tyerman, 2014). A similar effect of PIP2; 1 in lateral root emergence (LRE) and development was found in *Arabidopsis*, which is regulated by auxin (Péret et al., 2012). All the above data showed that aquaporin is involved in regulating cell hydraulic conductivity and turgor pressure during new lateral root emergence through overlaying tissues. Similar to PIPs, TIPs were also reported to affect LRE (Reinhardt et al., 2016).

Both root and shoot biomass were lower in RNAi plants than in WT plants in the greenhouse trials (Fig. 5). However, less difference in root and shoot biomass was observed between WT and PIP2; 1 RNAi plants when plants grew in the phytotron (Fig. 6b and d). In the greenhouse, the light intensity was much higher than in the phytotron, meaning that high evaporation occurred in the greenhouse. As a result, more water depletion occurred in the *PIP2;1* silenced plant than in the WT, and furthermore root and shoot biomass were lower in the RNAi plants than WT plants when plants grew in the greenhouse. Additionally, the yield production was affected by *PIP2;1* down regulation. In most studies, plant growth was inhibited by the down regulation of aquaporin expression, and there was a lower photosynthetic rate and CO_2 transport conductance (Maurel et al., 2016). In contrast, plant growth was improved by over expression of aquaporin in favorable growth conditions (Groszmann et al., 2017).

The present results showed that there was no significant difference in photosynthetic rate between RNAi plants and WT plants (Table 1). However, a higher stomatal conductance was observed in RNAi line 3 plants, indicating that plants attempted to increase transpiration to compensate for the low Lpr. Indeed, the transpiration rate was slightly higher in the PIP2; 1 RNAi line 3 than in the WT. In other studies, a 40% increase and a 30% decrease in stomatal conductance was observed in *NtAQP1* overexpression and antisense plants, respectively, when compared with the WT plants (Maurel et al., 2016). Moreover, Siefritz et al. (2002b) also showed a 42% decrease in Lpr in NtAQP1 antisense plants when grown in soil. In this study, a difference was observed in PIP2; 1 RNAi rice plants, indicating different behavior between rice and tobacco when aquaporin was silenced. Similar to rice, in the PIP1s RNAi *Populus canescens* plant, the stomatal conductance was increased by 47% (Bi et al., 2015). The stomatal conductance might be regulated by the interaction of abscisic acid (ABA) and aquaporin in the PIP2; 1 RNAi plants. In *Arabidopsis*, PIP2; 1 activation was required for stomatal closure in response to ABA treatment, and it was shown that the plant had a defect in stomatal closure in the *pip2;1* knockout line (Rodrigues et al., 2017; Grondin et al., 2015). In addition, Parent et al. (2009) showed that a high and low stomatal conductance was seen in the ABA

Table 1

Comparison of light saturated photosynthetic rate (A, $\mu molCO_2.m^{-2}.s^{-1}$), stomatal conductance (g_s, $molCO_2.m^{-2}.s^{-1}$), transpiration rate (Tr, $mmolH_2O.m^{-2}.s^{-1}$), intercellular CO_2 concentration (Ci, $\mu molCO_2.mol^{-1}$), chloroplast CO_2 concentration (Cc, $\mu molCO_2.mol^{-1}$), mesophyll conductance (g_m, $molCO_2.m^{-2}.s^{-1}$), total electron transport rate (J_T, μmol $photons.m^{-2}.s^{-1}$), the CO_2 compensation point (Γ*, $\mu molCO_2.mol^{-1}$) and the rate of dark respiration (Rd, $\mu molCO_2.m^{-2}.s^{-1}$) between WT and PIP2; 1 RNAi plants. The values indicated means ± SD from five plants. Different letters indicate different significance groups using Tukey's honest significant difference test ($P < 0.05$). Line 3 and line 4 are two independent lines of PIP2; 1 RNAi plants.

	A	g_s	Tr	Ci	Cc	g_m	J_T	Γ*	Rd
WT	26.6 ± 1.6a	0.19 ± 0.01b	11.0 ± 1.4a	246 ± 15b	167 ± 16a	0.35 ± 0.07a	219 ± 16a	41.6 ± 1.5a	0.76 ± 0.11a
Line 3	25.9 ± 0.8a	0.23 ± 0.02a	12.8 ± 2.4a	265 ± 10a	172 ± 9a	0.28 ± 0.02a	209 ± 13a	41.9 ± 0.7a	0.60 ± 0.06a
Line 4	25.8 ± 0.8a	0.20 ± 0.02b	12.4 ± 2.2a	248 ± 6 ab	168 ± 7a	0.33 ± 0.03a	211 ± 8a	41.9 ± 0.7a	0.60 ± 0.06a

Table 2
Comparison of root morphological parameters, total root length (L, cm), surface area (SA, cm^2), average diameter (AD, mm), root volume (RV, cm^3), and number of root tips (T) from WT and PIP2; 1 RNAi plants. The values indicate the mean ± SD from four plants. Different letters indicate different significance groups using Tukey's honest significant difference test ($P < 0.05$).

	L	SA	AD	RV	T
WT	1013 ± 48a	92 ± 5a	0.28 ± 0.02a	0.69 ± 0.05a	5272 ± 303a
Line 3	706 ± 44b	64 ± 6b	0.29 ± 0.02a	0.47 ± 0.06b	3075 ± 959b
Line 4	835 ± 165b	74 ± 16b	0.28 ± 0.01a	0.52 ± 0.13b	3902 ± 689b

antisense and sense maize lines, respectively, while low and high aquaporin abundance was observed in the two lines. These results indicate that ABA regulates the stomatal conductance in the PIP2; 1 RNAi rice line.

Moreover, it was demonstrated that aquaporins were responsible for the regulation of CO_2 transport during carbon fixation (Groszmann et al., 2017; Kaldenhoff, 2012). In this study, no significant difference in g_m was observed between WT and the PIP2; 1 RNAi plants. It has been shown that aquaporin, i.e., PIP1, could facilitate the transport of CO_2 through membranes in mesophyll cells (Uehlein et al., 2003, 2008), and g_m was further enhanced. In contrast, g_m decreased in NtAQP1 antisense tobacco (Uehlein et al., 2003; Flexas et al., 2006) and *AtPIP1;2* T-DNA insertion mutants of *Arabidopsis* (Heckwolf et al., 2011). In barley, CO_2 was transported by PIP2s (Mori et al., 2014). It is unclear whether PIP2 is a CO_2 transport facilitator in rice plants. Further evidence should be provided to elucidate the function of PIP2s (PIP2; 1) in the regulation of g_m.

In C3 plants, the light saturated photosynthetic rate was more restricted by the CO_2 transport resistance in mesophyll cells under favorable conditions (Kaldenhoff, 2012; Evans et al., 2009; Flexas et al., 2008; Ding et al., 2016b; Li et al., 2009). In this study, no significant difference in A was observed between WT and PIP2; 1 RNAi plants, which could be due to the equal g_m and Cc. A high g_s could not further enhance A in the PIP2; 1 RNAi plants.

More serious leaf rolling and lower shoot biomass were found in the PIP2; 1 RNAi plants than in WT plants under PEG treatment (Fig. 6). These results indicated that rice seedlings were more sensitive to drought and/or osmotic stress treatment when the expression of *PIP2;1* was silenced. It was reported that, in most cases, plant growth was improved by the overexpression of aquaporins under unfavorable growth conditions (drought, salt and cold), while plants were more sensitive to environmental stress in aquaporin downregulated plants (Groszmann et al., 2017; Sade and Moshelion, 2017). In rice, enhancement of salt resistance and chilling tolerance was observed from overexpression of *PIP1;1*, *PIP1;3*, and *PIP2;7*. Additionally, a correlation with root hydraulic regulation was assumed in this case (Li et al., 2008; Matsumoto et al., 2009; Liu et al., 2013). Under drought stress, less water was absorbed by roots, and more ABA was synthesized in roots (Ding et al., 2016a). Furthermore, stomatal closure occurred due to ABA functioning in guard cells. In the PIP2; 1 RNAi plants, more serious leaf rolling was observed under PEG treatment, indicating that less water was transported to the shoot than in WT plants. Indeed, Lpr was reduced by approximately four-fold in the PIP2; 1 RNAi plants in comparison with the WT under normal water conditions (Fig. 4), while Lpr was not measured under drought stress conditions. However, Grondin et al. (2016) demonstrated that the contribution of aquaporins to Lpr was up to 85% under drought stress in rice, and drought stress affects Lpr mainly through the regulation of aquaporin and root anatomy and morphology (Ding et al., 2018). Smaller roots were observed in the PIP2; 1 RNAi plants than in WT plants (Fig. 5c), and this

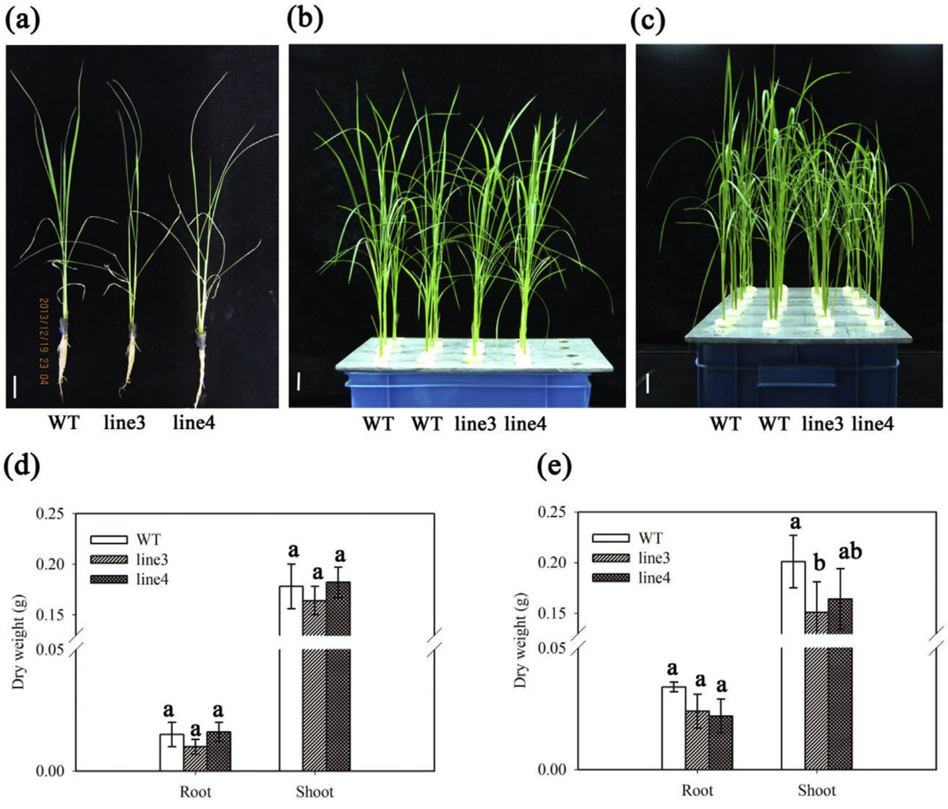

Fig. 6. Effect of drought stress on the growth of PIP2; 1 RNAi and WT plants. Comparison of leaf rolling under PEG treatment (a), shoot growth (b) and dry weight (d) under control water treatment and shoot growth (c) and dry weight (e) under PEG treatment, between WT and RNAi plants. For leaf rolling comparison, the rice plants were cultured with hydroponics in a greenhouse, and four-week-old plants were subjected to 15% (w/v) PEG6000 to simulate drought stress. After one week of treatment, leaf rolling was detected. For plant growth and dry weight comparison, the rice plants were cultured with hydroponics in a phytotron. Two-week old plants growing under control water conditions were imaged and harvested for dry weight comparison. Meanwhile, the two-week-old plants were subjected to 15% (w/v) PEG6000 to simulate drought stress. After one week of treatment, the plants were imaged and harvested for dry weight comparison. The values were from four replications, and the error bars indicate ± SD. Different letters indicate different significance groups using Tukey's honest significant difference test ($P < 0.05$). Bars indicate 2 cm in a, b and c.

could decrease the drought tolerance of the PIP2; 1 RNAi plants. As mentioned above, rice roots tend to have a higher root hydraulic resistance per root surface area, resulting from outer apoplastic barriers, sclerenchymatous tissue (Miyamoto et al., 2001), and aerenchyma formation (Yang et al., 2012), in comparison to other herbaceous species. Aquaporins may play more important roles in regulating Lpr in rice roots, especially under unfavorable growth environments.

5. Conclusion

The data presented in this study showed that PIP2; 1 has the most pronounced capacity in facilitating water diffusion in the heterological yeast system. Rice with inhibited *PIP2;1* expression showed a characteristic phenotype related to the reduction of root hydraulic conductivity. Conclusively, PIP2; 1 is one of the key components determining root water flux and plant growth.

Author contribution

L.Kai, S.Guo, R.Kaldenhoff, and Y.Zhu conceived and designed the research. L.Ding and N.Uehlein conducted the experiments. L.Ding and L.Kai analyzed the data. L.Ding, L.Kai and R.Kaldenhoff wrote the manuscript. All authors read and approved the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.03.017.

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