Journal of Plant Research (2026) 139:255–265
https://doi.org/10.1007/s10265-025-01691-z

REGULAR PAPER – PHYSIOLOGY/BIOCHEMISTRY/MOLECULAR AND CELLULAR BIOLOGY

Knockout of a single aquaporin, OsPIP2;4, decreases root water permeability in rice

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Received: 21 October 2025 / Accepted: 29 December 2025

Abstract
Aquaporins (AQPs) are membrane proteins that facilitate water transport and are present in nearly all bacterial, animal, and plant cells. In plants, AQPs are classified into four or more subfamilies, with plasma membrane intrinsic proteins (PIPs) playing a key role in root water uptake and cellular water regulation. Previous studies have demonstrated that PIPs contribute to root hydraulic conductivity (Lp_r) in various plant species. In this study, we examined the specific role of OsPIP2;4, one of the PIP-type aquaporins among 11 rice (*Oryza sativa*) PIP2s, in regulating Lp_r. Transgenic rice plants, including OsPIP2;4-knockout (KO) and overexpressing (Ox) lines, were used for this investigation. Two independent KO lines, generated via the CRISPR-Cas9 system and T-DNA insertion mutagenesis, respectively, showed significantly lower Lp_r compared to wild-type rice plants. The decrease in Lp_r in the T-DNA KO line was associated with reduced *OsPIP2;4* transcript levels, measured by real-time PCR, and lower OsPIP2;4 protein levels, as shown by immunohistochemical analysis. Conversely, no notable increase in Lp_r was observed in the Ox lines. These results suggest that OsPIP2;4 is expressed in appropriate tissues in rice roots and is a key factor influencing Lp_r. This research represents a significant step toward further understanding the physiological functions of OsPIP2;4 in rice.

Keywords PIP aquaporin · Rice · Root hydraulic conductivity · Root pressure chamber

Introduction

The first aquaporin (AQP) was discovered as a water transporter in human red blood cells (Preston et al. 1992). The functional presence of AQPs in nearly all bacterial, animal, and plant cells indicates their essential roles in living cells. The molecular mechanisms by which AQPs select and transport water molecules have been well studied (Murata et al. 2000). In plants, AQPs are categorized into subfamilies: 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) (Laloux et al. 2018). Among these, PIPs are crucial for maintaining cellular water balance (Maurel and Chrispeels 2001; Tyerman et al. 1999) and play a role in root water uptake (Gronden et al. 2016).

Three pathways are involved in radial water transport in roots: the apoplastic, symplastic, and cell-to-cell pathways (Steudle and Peterson 1998). In the apoplastic pathway, water moves through the cell walls or intercellular spaces. The symplastic pathway is a cytoplasmic continuum between adjusting cells mediated by plasmodesmata or a water transport path that includes a cytoplasmic continuum and vacuoles. In water transport, water permeability in the tonoplast is generally much higher than that in the plasma membrane because of the abundance of TIPs, and the

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Published online: 01 February 2026

tonoplast does not block symplastic water transport (Tyerman et al. 1999). The cell-to-cell pathway involves movement across the cell wall and membrane. In experiments, the latter two pathways are often difficult to measure separately and are usually combined into a single cell-to-cell/symplastic pathway. PIPs play a key role in water transport in the cell-to-cell/symplast pathway. Although the ratio of water flow between the apoplastic and cell-to-cell/symplastic pathways changes depending on environmental conditions (Steudle 2000), the cell-to-cell/symplastic pathway is critical for root water transport under normal growth conditions (Javot and Maurel 2002). PIPs are believed to be essential for achieving physiologically sufficient root hydraulic conductivity (Lp_r). To understand the water absorption of roots and plants' response and tolerance to water stress conditions, it is essential to measure Lp_r and determine its value quantitatively. As with other plants, several reports have suggested that PIPs are involved in the regulation of Lp_r in rice (Henry et al. 2012; Sakurai-Ishikawa et al. 2011). However, the specific PIP molecular species significantly involved in Lp_r have rarely been investigated. To determine which molecular species among these PIPs significantly contribute to Lp_r, it is necessary to measure Lp_r in knockouts and overexpression lines of specific PIP molecular species. Identifying PIPs that are significantly involved in Lp_r provides a molecular basis for understanding the physiology and regulation of Lp_r.

In the present study, Lp_r in rice was investigated among OsPIP2;4-knockout or -overexpressing lines. Most OsPIPs are expressed in the roots (Sakurai et al. 2008; Sakurai-Ishikawa et al. 2011). Among them, OsPIP2;3, 2;4, and 2;5 are less expressed in leaves and more specifically expressed in roots (Matsumoto et al. 2009). Among these three OsPIP2s, OsPIP2;4 showed the highest expression in roots (Matsumoto et al. 2009). Water transport activity of OsPIP2;4 has been measured in the heterologous expression systems using yeast spheroplast (Sakurai et al. 2005) or a frog *Xenopus leavis* (*X. leavis*) oocytes (Matsumoto et al. 2009; Tran et al. 2025). Only one study has reported OsPIP2;4 overexpressing rice with Lp_r estimation (Nada and Abogadallah 2020), and no study has reported OsPIP2;4-knockout rice with Lp_r measurements. Two independent knockout lines were prepared in the present study: one by CRISPR-Cas9 in the Nipponbare background and the other by T-DNA insertion in the Hwayong background. In addition, OsPIP2;4-overexpressing rice lines were examined in the present study and compared with previous reports on ZmPIP2;5-overexpressing maize (Ding et al. 2020) and OsPIP2;4-overexpressing rice (Nada and Abogadallah 2020). In *Poaceae*, the Casparian strips are present in the root exodermis and endodermis, where apoplastic water transport is limited, shifting water to the cell-to-cell/symplastic pathway via PIPs. Therefore, the exodermis and endodermis play a crucial role in Lp_r (Ding et al. 2020), and PIPs must function properly within these tissues. In the present study, we also examined and visualized how OsPIP2;4 is expressed in root tissues in knockout, overexpression, and wild-type lines.

Materials and methods

Plant materials and growth condition

Japonica rice (*Oryza sativa*) varieties, Nipponbare and Hwayoung as wild types, overexpression lines (Ox), and knockout (KO) with CRISPR-Cas9 or T-DNA insertion lines were subjected to investigation. Seeds were sterilized with 1 mL sodium hypochlorite solution (Nacalai, Kyoto, Japan), 5 µL Tween-20 (Wako, Osaka, Japan), and 4 mL distilled water (DW) for 50 min and then washed with DW five times. Seeds were incubated for 6–7 days to germinate in 1 mM $CaSO_4$ solution under dark conditions at a continuous temperature of 20 °C. Seedlings (6 or fewer for root water permeability measurements, or 10 or fewer for RNA extraction per pot) were transferred to 1/5,000 A Wagner pots filled with 3.5 L of nutrition solution (4 mM KNO_3, 1 mM $NH_4H_2PO_4$, 1 mM $CaCl_2 \cdot 2\ H_2O$, 0.42 mM $MgSO_4 \cdot 7\ H_2O$, 9.1 µM $MnCl_2 \cdot 4\ H_2O$, 0.32 µM $CuSO_4 \cdot 5\ H_2O$, 0.77 µM $ZnSO_4 \cdot 7\ H_2O$, 46.3 µM H_3BO_3, 97 µM Na_2MoO_4, 0.11 mM NaOH, 17.5 µM $FeCl_3 \cdot 6\ H_2O$, 19.6 µM $C_6H_8O_7$). Plants were grown hydroponically under a controlled environment (12-h dark at 25 °C and 12-h light at 28 °C, with fluorescent lamps of 150 µmol $m^{-2}\ s^{-1}$) with a nutrition solution changed every 2-3days.

Isolation of the T-DNA inserted OsPIP2;4 knockout mutant

T-DNA insertion rice mutants of the *OsPIP2;4* gene were surveyed using the Rice Functional Genomic Express Database (http://signal.salk.edu/cgi-bin/RiceGE). An ideal candidate for the null mutant was identified in the T-DNA population using japonica rice cultivars (PFG_1C-08304.L; cv. Hwayoung) (An et al. 2003). The homozygous allele was selected by genomic PCR using the following primers (5' to 3'): 08304_Forward, TGATCAACAGATATGGTGG TCC; 08304_Reverse, GTGCAAACACAAATCGATGG; Left border_GA2707: GGTGAATGGCATCGTTTGAA.

OsPIP2;4 knockout by CRISPR-Cas9

To knockout *OsPIP2;4* by CRISPR-Cas9, pZH_gYSA_ MMCas9 and pU6gRNA vectors were used (Mikami et

al. 2015). Candidate targeting sites in the *OsPIP2;4* gene were designated by using CRIPR-P (http://cbi.hzau.edu.cn/cgi-bin/CRISPR; Lei et al. 2014). The 45–67 bp region of the coding sequence of the gene was chosen as the target of the guide RNA: 5'-GCGGCGGGTCGATGTAGTCCCG G-3' (note that this sequence corresponds to the antisense strand of the *OsPIP2;4* gene). BbsI site-attached forward and reverse primers were prepared for annealing. Annealed double-strand fragments were subcloned into the BbsI site of pU6gRNA. Then the guide RNA expression cassette was subcloned into pZH_gYSA_MMCas9 by using AscI and PacI. The EHA105 strain of *Agrobacterium tumefaciens* harboring the constructed pZH_gYSA_MMCas9 was used to produce transgenic rice. Hygromycin-resistant calli were selected on N6D medium plates (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan). Organ redifferentiation of the selected calli was induced on redifferentiation medium, which contained 30 g L^{-1} sucrose, 30 g L^{-1} D(-)-sorbitol, 2 g L^{-1} casamino acids, 4.6 g L^{-1} MS powder (FUJIFILM Wako Pure Chemical Corp.), MS vitamins, 0.2 g L^{-1} NAA, 0.2 g L^{-1} kinetin, 0.1 g L^{-1} carbenicillin, and 0.05 g L^{-1} hygromycin. The pH of the medium was adjusted to 5.8, and 4 g L^{-1} gelrite (FUJIFILM Wako Pure Chemical Corp.) was used to solidify the medium. Redifferentiating calli were then transferred onto hormone-free medium, which contained 30 g L^{-1} sucrose, MS powder, MS vitamins, 0.1 g L^{-1} carbenicillin, and 0.05 g L^{-1} hygromycin. pH adjustment and solidification were performed as in the case of the redifferentiation medium. Each redifferentiated individual was eventually transferred to a plastic pot filled with soil, and all candidate plants were grown in a containment greenhouse for harvesting seeds.

Overexpression of *OsPIP2;4*

Overexpression (Ox) lines of OsPIP2;4 under the control of the actin promoter were generated in the Nipponbare background using the derivative vector pSMAHACTG2 (Fig. S1). This vector was constructed based on pSMAHdN636L-GateA (Hakata et al. 2010). The coding region of *OsPIP2;4* was subcloned into the pENTR™/D-TOPO-vector (Invitrogen, USA) using the pENTR™/D-TOPO cloning kit (Invitrogen). Then the coding region was cloned into the pSMAHACTG2 vector using the Gateway LR Clonase Enzyme Mix (Invitrogen). The pSMAHACTG2 vector is a binary Ti plasmid with an actin promoter and GATEWAY cassette. After the resulting construct was transformed into the EHA105 strain of Agrobacterium tumefaciens, the calli differentiated from embryos of Nipponbare seeds were infected with the strain. Hygromycin-resistant calli were selected on N6D medium plates, and the redifferentiating calli were then transferred to hormone-free medium. Each redifferentiated individual was eventually transferred to a plastic pot filled with soil, and all candidate plants were grown in a growth chamber for harvesting seeds.

RNA extraction and quantitative real-time qPCR

Roots and shoots were separately harvested from 14-day-old plants three hours after dawn. Roots and shoot tissues were frozen in liquid nitrogen and ground in mortar. RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Venlo, Netherlands), and the RNA concentration was measured using NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA).

The extracted RNA was reverse-transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) following the manufacturer's instructions, with 50 ng of total RNA and a random primer provided in the kit. PCR products were amplified using the Applied Biosystems 7300 Real-Time PCR System with Power SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA). The expression levels of *OsPIPs* were measured using the absolute quantification method (Sakurai-Ishikawa et al. 2011). The quantified data were normalized to the internal standard *eEF-1α* (Jain 2009; Li et al. 2011) transcript as a reference, if necessary. Primers for qPCR were designed in the UTR regions, as listed in Table S1. Because the 3'-UTRs of *OsPIP2;4* differ between Nipponbare and Hwayoung, Hwayoung-specific primer sets for *OsPIP2;4* were also designed. No UTRs were added in the case of overexpression. Therefore, another primer set for *OsPIP2;4* (OsPIP24C) was designed, with one primer in the coding region and the other near the stop codon of OsPIP2;4, including a few additional nucleotides from the vector. The amount of *OsPIP2;4* transcript in the Ox lines was calculated as the sum of the data from the PIP24C primers and regular primers (overexpression + endogenous).

Screening of mutagenized rice lines by DNA sequencing and CAPS analyses

Genomic DNA was extracted from the leaves of T0 transgenic candidate seedlings using a DNA extraction buffer composed of 100 mM Tris-HCl (pH 9.5), 1 M KCl, and 10 mM EDTA. The genomic fragment of the OsPIP2;4 gene in each candidate plant was amplified by PCR using the following primers: forward, 5'-AGGGAATATTAAGCTTAT GGGCAAAGAGGTG-3' and reverse, 5'-CGTTACTAGT GGATCCCTACGCGTTGCTCCG-3'. The resulting fragments were cloned into pYES2 using the In-Fusion cloning system (Takara Bio, Shiga, Japan), and the DNA sequences of the cloned fragments were analyzed by Sanger sequencing. C-deletion and T-insertion mutations in the g38-1-3 line

were detected using the CAPS method. T1 seeds of the line were germinated, and the seedlings were grown hydroponically. Genomic DNA was isolated from randomly selected T1 seedlings as described above and concentrated through ethanol precipitation. Genomic PCR was performed using PrimeSTAR Max DNA Polymerase (Takara Bio, Shiga, Japan) with the following specific primers: forward, 5'-AGTACCACTGAAACCCCTATAAATATA-3' and reverse, 5'-AAAGTTTATGCACTTACGGGAACAT-3'. The amplified fragments were purified using the NucleoSpin Gel and PCR Cleanup Kit (Takara Bio, Shiga, Japan). Mutations involving C-deletion and T-insertion were subsequently examined by restriction enzyme digestion with BciVI (for C-deletion, 37 °C for 1 h) and BsaBI (for T-insertion, 60 °C for 1 h), respectively. Restriction polymorphisms were visualized through gel electrophoresis using a 2.5% agarose gel.

Measurement of osmotic water permeability (*Pf*) using the oocytes heterologous expression system

The coding regions of *OsPIPs* were subcloned into the oocyte expression vector (Tran et al. 2025). Isolation of *X. laevis* oocytes and preparation of capped RNAs (cRNAs) were carried out as described previously (Katsuhara et al. 2002). Each oocyte was injected with 50 ng of cRNA in 50 nLof solution or only 50 nL of water (as negative control, NC). After a one-day incubation to allow OsPIP protein expression, oocytes were subjected to the swelling assay. Oocytes were transferred from culture medium (200 mOsm) to the diluted solution 5-fold (40 mOsm) to induce water influx. Osmotic water permeability (*P*f) values were calculated based on changes in cell volume (water influx), treatment duration, osmotic difference between solutions, average oocyte surface area, and average oocyte volume (Katsuhara et al. 2002).

Measurement of the root hydraulic conductivity (Lp_r)

We measured Lp_r using the pressure chamber method, as previously described by Horie et al. (2011), with some modifications. In the present study, Lp_r was measured in 14-day-old plants for three–six hours after dawn. The roots were enclosed in a pressurized hydroponic solution, and exudates were collected from the cut surface of the shoot using cotton balls at 0.12 and 0.08 MPa, every 80 s, eight times at each pressure. The collected exudates were weighed (AE163, Mettler Instruments, Switzerland). The exudation rate normalized by root surface area (Jv, m s^{-1}) at one pressure condition was calculated from the exudate volume (converted from weight) using Eq. 1. The root-projected area was measured using the WinRHIZO system (Regent Instruments, Canada). The difference of two Jv (ΔJv) from two different pressures and the pressure difference (ΔP) was estimated to calculate the root hydraulic conductivity (Lp_r, m sec^{-1} MPa^{-1}, Eq. 2).

$$Jv\,(\mathrm{m\,sec^{-1}}) = \mathrm{Exudate}\,(\mathrm{m^3}) \,/\, (\mathrm{root\ projection\ area}\,(\mathrm{m^2}) \times \pi \times \mathrm{time}\,(\mathrm{sec})) \quad (1)$$

$$Lp_r\,(\mathrm{m\,sec^{-1}MPa^{-1}}) = \Delta Jv/\Delta P\,(\mathrm{MPa}) \quad (2)$$

Immuno-histochemical staining

The localization of OsPIP2;4 was analyzed using the immunohistochemical staining method described previously (Tran et al. 2025). The anti-OsPIP2;4 antibody was designed to recognize the amino acid sequence "VDVSTLEAGGAR" at the N-terminus of OsPIP2;4. In the present study, secondary antibodies (anti-rabbit IgG goat antibody) conjugated with Alexa 488 (Invitrogen) were used. After washing five times with PBS, the samples were observed using a confocal laser microscope (FV1000-D, OLYMPUS, Tokyo, Japan).

Statistical analysis

All data were analyzed by MS Excel and R studio (ver.3.5.3). One-way ANOVA and Tukey–Kramer or Tukey–HSD methods, or Student's *t*-test were applied to detect significant differences.

Results

Expression properties of *OsPIP* genes in rice and water transport activity of each OsPIP in the oocyte heterologous expression system

The amount of 11 OsPIP transcripts was quantified in the Nipponbare cultivar using real-time PCR (Fig. 1a). Each expression level was normalized to that of the transcriptional factor eEF1-α. The expression levels of seven genes—*OsPIP1;2*, *OsPIP1;3*, *OsPIP2;1*, *OsPIP2;3*, *OsPIP2;4*, *OsPIP2;5*, and *OsPIP2;6*—in the roots were significantly higher than in the shoots. Notably, *OsPIP2;4* showed the highest expression in roots, with a 14-fold increase compared to leaves (0.83 in roots vs. 0.06 in shoots), consistent with a recent report (Tran et al. 2025). The *OsPIP2;4* transcript was the most abundant among *OsPIP2s* in the roots of 14-day-old Nipponbare plants (Figs. 1a, S2). Although transcripts of *OsPIP1s* were abundantly detected in roots (Fig. 1a), the water transport activity of OsPIP1s was not observed in the oocyte system when expressed alone (Fig. 1b). In contrast, OsPIP2;4, OsPIP2;5, and some other

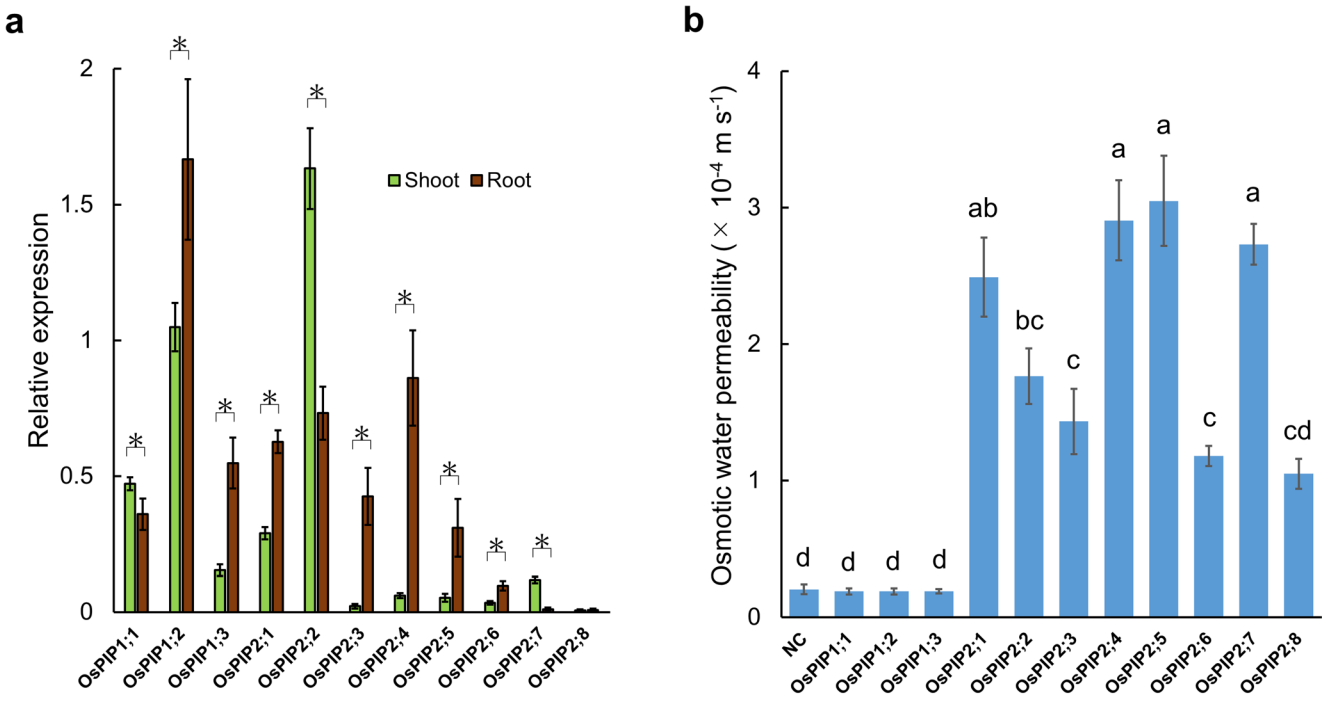

Fig. 1 Expression and water transport activity of OsPIPs. **a** Transcript levels of each *OsPIP* in the roots and shoots of 14-day-old rice are shown. Each data was normalized by *eEF1-α*, and each bar represents the mean ± SD ($n = 3$). Significant differences (*: $p < 0.05$) were determined by Student's *t*-test between roots and shoots for each *OsPIP* transcript. **b** The osmotic water permeability (*Pf*) of *X. laevis* oocytes expressing OsPIPs was analyzed using a swelling assay (see Materials and Methods for details). Water (no cRNA, NC) or 50 ng/50 nL of each cRNA was injected into the oocytes ($n = 6$–12). Each bar shows the mean ± SE. Different letters indicate significant differences determined by one-way ANOVA and Tukey–Kramer test

PIP2s induced a high *Pf* in oocytes (Fig. 1b). Therefore, we focused on analyzing the *OsPIP2;4* gene in the following sections.

Characterization of the *OsPIP2;4* knockout line harboring T-DNA insertion

The rice mutant, in which the *OsPIP2;4* gene was disrupted by the insertion of the T-DNA (> 10,000 bp long) in its first exon, was found in the database of the rice T-DNA mutant population (An et al. 2003). We identified and selected a homozygous knockout (KO) line (Fig. 2a). In this *OsPIP2;4* KO (T-DNA), almost no *OsPIP2;4* transcripts were detected in the roots (Fig. 2b). The expression of most other *OsPIP2* genes remained unchanged in the KO (T-DNA) line. Although there was a slight increase in *OsPIP1;2* expression in the KO (T-DNA) line, the total *OsPIP* transcripts were slightly lower than those in the WT (Hwy) (Fig. 2b). In immunohistochemical analysis (Fig. 2c), only very weak fluorescence was detected in the roots, especially sclerenchyma, endodermis, pericycle, and exodermis, of the KO (T-DNA) line compared to the WT (Hwy), indicating a substantial decrease in the OsPIP2;4 protein level in KO (T-DNA) roots, consistent with the reduction in *OsPIP2;4* transcripts. Corresponding to the decreased transcript and protein levels, pressure chamber analysis using rice seedlings revealed that the Lp_r of KO (T-DNA) plants was significantly lower than that of WT (Hwy) plants (Fig. 2d).

Characterization of the CRISPR-Cas9-mediated *OsPIP2;4* knockout line

We generated another *OsPIP2;4* mutant allele using the CRISPR-Cas9 system, targeting the first exon of the *OsPIP2;4* gene in cv. Nipponbare. As a result, two independent mutations were identified in T0 plants: one was a C-deletion that created a stop codon, resulting in the production of only 40 amino acids that were entirely different from the original 286 amino acids in the full-length OsPIP2;4 protein (Figs. 3a and S3). The other is a T-insertion, which produces 248 amino acids, forming an artificial hydrophilic sequence without a transmembrane domain at the target site (Figs. 3a and S3). Sequence analysis of the T0 generation suggested that both null mutations could have occurred simultaneously in a single mutant line. We then examined the genotype of the next generation (T1).

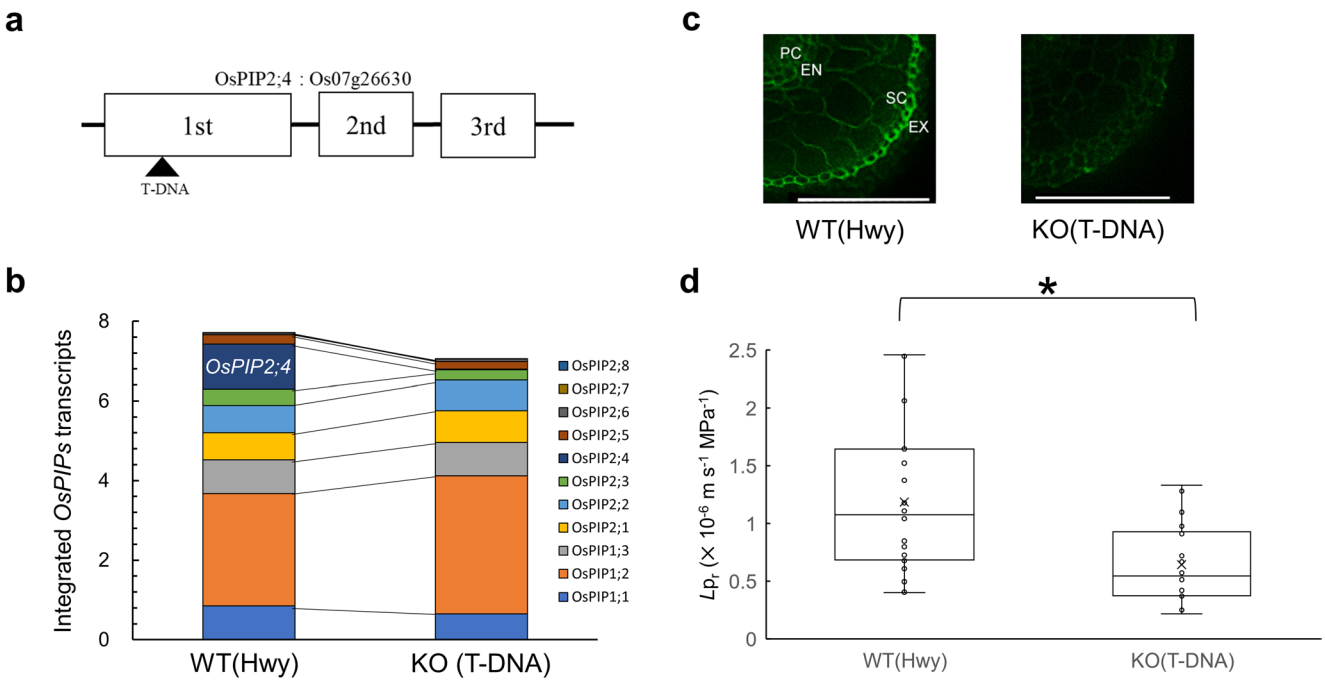

Fig. 2 Characterization of the *OsPIP2;4* knockout line harboring the T-DNA insertion. **a** Molecular structure of the *OsPIP2;4* gene in the T-DNA-insertion line. T-DNA was inserted into the 1st exon, as indicated by a black triangle. **b** Total *OsPIPs* expression levels in the roots of Hwayoung and KO plants. Each *OsPIP* transcript amount was normalized to the expression level of the transcriptional factor *eEF1-α* and was integrated. **c** Immunohistochemical analysis using root cross-sections derived from the mature root region (approximately 40 mm from the root tip) of 14-day-old plants. OsPIP2;4 proteins were visualized using green fluorescence. The abbreviations in the left image are as follows: EX, exodermis; SC, sclerenchyma; EN, endodermis; and PC, pericycle. The bar represents 100 µm. **d** Lp_r of WT (Hwy) ($n=27$) and KO (T-DNA) ($n=22$). Significant differences (*: $p<0.01$) were determined by Student's *t*-test between WT (Hwy) and KO (T-DNA)

Figure 3b and c show the results of CAPS analysis of randomly selected T1 seedlings (lanes 1–9). Homozygous and heterozygous C-deletions were identified by two and three bands, respectively, while a single band represented the WT allele after digestion with BciVI (Fig. 3b). Homozygous and heterozygous T-insertions appeared as three and four bands, respectively, in contrast to two bands for the WT allele after digestion with BsaBI (Fig. 3c). Based on these results, the lines selected for subsequent analysis included lines 5 and 9 as C-deletion homozygotes, lines 2 as a T-insertion homozygote, and lines 6 and 8 as heterozygotes with both a C-deletion and a T-insertion (also OsPIP2;4 null). Line 4 is the heterozygote with a T-insertion (Fig. 3c), still, no C-deletion (Fig. 3b), indicating it is not null. When the pressure chamber method was applied to these mutants and WT, the results revealed that the Lp_r of KO (CRISPR-Cas9) plants was significantly lower than that of WT (Nip) (Fig. 3d). The difference between the mutants and WT was statistically significant ($p<0.01$).

Characterization of *OsPIP2;4* Ox lines

Under the control of the actin promoter (Fig. 4a), two independent *OsPIP2;4*-overexpression lines (Ox#1 and Ox#2) were generated. Quantification of *OsPIPs* transcripts in Ox#1 confirmed an increase in *OsPIP2;4* transcripts (Fig. 4b). The transcripts of some other *OsPIPs*, such as *OsPIP1;2*, *OsPIP1;3*, and *OsPIP2;2*, were decreased, but the total transcripts of *OsPIPs* in Ox#1 accumulated more than in WT (Nip) (Fig. 4b). Immunostaining of OsPIP2;4 revealed ubiquitous and high fluorescence in the roots of both Ox lines (Fig. 4c), suggesting that OsPIP2;4 proteins were highly expressed in all root tissues. These data indicate the abundant presence of *OsPIP2;4* transcripts and proteins in Ox roots. However, Lp_r measurements indicated no significant increase in Lp_r compared to that in WT (Fig. 4d). Surprisingly, the Ox#2 line rather showed significantly lower Lp_r than WT, even though a much higher amount of the *OsPIP2;4* transcript was detected in Ox#2 than in Ox#1, and ubiquitous overexpression of the OsPIP2;4 protein was found in Ox#2 (Figs. 4c and d, S4). Unknown dominant-negative effects might have reduced the Lp_r of OX#2. These results indicate that no significant enhancement of Lp_r

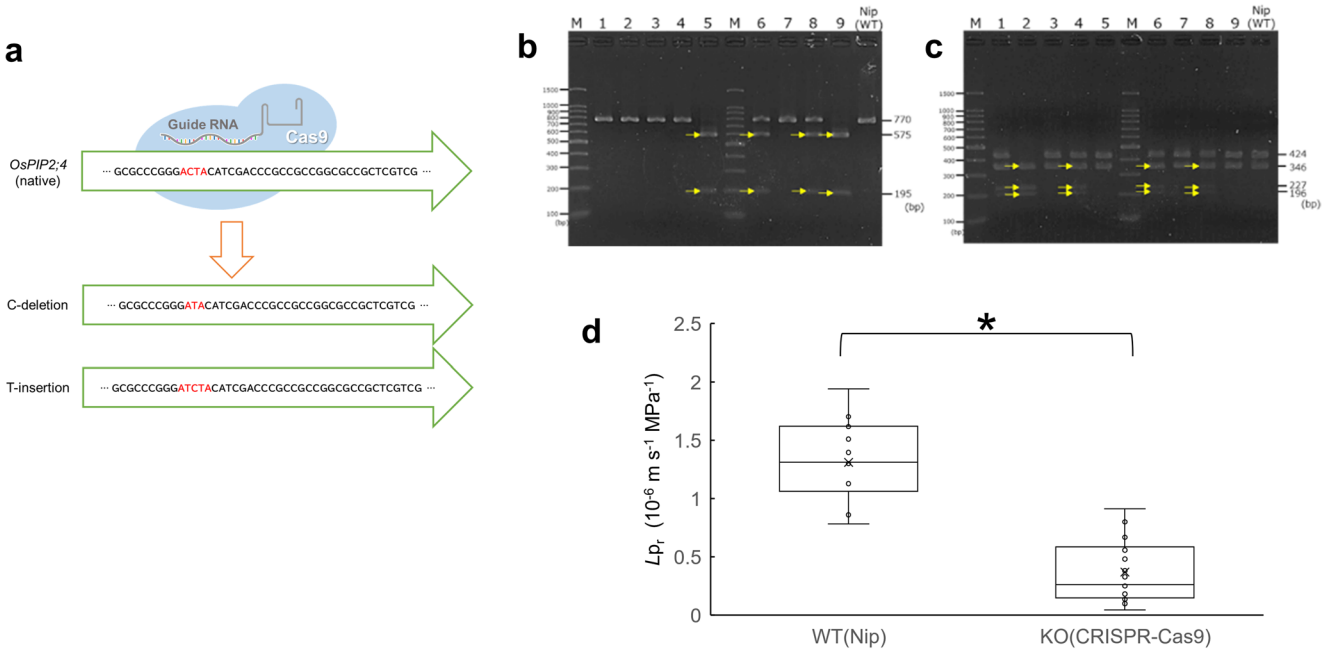

Fig. 3 Characterization of the CRISPR-Cas9-mediated *OsPIP2;4* knockout line. **a** Schematic drawing of the target site in the *OsPIP2;4* gene (the N-terminal) for CRISPR-Cas9-based mutagenesis: Nipponbare background (native) and two mutant (C-deletion, T-insertion) lines. The WT and mutated sequences are shown in bold red. **b**, **c** CAPS analysis of C-deletion and T-insertion mutations in the *OsPIP2;4* gene in randomly selected T1 seedlings. Restriction polymorphisms after gel electrophoresis are shown. (b) BciVI digestion for C-deletion: Homozygous and heterozygous seedings are supposed to show mainly two and three bands, respectively, in comparison with a single band of the WT allele. **c** BsaBI digestion for T-insertion: Homozygous and heterozygous seedings are supposed to show mainly three and four bands, respectively, in comparison with two bands of the WT allele. Lanes 1–9 represent randomly selected T1 seedlings. Nip (WT): Nipponbare wild type. M: Marker. **d** Lp_r of WT (Nip) ($n=14$) and KO (CRISPR-Cas9) ($n=18$). Significant differences (*: $p<0.01$) were determined by Student's *t*-test between wild-type Nipponbare and KO

occurred in the *OsPIP2;4*-overexpressing lines examined in the present study.

Discussion

In addition to water, several AQPs transport low-molecular-weight substances (Sun et al. 2024). However, in the present study, the primary focus was on the physiological water transport in rice roots. Among the *OsPIP2* genes highly expressed in rice roots, *OsPIP2;4* showed the highest expression in the cultivar Nipponbare in the present study (Figs. 1a, S2), as well as in a previous study using the cultivar Somewake (Matsumoto et al. 2009). The high root-specificity of OsPIP2;4 expression may indicate that OsPIP2;4 has an important role in root function, and implies that OsPIP2;4 may be a key player in Lp_r formation in rice.

Two types of conductivities have been measured to study root water transport: the root single-cell hydraulic conductivity (Lp_c) and the root hydraulic conductivity (Lp_r), related to the radial water transport pathway of the whole root. Lp_c is measured using the root pressure probe method (Javot et al. 2003), while Lp_r is measured by the pressure chamber method (see Materials and Methods, this paper). There are two reports of Lp_c measurements using a single *PIP* knockout mutant line. One is *AtPIP2;2*-KO in *Arabidopsis thaliana* (Javot et al. 2003), and the other is *ZmPIP2:5*-KO in maize (Ding et al. 2020). Both studies reported a decrease in Lp_c in root cortex cells. In two AtPIP2;2-KO lines (Javot et al. 2003), reductions of 28 or 27% were observed in Lp_c, with a significant difference from the wild type ($p<0.05$). In a *ZmPIP2:5*-KO line, Lp_c decreased significantly ($p<0.001$) by 63% compared to the wild type (Ding et al. 2020). However, Lp_c may not necessarily be correlated with Lp_r (Hachez et al. 2012), and the effect of these genes on Lp_r was difficult to evaluate in theory.

As for the Lp_r measurements of knockdown and knockout lines of PIP2s and PIP1s, some reports have been published as follows. An example of the knockdown of the rice *OsPIP2;1* gene by RNAi has been reported (Ding et al. 2019). *OsPIP2;1* showed the highest expression among *OsPIP2s* in roots in the study of Ding et al. (2019). In the present study, the gene expression level was the third highest among OsPIP2s in roots (Fig. 1a). Lp_r was significantly

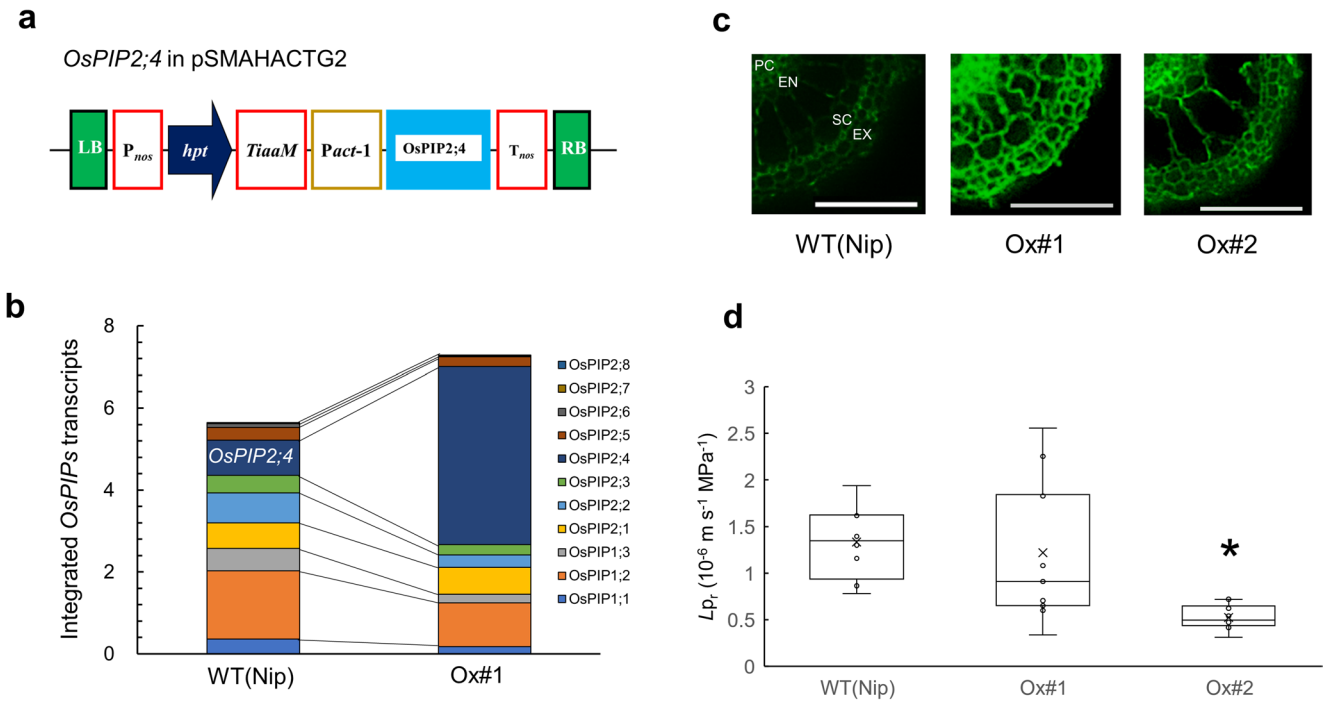

Fig. 4 Characterization of *OsPIP2;4* overexpressing (Ox) line. **a** Key construct region of the vector "pSMAHACTG2" used for the production of *OsPIP2;4*-overexpressing lines. **b** Total *OsPIPs* expression levels in the roots of WT (Nip) and Ox#1. Each *OsPIP* transcript level was normalized to the expression level of the transcriptional factor *eEF1-α* and integrated. **c** Immunohistochemical analysis of the root cross-section (mature region) derived from 14-day-old WT(Nip), Ox#1, and Ox#2 plants. OsPIP2;4 proteins were visualized with green fluorescence. Abbreviations in the left image were as follows: EX, exodermis; SC, sclerenchyma; EN, endodermis, and PC, pericycle. The bar represents 100 µm. **d** Lp_r of WT(Nip) ($n=8$), Ox#1 ($n=11$), and Ox#2 ($n=10$). Significant differences (*: $p<0.01$) were determined by Student's *t*-test between WT(Nip) and Ox#2

($p<0.05$) decreased by approximately fourfold in the two *OsPIP2;1* RNAi lines, with a decrease in *OsPIP2;1* expression levels of 70% or 50% compared with wild-type plants (Ding et al. 2019). To measure the Lp_r of *PIP1* KO plants, *AtPIP1;2* in *A. thaliana* (Postaire et al. 2010) and *SvPIP1;6* in green foxtail (*Setria viridis*) have been targeted (Gal et al. 2023). In the case of *AtPIP1;2*, significant reductions in Lp_r ($p<0.02$) of 21% or 33% were observed in two independent KO lines (Postaire et al. 2010). Two independent *SvPIP1;6*-KO lines also showed significant reductions (approximately 50%) in Lp_r (Gal et al. 2023).

The first KO plants of the *PIP2* subfamily were derived from the *AtPIP2;2* gene in *A. thaliana*. However, it has been reported that *AtPIP2;2*–KO plants showed no significant change in Lp_r (Javot et al. 2003). Conversely, in the case of the *ZmPIP2;5* gene, which is the most highly expressed *PIPs* in maize roots and is mainly expressed in the exodermis and endodermis (Fetter et al. 2004; Hachez et al. 2012), the KO plant exhibited a significant reduction in Lp_r of approximately 40% compared with wild-type plants (Ding et al. 2020). However, only one KO T-DNA insertion line was measured in *ZmPIP2;5*-KO plants (Ding et al. 2020).

In our study, we examined two independent OsPIP2;4 KO lines, T-DNA-based and CRISPR-Cas9-mediated mutants. Significantly lower Lp_r values than those of WT were observed in both KO lines (Figs. 2 and 3); that is, the Lp_r values of *OsPIP2;4* knockout lines were approximately 28% (CRISPR-Cas9 line) and 54% (T-DNA line) compared to those of WT. It is currently unclear why the effect of *OsPIP2;4* knockout in Lp_r led to a large difference between T-DNA-based and CRISPR-based mutants. There might be a systematic difference in the composition and regulation of Lp_r between the cultivars used. These results indicate that OsPIP2;4 is a major determinant of Lp_r in rice plants. However, water channel aquaporins are redundant in rice, and it is unlikely that OsPIP2;4 exclusively determines Lp_r. A similar large negative impact of the knockout of a single *PIP2* gene, *ZmPIP2;5*, on Lp_r was reported in maize, in which the authors suggested a possible reason that PIP2 is a key actor facilitating radial water flow and controlling whole-root conductivity (Ding et al. 2020). A similar explanation could be applied to the role of OsPIP2;4 in the regulation of Lp_r in rice plants. Notably, *OsPIP2;5* was reported to be expressed at higher levels than *OsPIP2;4* in the cultivar Akitakomachi (Sakurai et al. 2011). Therefore, it will also be important to

dissect at least *OsPIP2;5* mutants of rice to further understand the OsPIP-mediated Lp_r composition in rice.

Many recombinant plants with AQP overexpression have been produced (Ren et al. 2021). These plants usually grew well under non-stress conditions. However, conflicting results have been reported regarding drought response, with some plants increasing their tolerance while others increasing their sensitivity (Ren et al. 2021). However, few studies have examined Lp_r in plants overexpressing aquaporins. *OsPIP1;3*-overexpressing lines showed higher Lp_r than WT under osmotic stress conditions, although the Lp_r of the plants was similar to that of WT under normal conditions (Liu et al. 2020). *OsPIP2;4*-overexpressing rice plants produced in a previous study resulted in only a slight increase in Lp_r (10–30% increase) compared with WT (Nada and Abogadallah 2020). In the present study, however, Lp_r did not increase in *OsPIP2;4* Ox lines (Fig. 4d). In addition, no increase in Lp_r has been reported in *ZmPIP2;5*-overexpressing maize (Ding. et al. 2020). As a possible explanation, Ding et al. (2020) suggested that the gatekeeper cells of wild-type plants have a saturated membrane permeability, which prevents a further increase in Lp_r. A similar explanation could be given for the results presented in this study: OsPIP2;4 is specifically expressed in the tissues regulating root water permeability (i.e., the endodermis and exodermis) in WT. In fact, our previous work (Tran et al. 2025) and Fig. 2c in this study support this notion. In such conditions in WT, the expression of the OsPIP2;4 protein might be saturated at the rate-limiting sites for water transport. Therefore, the ubiquitous overexpression of OsPIP2;4 in tissues other than the endodermis and exodermis could not lead to enhancement of the Lp_r of the entire root. Intriguingly, overexpression of OsPIP2;4 in Ox#2 resulted in a lower Lp_r than that in wild-type Nipponbare (Fig. 4d). The transcript level of *OsPIP2;4* in Ox#2 was 10 times higher than that in Ox#1 (Fig. S4), and some Ox#2 plants showed poor growth in the early stages of development (data not shown). Thus, overexpression of OsPIP2;4 might cause some dominant-negative inhibition of Lp_r and growth through unknown mechanisms.

OsPIP2;4 KO by T-DNA insertion showed a slight increase in *OsPIP1;2* expression (Fig. 3b). OsPIP1s show no or very low water transport activity when expressed alone in heterologous expression systems using oocytes (Fig. 1b), consistent with previous reports (Ding et al. 2019; Matsumoto et al. 2009; Sakurai et al. 2005), however, heterotetramerization of some PIP1s with PIP2s enhances water transport activity (Bienert et al. 2018; Fetter et al. 2004; Horie et al. 2011; Liu et al. 2013; Matsumoto et al. 2009; Yaneff et al. 2015). Increased *OsPIP1;2* levels in the current KO plants might partially compensate for Lp_r in *OsPIP2;4* KO plants; however, whether this occurs in plants remains unclear. Although our study demonstrated that OsPIP2;4 significantly contributes to Lp_r, it is currently not possible to identify all AQP molecular species involved in Lp_r. Future studies should examine the roles of OsPIP2s and OsPIP1s, particularly their interactions with OsPIP2s, in regulating Lp_r in rice.

This study showed that OsPIP2;4 plays a crucial role in water transport in rice. However, since overexpression did not increase Lp_r, it is likely that a "safety mechanism" is in place, indicating that unnecessary boosting water transport activity could threaten plant survival. In other words, there must be an optimal amount and tissue-localization of AQP expression to achieve optimal water permeability in plants. Water transport activity within plants involves various factors, including interactions with other AQP molecular species (heteromerization) and phosphorylation (Chaumont and Tyerman 2014), which regulate AQP activity and intracellular trafficking (Chaumont and Tyerman 2014). Tissue-specific suberization other than the AQP amounts may be also included in water transport activity in roots (Kreszies et al. 2019). To understand the physiological mechanisms connecting AQP expression levels and water transport activity, further analysis is needed. In addition, future studies using various *OsPIP2;4* mutant alleles will be required to fully elucidate the physiological roles of OsPIP2;4 in rice.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10265-025-01691-z.

Acknowledgements The authors would like to thank Dr. Sasaki and Mr. Tsuchiya (IPSR, Okayama University, Japan) for their technical assistance. The authors appreciate Dr. G. Horiguchi (National Institute for Basic Biology, Japan) and Dr. H. Ichikawa (Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Japan) for providing the vector for Ox rice lines (their affiliations were at the time of providing). We would also like to thank Drs. Seiichi Toki (Ryukoku University, Japan), Hiroaki Saika (NARO, Japan), and Masaki Endo (NARO, Japan) for providing us with the pZH_OsU6gRNA_MMCas9 vector. The authors also appreciate Dr. Hatano-Murai (NARO, Japan) for the critical discussion on rice AQPs.

Author contributions A.O.: Root hydraulic conductivity analysis, expression analysis (tissue localization), data curation, writing – original draft; T.H.: Conceptualization, Rice mutant line preparation, writing – review and editing: R.I.: Expression analysis (qPCR); S.S.: Water permeability analysis (swelling assay); R.H.: Construction of the plasmid DNA to produceCRISPR-Cas9 rice mutants; Y.M.: Selection and analysis of CRISPR-Cas9 rice mutants for *OsPIP2;4*; J.I.: Ox line preparation, Supervision, Writing –review, and editing; M.M: Root hydraulic conductivity analysis; S.U.: Supervision; Writing, review, and editing; M.K.: Conceptualization, Project administration, Supervision, Data curation, writing –review, and editing.

Funding Open Access funding provided by Okayama University. This work was supported by JSPS KAKENHI Grant Number JP20K06708 to M.K. and T.H., and the Ministry of Education, Culture, Sports, Science and Technology (MEXT) as part of the Joint Research Program implemented at the Institute of Plant Science and Resources, Okayama University, Japan (R415, R515, and R619 to T.H.). Public Interest In-

corporated Foundation Ohmoto Ikueikai to A.O.

Data availability The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest All authors of this study declare no conflict of interest or personal relationships that could influence the work presented in this paper.

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