Rice aquaporin OsPIP2;2 is a water-transporting facilitator in relevance to drought-tolerant responses

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Abstract
In rice (Oryza sativa), the PLASMA MEMBRANE INTRINSIC PROTEIN (PIP) family of aquaporin has 11 members, OsPIP1;1 to OsPIP1;3, and OsPIP2;1 to OsPIP2;8, which are hypothesized to facilitate the transport of H2O and other small compounds across cell membranes. To date, however, only OsPIP1;2, OsPIP2;1, and OsPIP2;4 have been demonstrated for substrate selectivity in their source plant (rice). In this study, OsPIP2;2 was characterized as the most efficient facilitator of H2O transport across cell membranes in comparison with the other 10 OsPIPs. In concomitant tests of all OsPIPs, four genes (OsPIP1;3, OsPIP2;1, OsPIP2;2, and OsPIP2;4) were induced to express in leaves of rice plants following a physiological drought stress, while OsPIP2;2 was expressed to the highest level. After de novo expression in frog oocytes and yeast cells, the four OsPIP proteins were localized to the plasma membranes in trimer and tetramer and displayed the activity to increase the membrane permeability to H2O. In comparison, OsPIP2;2 was most supportive to H2O import to oocytes and yeast cells. After de novo expression in tobacco protoplasts, OsPIP2;2 exceeded OsPIP1;3, OsPIP2;1, and OsPIP2;4 to support H2O transport across the plasma membranes. OsPIP2;2-mediated H2O transport was accompanied by drought-tolerant responses, including increases in concentrations of proline and polyamines, both of which are physiological markers of drought tolerance. In rice protoplasts, H2O transport and drought-tolerant responses, which included expression of marker genes of drought tolerance pathway, were considerably enhanced by OsPIP2;2 overexpression but strongly inhibited by the gene silencing. Furthermore, OsPIP2;2 played a role in maintenance of the cell membrane integrity and effectively protected rice cells from electrolyte leakage caused by the physiological drought stress. These results suggest that OsPIP2;2 is a predominant facilitator of H2O transport in relevance to drought tolerance in the plant.
1 | INTRODUCTION

Aquaporins (AQPs) are integral membrane proteins initially defined as “water channels” in all living organisms (Agre, 2004; Agre et al., 1993; Brown, 2017; Preston et al., 1992) but subsequently found to have a broader spectrum of cargo (substrate) selectivity among about 20 compounds (Laloux et al., 2018; Rhee et al., 2017). By the substrate-transporting role, AQPs participate in many physiological and pathological processes in animals (Brown, 2017; He & Yang, 2019; Bollag et al., 2020; Wang, Schoebel, et al., 2020; Wang, Zhang, et al., 2020) and plants (Laloux et al., 2018; Li et al., 2019; Singh et al., 2020; Tian et al., 2016). For example, if an AQP serves as a H2O-transporting channel, it will be associated with water relations and drought tolerance under most circumstances, possibly in all organs or throughout full life cycle, either in animals (Brown, 2017; de Laurentis et al., 2020; Li & Wang, 2017) or in plants (Hoai et al., 2020; Li & Wang, 2017; Plett et al., 2020; Vishwakarma et al., 2019). In plants, AQPs fall into five major phylogenic families, including the plasma membrane (PM) intrinsic protein (PIP) family. The PIP family is further divided into the PIP1 subfamily, which contains a varied number of PIP1 proteins from PIP1;1 to PIP1;12, and the PIP2 subfamily, which comprises several PIP2 isoforms from PIP2;1 to PIP2;12, in different plant species (Laloux et al., 2018). These proteins are believed to facilitate the transport of different substrates across PMs in an overlapping or redundant manner (Brown, 2017; Maurel et al., 2015a, 2015b). However, substrates transported by only a small number of PIPs have been determined so far, indicating the imminent necessity to characterize substrate selectivity of most PIPs in most plant species, especially food crops (Laloux et al., 2018; Singh et al., 2020).

In food crops, rice (Oryza sativa) occupies a prominent position in global food security due to its vast production that is used to feed a huge population in the world. Rice also is representative of cereal crops with respect to the function of PIPs/AQPs in H2O transport tightly associated with water utility, osmotic response, and drought tolerance (Afzal et al., 2016; Barzana et al., 2020; Lee et al., 2003; Shekoofa & Sinclair, 2018; Vishwakarma et al., 2019). Drought tolerance is important not only to drylands agriculture (Ayadi et al., 2019; Zhang, Hu, et al., 2019) but also for flooding crops, typically like rice planted in the conventional agriculture (Ding et al., 2015, 2019; Grondin et al., 2016; Henry et al., 2012; Oladosu et al., 2019; Sriskantharajah et al., 2020; Vinnakota et al., 2016). In many countries that possess a large population with relatively little arable land, coastal wetland and salt licks have been reclaimed for rice planting (Chen et al., 2015; Zong et al., 2007; Mañosa et al., 2001; Withers, 2002). Compared with other cereal crops, rice has more excessive transpiration from leaves and lower hydraulic conductance of roots (refer to Nada & Abogadallah, 2020). Therefore, rice is more sensitive to water deficit, to which the water-transporting role of AQPs/PIPs provides an effective tolerance for survival (Ding et al., 2015, 2019; Grondin et al., 2016; Oladosu et al., 2019; Vinnakota et al., 2016).

Functional multiplicity is a common characteristic of eukaryotic AQPs (Brown, 2017), while PIPs may be more multifaceted in rice than in other plants, especially the biological model Arabidopsis thaliana. Rice has almost 36-fold larger genome size than Arabidopsis but does not have a homolog of AtPIP1;4 and AtPIP1;5 (Sakurai et al., 2005). Both rice and Arabidopsis have the same number of members (PIP2;1 to PIP2;8) in the PIP2 subfamily, but the number of members is different in the PIP1 subfamily. The PIP2 subfamily is consisting of AtPIP1;1 to AtPIP1;5 in Arabidopsis but only has OsPIP1;1 to OsPIP1;3 in rice (Laloux et al., 2018). Presumably, OsPIPs have a higher degree of

| OsPIPs | Substrates | Study systems | Evaluation criteria | Regulated processes | References |
|--------|------------|---------------|---------------------|-------------------|------------|
| 1;1, 1;2, 1;3, 2;1, 2;2, 2;4, 2;6, 2;8 | None tested | Rice | Gene expression levels | Drought tolerance assumed | Grondin et al., 2016 |
| 2;1, 2;1, 2;2, 2;3, 2;4, 2;5 | H2O | Yeast | Stopped-flow spectrometry | Osmotic response | Sakurai et al., 2005, 2008 |
| 1;3, 2;3, 2;4, 2;5 | None tested | Rice | Gene expression levels | Chilling response | Sakurai et al., 2005, 2008 |
| 2;1 | CO2 | Rice | Curve-fitting | Photosynthesis | Xu et al., 2019 |
| 1;2 | H2O | Yeast | Membrane water permeability | Water relations | Ding et al., 2019 |
| | Rice | Root hydraulic conductivity | Water relations | | |
| 2;4 | H2O | Rice | Root hydraulic conductivity and xylem sap flow | Water relations | Nada & Abogadallah, 2020 |

Abbreviation: PIP, plasma membrane intrinsic protein.
functional multiplicity. To date, however, OsPIPs have received poor attentions in contrast to extensive understandings of the homologs in other plants with respect to the fundamental importance for substrate transport (Bezerra-Neto et al., 2019; Kromdijk et al., 2020) and functional regulation mechanisms (Kukulski et al., 2005; Kirscht et al., 2016; Wang, Wang, et al., 2018; Singh et al., 2020). While several PIPs have been characterized as transport channels for specific substrates in biological model plants like Arabidopsis and cereal crops like barley (Fox et al., 2017; Sadura et al., 2020), only three OsPIPs were studied with respect to substrate selectivity in their source plant (rice) (Table 1). OsPIP1;2 was recently shown to support mesophyll CO₂ transport and phloem sucrose transport (Xu et al., 2019). This protein was identified as a physiologically relevant facilitator of CO₂ transport across PMs of rice cells based on gas exchange measurements performed on leaves of the wild-type (WT) and OsPIP1;2-overexpressing rice plants (Xu et al., 2019). However, direct evidence is still lacking to demonstrate the role of OsPIP1;2 in mediating sucrose transport through the phloem PMs or sieve plates. OsPIP1;4 was characterized as an H₂O-transporting channel by analyzing root hydraulic conductivity and xylem sap flow in the WT and OsPIP2;4-overexpressing rice plants (Nada & Abogadallah, 2020). OsPIP2;1 was demonstrated to be a H₂O-transporting facilitator based on measurements of hydraulic conductivity in root cells of the WT and OsPIP2;1-silenced rice plants (Ding et al., 2019). In contrast, substrate selectivity of OsPIP1;1, 1;3, 2;2, 2;3, 2;5, 2;6, and 2;8 was tested in yeast but not in their source plant (Table 1).

In the present study, we analyzed OsPIPs with respect to their presence or absence in H₂O transport across living cell PMs according to the original definition of “water channels” (Agre, 2004; Agre et al., 1993; Brown, 2017; Preston et al., 1992). We further determined the role of OsPIP2;2 in the plant resistance to a physiological drought stress caused by Polyethylene glycol 6,000 (PEG₆₀₀₀). PEG with molecular mass 4,000–8,000 effectively induces physiological drought in a variety of plant species, and PEG₆₀₀₀ has been mostly used (Arisha et al., 2020; Dong et al., 2005; Hajihashemi & Geuns, 2016; Huang et al., 2019; Ren et al., 2019; Tiwari et al., 2020; Zhang et al., 2017). While PEG induces drought syndromes from leaf wilting to plant collapse, plants in this process may defend themselves by activating the drought tolerance pathway to preserve water relations (Dong et al., 2005; Zhang et al., 2017). By cytological, physiological, and molecular assays, we show that OsPIP2;2 is a predominant facilitator of H₂O transportation in relevance to drought-tolerant responses in rice cells.

2 | MATERIALS AND METHODS

2.1 | Plant growth and treatment

Seeds of the rice variety Nipponbare were initially provided by Professor Hongsheng Zhang (Nanjing Agricultural University) and then reproduced and maintained in this lab. Seeds of tobacco (Nicotiana benthamiana) were reproduced and maintained in this lab. Rice and tobacco seeds were germinated in flat plastic trays filled with a substrate containing an industrial soil branded as PINSTRUP. Five days later, the germinal seedlings were moved into 12-L pots (two plants per pot) filled with the same soil. Seeds were incubated, and the plants were grown in environment-controlled chambers under 28°C, 12-h light at 250 ± 50 μmol quanta/m²/s and a relative humidity of 80%.

PEG₆₀₀₀ was used as an inducer of physiological drought stress (Dong et al., 2005; Dubois & Inzé, 2020) and applied to 15-day-old rice seedlings. Usually, PEG₆₀₀₀ is prepared as an aqueous solution at a certain percent concentration (w/v) and applied in parallel to the deionized water control to incubate plants by immersing the root system. In this study, 15-day-old rice seedlings growing in the PINSTRUP soil were taken out carefully by pushing the soil away using a finger, cleaned gently by soft washing with tap water to remove the surrounding soil scraps, and then placed into plastic tubes containing deionized water. After a 12-h acclimation, seedlings were moved into new tubes containing deionized water (control) or an aqueous solution of PEG₆₀₀₀ justified to 0%, 5%, 10%, 15%, 20%, 25%, and 30% w/v, respectively. Seedlings were monitored in the subsequent 10 h by automatic photography at 10-min intervals. Chronological development of drought syndromes was analyzed.

2.2 | Quantification of OsPIP expression levels

Total RNA was isolated from leaves and subjected to real-time quantitative reverse polymerase chain reaction (qRT-PCR). All the qRT-PCR
experiments were performed with the QuantStudio®3 Real-Time PCR Instrument (ThermoFisher Scientific) and using the constitutively expressed EF1α gene as a reference (Liu et al., 2011). Relative expression level of a tested gene was quantified as the tested gene to EF1α transcript quantity ratio, which was determined by the $2^{-\Delta \Delta C_t}$ method (Livak & Schmittgen, 2011).

### 2.3 OsPIP de novo expression

#### 2.3.1 Expression in oocytes

Based on gene expression analyses (Figure 1), OsPIP1;3, OsPIP2;2, OsPIP2;2, and OsPIP2;4 were further investigated. Complementary
DNA tagged cDNA libraries of OsPIPs were obtained from RT-PCR amplification of total RNAs isolated from leaves of 15-day-old rice plants. For use in transformation of African clawed frog (*Xenopus laevis*) oocytes, every OsPIP cDNA was fused to the enhanced green-fluorescent *YFP* gene or a six-his tandem sequence by conventional recombination methods (Li et al., 2015, 2019). Commercial nursery *X. laevis* was obtained from Stem Cell Bank of Chinese Academy of Sciences and maintained in an 18°C water incubator supplied twice a week with blood worm and ox heart, liver, and lung. Mature oocytes at V to VI stages were excised and used in transformation. Briefly, the binary vector pGH19 and an eGFP-fused OsPIP cDNA were digested with *XbaI* and *EcoR1* (TAKARA) and then glued with T4 ligase (Thermo). The recombinant vector was linearized with restriction enzyme NotI (TAKARA) and purified in RNase-free water. A constant amount of 1 μg linearized DNA was applied for an in vitro transription using RiboMAX™ Large-Scale RNA Production Systems-T7 (Promega). The resulting cRNA was injected into oocytes. The transformed oocytes were incubated in sterile ND96 solution (96-mM NaCl, 2.0 mM KCl, 1.8 mM CaCl2, 1.0 mM MgCl2, 1.0 mM HEPES, 2.0-mM Na-pyruvate, pH 7.6) amended with penicillin and streptomycin. After incubation for 36 h, oocytes were observed between 495 and 520 nm using 488-nm argon-ion laser excitation with a Zeiss LSM700 laser scanning confocal microscope. Fusion proteins were analyzed by immunoblotting of membrane proteins isolated from the transformed oocytes using a previously described protocol (Jørgensen et al., 2016). Similar procedure was used in construction, de novo expression, and analyses of the OsPIP-his fusion protein except for laser scanning confocal microscopy (LSCM).

2.3.2 | Expression in yeast

Each of the OsPIP-his fusion genes was cloned into the yeast binary vector pYES2 (Wang, Zhang, et al., 2020) with the help of restriction enzymes *HindIII* and *EcoR1* (TAKARA). The recombinant vector was transformed into competent cells of the wine-brewing yeast (*Saccharomyces cerevisiae*) strain NMY51 in transformation solution (0.1 M LiCl, 2.0 mM NaCl, 2.0 mM KCl, 5.0 mM MgCl2 and 5.0 mM HEPES, 2.0-mM Na-pyruvate, pH 7.6) amended with penicillin and streptomycin. After incubation for 36 h, oocytes were observed between 495 and 520 nm using 488-nm argon-ion laser excitation with a Zeiss LSM700 laser scanning confocal microscope. Fusion proteins were analyzed by immunoblotting of membrane proteins isolated from the transformed oocytes using a previously described protocol (Jørgensen et al., 2016). Similar procedure was used in construction, de novo expression, and analyses of the OsPIP-his fusion protein except for laser scanning confocal microscopy (LSCM).

2.3.3 | Expression in tobacco plants

De novo expression of the selected OsPIPs (OsPIP1;3, OsPIP2;1, OsPIP2;2, and OsPIP2;4) in leaves of tobacco was performed by the agroinoculation method (Liu et al., 2011). Each of the OsPIP genes was combined at the N-terminus with the constitutive 35S promoter from cauliflower mosaic virus and at the C-terminus with the YELLOELOW-FLUORESCENT PROTEIN (YFP) gene from a previously used yeast vector (Li et al., 2015). The fusion gene was inserted into the plant binary vector pCAMBIA1301 (Liu et al., 2011). The recombinant vector was transferred into competent cells of the *Agrobacterium tumefaciens* strain GV3101. A bacterial suspension of the recombinant GV3101 cultures was infiltrated into intercellular spaces of fully expanded leaves of 30-day-old tobacco plants. In the subsequent 48–60 h, transinfected leaves were excised and observed by LSCM to reveal the subcellular localization of OsPIP-YFP fusion proteins.

2.3.4 | Expression in plant protoplasts

This was performed by the chemical mediation method (Shen et al., 2014). Protoplasts were isolated from strips of fully expanded leaves of 30-day-old tobacco seedlings or from stems and leaf sheaths of 30-day-old rice plants, using reagents from Sigma-Aldrich and a previously described protocol (Shen et al., 2014; Yoo et al., 2007). Cell walls were removed by fungal cellulase and macerozyme used in an aqueous solution. The enzyme solution containing released protoplasts was diluted with equal volume of aqueous washing solution (154-mM NaCl, 125-mM CaCl2 and 5-mM KCl) and filtered with a 75-μm nylon mesh. The filtrate collection was centrifuged at 100g and 4°C for 2 min. The supernatant was discharged. The precipitate was resuspended with the washing solution and centrifuged at 1000g and 4°C for 5 min. The supernatant was removed again. Tubes containing the protoplasts were placed on ice bath and suspended with the MMg solution (4-mM 2,4-morpholinino-ethanesulfonic acid, 4-M mannitol, and 15-mM MgCl2) and adjusted to 5 × 10⁶ protoplasts/ml. This protoplast suspension was supplied with 10 μl of the recombinant vector that carries an OsPIP insert, 110 μl of PEG-CaCl2 (40% w/v PEG4000, 2-M mannitol, and 1-M CaCl2), followed by incubation on ice bath for 30 min. At this time point, the transformation was terminated by adding 440 μl of the W5 solution (4-mM MES, 5-M mannitol, and 5-mM KCl). The suspension of transformed protoplasts was centrifuged at 1000g and 4°C for 5 min, the supernatant was removed, and the WI solution (4-mM MES, 5-M Mannitol, and 5-mM KCl)0) was gently added. The resulting protoplast suspension was shifted into wells of a 96-well plate, incubated for 12 h under room temperature and weak illumination, and used in further tests.

2.4 | OsPIP2;2 silencing and overexpression

The rice tungro bacilliform virus (RTBV) vector pRBDV (Kant & Dasgupta, 2017; Purkayastha et al., 2010) was used in hairpin and overexpression constructions (Li et al., 2019). The hairpin unit was constructed using the gene internal 300-bp fragment from the less

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conserved 241–553 region of the OsPIP2;2 coding sequence (Figure S1). The specificity of the OsPIP2;2-241–553 fragment in targeting the endogenous gene was verified by hybridization predominantly with OsPIP2;2 over the other 10 OsPIPs (Figure S2). The OsPIP2;2-241–553 sequence was cloned by RT-PCR using RNA isolated from leaves of rice plants 3 h after incubation in 30% w/v PEG6000 and then inserted into pRBDV. This yielded the recombinant vector carrying the pRBDV:OsPIP2;2-241–553 sense and pRBDV: OsPIP2;2-241–553 antisense sequences, respectively. Overexpression of OsPIP2;2 was constructed by inserting the 35S promoter and OsPIP2;2 CDS fusion gene into pRBDV, generating the recombinant pRBDV:OsPIP2;2 vector. Subsequently, each recombinant vector was transferred into the tobacco genome by transfecting leaves of 20-day-old tobacco plants. Fifteen days later, the gene silencing and overexpression efficiencies were confirmed by RT-PCR analyses using RNA isolated from leaves newly growing in the transfected plants. Leaf transformation with the hairpin construct resulted in 40%–80% fall in the OsPIP2;2 transcript quantity in experimental replicates. The OsPIP2;2-silenced plants were used in protoplast preparation and water permeability assays only when the gene silencing efficiency reached to 70% or higher. In experimental replicates, leaf infection with the overexpression construct caused 55%–87% enhancement in OsPIP2;2 expression. The OsPIP2;2-overexpressing plants were used in protoplast preparation and water permeability assays only when the gene overexpression efficiency reached to 75% or higher.

2.5 | Physiological measurements

2.5.1 | Water permeability measurements

Water permeability of the directly transformed rice and tobacco protoplasts, protoplasts from the transformed oocytes and yeast, and protoplasts from the OsPIP2;2-silenced and -overexpressing rice plants was measured by microscopy. These protoplasts were separately incubated in the ND96 solution, which provides a low osmotic gradient from exteriors to interiors of the incubated protoplasts. Ten minutes later, the osmotic water permeability coefficient (PI) was determined by measuring volume changes of the protoplasts as previously described (Ding et al., 2019; Li et al., 2015).

2.5.2 | Proline measurement

Proline concentrations in tobacco and rice protoplasts were determined with a plate reader. Before testing, a standard curve was established using a commercial proline standard of known concentrations and the corresponding absorbance readings. To isolate proline from protoplasts, 5 ml of 3% sulfosalicylic acid solution was added into a tube containing a protoplast suspension and then the sample was setting for 10 min in a boiling water bath. The extraction solution was filtered into a clean test tube after cooling and the supernatant was regarded as a proline extract. This proline extract solution was regarded as a proline extract. This proline extract solution was filtered into a clean test tube after cooling and the supernatant was setting for 10 min in a boiling water bath. The extraction was mixed with equal volumes of glacial acetic acid and acid ninhydrin. After the mixture was phased automatically, solution of the upper phase was collected and centrifuged at 3,500 g for 5 min. Supernatant was assayed for absorbance at 520 nm in the plate reader. Proline concentration in the supernatant was estimated with reference to gradients of the absorbance by methylbenzene. The proline content was given as micrograms per gram protoplasts.

2.5.3 | Polyamine measurement

A previously described protocol (Zhu et al., 2020) was used to extract polyamines (PAs) from rice and tobacco protoplasts, respectively. Soluble conjugated PAs were calculated by subtracting the free PAs from the acid-soluble PAs. Precipitated protoplasts were suspended with 4 ml of v/v 5% cold perchloric acid (PCA) and incubated at 4°C for 1 h, followed by sonication in the presence of lyase and antifoam. The resulting suspension was centrifuged at 12,000 g and 4°C for 10 min. The supernatant was supplied with the internal standard 1,6-hexanediamine, and the mixture was centrifuge at 12,000 g and 4°C for 30 min. The resulting supernatant was blended with 6-N HCl at a 1:5 volume rate and hydrolyzed at 110°C for 18 h in flame-sealed glass ampules. After acid hydrolysis, HCl was evaporated by heating at 70°C, and the residue was suspended in 2 ml of 5% PCA, followed by centrifugation at 12,000 g and 4°C for 30 min. The supernatant contained the acid-soluble PA fraction and free PAs liberated from PA conjugates. To obtain the insoluble bound PA, the pellets were rinsed four times with 5% PCA to remove any traces of soluble PAs and were suspended in 5 ml of 6 N HCl. This solution was hydrolyzed by the same procedure as described above. PAs recovered from the non-hydrolyzed supernatant, hydrolyzed supernatant, and the pellet were benzoylated as follows. An aliquot of the supernatant was treated with 2 ml of 2 N NaOH and 15 ml of benzoyl chloride, vortexed vigorously, and incubated for 30 min at 37°C. The reaction was terminated by adding 4-ml saturated NaCl solution. Thereafter, the benzoyl PAs were extracted with 3-ml cold diethyl ether. Finally, 1.5 ml of the ether phase was evaporated to dryness and redissolved in 1-ml methanol. PAs in the final solution were assayed by high-performance liquid chromatography (HPLC) (Agilent 1220, America). Ten microliters of benzoyl PAs in methanol solution was injected into a 4.6 × 250-mm reverse-phase Kromasil C18 column (Agilent, Sweden) at 25°C. PAs were eluted from the column with 64% methanol at a flow rate of .7 ml/minute. PA peaks were detected with the plate reader at 254 nm.

2.5.4 | Electrolyte leakage measurement

Electrolyte leakage from leaves of rice seedlings was measured using a conductivity meter. Briefly, 1-cm leaf segments were immersed into deionized water by the aid of a vacuum pump, followed by incubation for 1 h under 25°C. The resulting solution containing electrolytes leaked from leaf segments were subjected
to measurements with the Leitz DDS-307 conductivity meter (Shanghai Right-One Instruments Company, LTD) under 220 V, 50 Hz, and 1.021 Kohlrausch coefficient. Similar measurements on deionized water were conducted in control. Extents of electrolyte leakage from WT, OsPIP2;2-Si, and OsPIP2;2-OE were given as the measured conductivity (μS/cm) values.

2.6 | Data treatment

All experiments were repeated at least three times with similar results. Quantitative data were analyzed using the commercial IBM SPSS19.0 software package (Shi, 2012). Homogeneity-of-variance in data was determined by the Levene test. The formal distribution pattern of the data was confirmed by the Kolmogorov–Smirnov test and P–P Plots. Fisher’s data-pairing test or Duncan’s new multiple range test was performed along with analysis of variance (ANOVA) on data from at least three independent experiments, each involving three repetitions, unless specified elsewhere, such as when a leaf was treated as a statistical unit.

3 | RESULTS

3.1 | OsPIP2;2 highly responds to a physiological drought stress

To identify OsPIP candidates implicated in water relations of rice, we analyzed expression levels of 11 OsPIP genes in plants of the rice variety Nipponbare following PEG6000 treatment (Figure 1). We applied a range of PEG6000 concentrations, 0%–30% at 5% gradients, to 15-day-old rice seedlings in liquid culture, and monitored

| TABLE 2 | Chronological changes in response of 15-day-old rice seedlings to a range of PEG6000 dosage in liquid culture |
|-------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| Drought syndrome development timing (min) | 0 | 5 | 10 | 15 | 20 | 25 | 30 |
| PEG6000 concentration (%) w/v | 0 | 10 | 15 | 20 | 25 | 30 |
| Time to first occurrence of leaf lengthening | NO | NO | 110 | 100 | 90 | 60 | 50 |
| Time to first occurrence of leaf wilt | NO | NO | 250 | 230 | 120 | 100 | 70 |
| Time to wilting of all leaves | NO | NO | NO | NO | 220 | 180 | 150 |
| Time to plant collapse | NO | NO | NO | NO | NO | 320 | 280 |

Abbreviation: NO, not occur.
chronological development of drought syndromes in the subsequent 10 h (Figure 1; Table 2). In that period, the lowest PEG$_{6000}$ dosage (5%) caused no responses, but drought syndromes were induced when PEG$_{6000}$ concentration was increased to 10% and higher (Figure 1). Leaf lengthening and narrowing (Figure 2, insets) were found to be the first sign of drought syndromes, occurred mostly on the second leaves (Figure 2, insets) in 50–110 min after PEG$_{6000}$ application at 10%–30% (Table 2). The formation of slender leaves was thought to indicate plant transition from the normal physiological status to drought response. This transition was followed by wilt of partial to all leaves and plant collapse in the end (Figures 1 and 2). All these syndromes occurred in 6 h after plant incubation with 25% and 30% PEG$_{6000}$ supplies, respectively (Table 2), but concentrations of 15% and 10% only caused partial leaves to wilt.

To evaluate the effects of the physiological stress on OsPIP expression, an aqueous solution of 15% or 30% PEG$_{6000}$ was applied to 15-day-old rice seedlings by immersing roots. These plants (+PEG$_{6000}$) and those incubated in deionized water (−PEG$_{6000}$) were used 3 days later in OsPIP expression assessments by qRT-PCR. The qRT-PCR analyses using total RNA isolated from the leaves indicated that all OsPIPs responded to the physiological drought stress, displaying significantly ($P = 1.2 \times 10^7$) enhanced expression in contrast to the steady-state expression levels in control (Figure 2, graph).

Extents by which the physiological drought-stress-induced gene expression were 3.6, 2.5, and 4.1 times for OsPIP1;3, OsPIP2;1, and OsPIP2;4, respectively, reached 12.9-fold for OsPIP2;2, but the extents were much smaller (1.7–88.7% times) for additional OsPIPs. Evidently, OsPIP2;2 exceeds all the other OsPIP genes in response to the physiological drought stress and displays the highest level of induced expression under the stress condition, indicating that OsPIP2;2 is likely to take part in water relations of rice.

### 3.2 De novo expression of OsPIP2;2 enhances water permeability of oocyte and yeast membranes

Based on the conceptual function of AQPs initially defined for water transport (Agre, 2004), we tried to determine if OsPIP1;3, OsPIP2;1, OsPIP2;2, and OsPIP2;4 have a role in mediating H$_2$O transport in living cells of both the wine-brewing yeast and African clawed frog oocytes. LSCM performed on oocytes 48 h after transformation indicated that all the fusion proteins (OsPIP1;3-eGFP, OsPIP2;1-eGFP, OsPIP2;2-eGFP, and OsPIP2;4-eGFP) were localized to oocyte membranes, in contrast to the absence of any protein fluorescence in control (Figure 3a). Every OsPIP-eGFP fusion protein was plentiful in the transformed oocytes, as determined by stereomicroscopic
observations (Figure 3a) and immunoblotting analyses (Figure 3b), respectively. These proteins were detected mainly as tetramer, accompanied by trimer at a small quantity (Figure 3b).

Based on the measured Pf values measured under a low extracellular osmotic gradient, OsPIP2;2-eGFP greatly promoted transport of the environmental H2O into cells of the oocytes transformed with OsPIP2;2-eGFP in contrast to eGFP (Figure 3c). OsPIP2;1-eGFP was weaker in this function, while neither OsPIP1;3-eGFP nor OsPIP2;4-eGFP exhibited evident effect on the water permeability of oocyte membranes (Figure 3c). In line with this result, plentiful production of the OsPIP-His fusion proteins was detected from the OsPIP1;3-his–transformed oocytes (Figure 4a). Once again, all the OsPIP-His fusion proteins existed in both tetramer and trimer (Figure 4a). While OsPIP2;2-His and OsPIP2;1-His caused a high and a moderate elevation in Pf, respectively, neither OsPIP1;3-His nor OsPIP2;4-His caused evident changes in Pf values over the basal level found in control (Figure 4b). Then, the OsPIP-His genes were expressed (Figure 5a) and the fusion proteins produced (Figure 5b) in yeast protoplasts. When the recombinant yeast protoplasts were incubated under a low osmotic gradient, Pf was increased by each OsPIP-His compared to His only, but the increase level was greater in yeast protoplasts that produced OsPIP2;2-His than those that produced the other OsPIP-His proteins (Figure 5c). These results suggest that de novo expression of OsPIP2;2 in oocyte and yeast cells facilitates H2O transport across the cell PMs.

3.3 | OsPIP2;2 de novo expression promotes H2O transport across tobacco PMs

We turned to study cytological and physiological performances of OsPIP1;3, OsPIP2;1, OsPIP2;2, and OsPIP2;4 in plants. LSCM performed on tobacco leaves 48 h after transformation with the OsPIP-YFP genes clearly detected the production of OsPIP1;3-YFP, OsPIP2;1-YFP, OsPIP2;2-YFP, and OsPIP2;4-YFP fusion proteins in the leaf cells (Figure 6a). The four OsPIP-YFP fusion proteins were all localized to PMs of the leaf cells, in contrast to apparently triple distributions of YFP only in the PMs, cytoplasmic spaces, and nuclei (Figure 6a). The protein production was correlated with expression of the YFP and OsPIP1;2-YFP genes (Figure 7a).

To assess if OsPIP1;3, OsPIP2;1, OsPIP2;2, and OsPIP2;4 affect H2O transport through plant PMs, tobacco protoplasts were transformed with the recombinant pCAMBIA1301 vector containing YFP (control) and OsPIP-YFP, respectively. When transformed protoplasts were incubated in the ND98 medium that held an extracellularly low osmotic gradient, Pf increases over the control level were substantially induced by OsPIP2;1-YFP, OsPIP2;2-YFP, and OsPIP2;4-YFP (Figure 6b). In comparison, OsPIP2;2-YFP displayed the most vigorous activity, providing the highest level in Pf elevation. On the contrary, OsPIP1;3-YFP did not cause evident effect on Pf (Figure 6b). Thus, OsPIP2;2 is a vigorous performer in mediating H2O transport across PMs of tobacco cells under the de novo expression condition.

3.4 | OsPIP2;2 de novo expression contributes to drought-tolerant responses in tobacco protoplasts

We analyzed if any of OsPIP2;1-YFP, OsPIP2;2-YFP, and OsPIP2;4-YFP affects drought-tolerant responses in tobacco protoplasts incubated with PEG6000. Increases in concentrations of proline and total PAs (including putrescine, spermidine, and spermine) are regarded as a physiological indicator of drought tolerance activation in plants (Oladosu et al., 2019; Vinnakota et al., 2016; Zhu et al., 2020). Thus, the transformed protoplasts were incubated with 10% PEG6000, and proline and PA concentrations were measured 10 min later. At this time point, proplast concentrations of both proline (Figure 6c)
and PAs (Figure 6d) were significantly increased by OsPIP2;1-YFP, OsPIP2;2-YFP, and OsPIP2;4-YFP in contrast to YFP only. In these fusion proteins, OsPIP2;4-YFP displayed the greatest activity in increasing proline and PAs, but OsPIP1;3-YFP had little effect on concentrations of both compounds (Figure 6c,d).

In addition to the physiological responses, cytological variations also occur in plants under a drought stress (Dong et al., 2004; Oladosu et al., 2019). We confirmed that the PEG6000 treatment (tobacco protoplast incubation in the presence of 10% PEG 6000) caused considerable reductions in protoplast size, indicating protoplast contraction due to water outflux (Figure 7b,c). Protoplast contraction became evident in 10 min posttreatment (mpt) with PEG6000, as compared to PEG-absent control (Figure 7b). Compared to control, the PEG treatment induced significant decreases in diameters of the protoplasts no matter if they had been transformed with YFP only or with the OsPIP2;2-YFP fusion gene (Figure 7c). However, de novo expression of OsPIP2;2-YFP (Figure 7a) aggravated protoplast contraction to a higher level, reducing protoplast diameter by a 20% higher degree than that of YFP only (Figure 7c). Intriguingly, de novo expression of OsPIP2;2-YFP seemed to intensify the integrity of protoplast membranes (Figure 7d,e). Protoplasts started to swell after 10 mpt, when the cytoplasm was congregated at one side, and partially collapsed not until 30 mpt (Figure 7d). At 30 mpt, the YFP-expressing protoplasts were malformed at a 61.2% proportion on average, whereas average collapse proportion remained as low as 25.9% in
the protoplasts expressing OsPIP2;2-YFP (Figure 7e). These analyses suggest that de novo expression of OsPIP2;2 enhances drought-tolerant responses in tobacco cells.

### 3.5 OsPIP2;2 promotes H$_2$O transport and drought-tolerant responses in rice protoplasts

We analyzed H$_2$O transport and drought-tolerant responses in rice protoplasts prepared from the Nipponbare plants that express the canonical OsPIP2;2 gene (WT) or display OsPIP2;2 silencing (OsPIP2;2-Si) or overexpression (OsPIP2;2-OE) mediated by the RBDV vector (Kant & Dasgupta, 2017; Li et al., 2019). By measuring Pf in protoplasts incubated in the liquid medium with a low osmotic gradient, we verified that considerable amounts of H$_2$O moved into the protoplast interiors over 10 min. In that period, quantities of H$_2$O transport across rice protoplast PMs were significantly ($P = 1.235 \times 10^{-16}$) increased by OsPIP2;2-OE but decreased by OsPIP2;2-Si compared to the canonical expression (Figure 8a). After 10% PEG$_{6000}$ was supplied to the medium, rice protoplasts became...
malformed in 30 min. In comparison to the WT, the OsPIP2;2-Si protoplasts collapsed at a higher proportion, but the collapse rate was substantially reduced by OsPIP2;2-OE (Figure 8b). Consistently, drought-tolerant responses, indicated by protoplast concentrations of proline (Figure 8c) and PA (Figure 8d), were induced at a greater level in the OsPIP2;2-OE protoplasts compared to the WT. On the contrary, OsPIP2;2-Si caused significant reductions in concentrations of proline (Figure 8c) and PA (Figure 8d). Clearly, OsPIP2;2 functions in protoplasts of its source plant to facilitate H2O transport across the PMs and contributes to defense responses against the physiological drought stress.

### 3.6 OsPIP2;2 conduces to membrane integrity of rice protoplasts

In plants, one of PEG-induced responses is cellular ion efflux known as electrolyte leakage, which occurs due to injured integrity of the PMs (Dong et al., 2005). We analyzed whether the role of OsPIP2;2 in reducing rice protoplast collapse (Figure 8b) relates to its effect on electrolyte leakage from the protoplasts in response to PEG6000. When the WT, OsPIP2;2-Si, and OsPIP2;2-OE plants incubated in deionized water were supplied with 30% PEG6000 and measured 3 h later, electrolyte leakage from leaves was detected at different extents. Compared to the WT, the OsPIP2;2-OE plants had much less electrolyte leakage, but electrolyte leakage from OsPIP2;2-Si plants was significantly increased (Figure 9a). Conveying the conductivity values to thousand times of their reciprocals reflects integrity degrees of the protoplast membranes (Figure 9b), suggesting that OsPIP2;2 is conducive to maintenance of the membrane integrity in response to the physiological drought stress.

### 3.7 OsPIP2;2 imparts activation of the drought tolerance pathway

In rice, activation of the drought tolerance pathway by PEG treatment essentially involves induced expression of the pathway-marker gene COR413-TM1 and the pathway-regulatory genes OsABF1, OsPP48, and OsPP108 (Zhang et al., 2017). We determined that these genes were induced by PEG6000 treatment to markedly express in leaves of the WT, OsPIP2;2-Si, and OsPIP2;2-OE plants (Figure 10). As
FIGURE 9 The effects of OsPIP2;2 silencing and overexpression on cell membrane integrity of 15-day-old rice seedlings growing in the absence (−) and presence (+) of PEG6000 treatment. (a) Electrolyte leakage from leaf segments of the plants 3 h after PEG6000 treatment or remained free from PEG6000. Data shown are mean values ± SDs of results from six independent experiments. Different letters on graphs indicate significant differences based on ANOVA and Duncan’s multiple new multiple range test of the data ($P = 1.8 \times 10^{-7} - 1.2 \times 10^{-15}$). (b) Rice cell membrane integrity given as reciprocals of electrolyte leakage calculated as averages based on data from a
quantified at 3 h after PEG$_{6000}$ application, levels of COR413-TM1, OsABF1, OsPP48, and OsPP108 expression were significantly ($P < .001$) induced in contrast to the steady-state expression levels found in the PEG-absent control plants. Moreover, COR413-TM1 exceeded OsABF1, OsPP48, and OsPP108 in expression extents in all plants. Noticeably, the OsPIP2;2-OE plants highly exceeded but OsPIP2;2-Si was considerably inferior to the WT in supporting PEG$_{6000}$-induced expression of all the genes (Figure 10). Clearly, OsPIP2;2 imparts the drought tolerance pathway toward activation in response to the physiological drought stress.

4 | DISCUSSION

Rice has a total of 33 AQPs including 11 PIP homologs (Sakurai et al., 2005) that mostly have not been characterized regarding substrate selectivity and specificities, as well as associated bioprocesses (Table 1). By quantifying differential expression of all the OsPIP homologs in rice plants responding to the physiological drought stress induced by the externally applied PFG$_{6000}$-we elucidated that OsPIP1;3, OsPIP2;1, OsPIP2;2, and OsPIP2;4 are potentially involved in water relations and drought tolerance in the plant. Based on measurements of membrane permeability to H$_2$O in the OsPIP-transformed oocyte and yeast cells, we showed that OsPIP2;2 is a predominant facilitator for H$_2$O transport across living cell membranes. By physiological and cytological analyses of plant protoplasts, we demonstrated that OsPIP2;2 is an effective implementor of H$_2$O transport across PMs in its source plant (rice). This is a small but substantial advance in exploring substrate selectivity and specificities of rice PIPs. The paucity in understandings of rice PIPs is incredible if we do not have the opportunity to scrutinize previous literatures. Previously to the present study, only three OsPIPs have characterized clearly to execute the substrate-transporting function in their source plant (Table 1).

Previously, OsPIP2;1 was identified as a physiologically relevant CO$_2$-transporting implementor in rice (Xu et al., 2019). In rice, moreover, both OsPIP1;2 (Ding et al., 2019) and OsPIP2;4 (Nada & Abogadallah, 2020) were characterized to support root hydraulic conductivity. It is unclear whether OsPIP1;2 and OsPIP2;4 also contribute to leaf hydraulic conductivity, which can be detected by cell pressure probing measurements (Hachez et al., 2011) as reliable as for the root (Li et al., 2015). No evidence has been obtained to elucidate whether

![Figure 10](attachment:image.png)

**Figure 10** The effects of OsPIP2;2 silencing and overexpression on expression of response genes regarded as molecular makers of the drought tolerance pathway. Rice seedlings already growing for 15 days in pot soil in a plant growth chamber were shifted into tubes containing deionized water. After 12-h acclimation, these plants were transferred into new tubes containing deionized water only or an aqueous solution of 30% PEG$_{6000}$. Three hours later, gene expression was analyzed by QRT-PCR performed with total RNAs isolated from the aerial parts of the plants. The constitutively expressed OsEF1α gene was used as a reference to quantify relative expression level of an OsPIP. Data show are mean values ± SD estimates. Different letters on graphs indicate significant differences by analysis of variance (ANOVA) and Duncan’s multiple new multiple range test of the data ($P = .0001 - 1.7 \times 10^{-11}$; $n = 6$ independent experiments each involving 15 plants tested in three biological repeats)
an OsPIP functions for substrate transport in both roots and leaves, but it is frequent that a particular AQP plays a same role in different organs of plants (Gomes et al., 2009; Li et al., 2015; Maurel et al., 2008). Our study identifies OsPIP2;2 to be the third recognized but most vigorous H₂O-transporting facilitator among 11 PIP homologs of rice. In yeast and oocytes, OsPIP2;2 and OsPIP2;4 contribute relatively smaller parts to H₂O transport in contrast to the prominent role of OsPIP2;2. This result is consistent with the regular pattern of functional redundancy when two or more AQP homologs fulfill a same function but functional intensities are different one from another (Gomes et al., 2009; Maurel et al., 2008; Péret, et al., 2012). Our results also agree with previous studies by Sakurai and colleagues (Sakurai et al., 2005, 2008). They did not test substrate-importing functions of any OsPIPs in rice. Instead, they analyzed differential expression of 33 AQP-encoding genes including 11 OsPIPs, which constitute the full repertoire of AQPs in the rice genome, in different

**FIGURE 11** Model of OsPIP2;2 functions in H₂O transport and drought tolerance. When plants are growing regularly without drought stress, OsPIP2;2 functions to facilitate H₂O transport in and out of the plant cells in response to a hydraulic gradient generated by natural metabolism in the apoplastic or cytoplasmic space. This function is assumed to play a role in maintenance of water relations. When rice plants incur osmotic stress from environment, such as the physiological drought stress caused by externally applied PEG, OsPIP2;2 turns to function to support drought tolerance possibly by physical and physiological regulatory mechanisms. In the assumed physical mechanism, the drought stress is inevitable to injure the cell membranes, causing electrolyte leakage for example, while the presence of OsPIP2;2 serves as an encountering force to help preserve the membrane integrity. The physiological mechanism, including increases in proline and polyamine concentrations, is used by OsPIP2;2 to maintain the cellular water homeostasis. Water homeostasis may also come from the role of OsPIP2;2 in modulating H₂O transportation shuttles in and out of the cells, instead of a single direction, depending on hydraulic gradient changes by electrolyte leakage. Both physical and physiological mechanisms could be integrated to increase drought tolerance intensity. Abscisic acid (ABA) signaling may partake in the regulation of OsPIP2;2-mediated drought tolerance, which involves the ABA-responsive transcription factor COR413-TM1. The OsPIP2;2-dependent tolerance pathway is also likely to have a crosstalk with signaling by H₂O₂ if it is induced by a particular stimulus (plant infection by a plant pathogen, for example) to accumulate in plant apoplastic spaces and to be transported by OsPIP2;2 to enter the cytoplasm
organs of rice plants after chilling treatment (Sakurai et al., 2005). Then, they performed de novo expression assays in a yeast (S. cerevisiae strain BJ5458) system and characterized OsPIP2;2 as a strong moderator for \( H_2O \) import into cells of the PIP-transformed yeast cultures (Sakurai et al., 2008). Despite of these advances regarding the four OsPIPs (1;2, 2;1, 2;2, and 2;4) and the predominant role of OsPIP2;2 in \( H_2O \) transport and drought tolerance, little has been known about substrate selectivity of additional seven homologs and specificities in mediating substrate transportations. The specificity of a PIP in transporting a substrate is determined by phosphorylation of the PIP at a specific serin resides in response to the substrate gradient between plant PMs (Törnroth-Horsefield et al., 2006). Identifying the site of OsPIP2;2 phosphorylation induced by the physiological stress could explain why OsPIP2;2 is so distinct from its homologs in the physiological function.

By genetic, molecular, and physiological analyses, we elucidated that the substrate-transporting role of OsPIP2;2 closely associates with drought tolerance in rice protoplasts and seedlings. The role of OsPIP1;2 in drought tolerance is attributable to improved maintenance of the membrane integrity and the activation of the drought tolerance pathway. Evidence has been provided as the negative effect of OsPIP2;2 on electrolyte leakage and the positive effect of OsPIP2;2 on the expression of response genes characteristic of the pathway. However, evidence obtained to date is insufficient to demonstrate molecular mechanisms underlying the functional relationship of OsPIP2;2 between \( H_2O \)-transporting regulation and drought-tolerant responses. Indeed, the physiological connection between substrate-transporting role of PIPs and their function in drought tolerance has not been established (Oladosu et al., 2019; Shekoofa & Sinclair, 2018; Singh et al., 2020; Vinnakota et al., 2016; Vishwakarma et al., 2019). A PIP channel for water transportation mediates \( H_2O \) trafficking in and out of plant cells, instead of one direction only inwards to the cellular interior. The assumed dynamics of oppositely directional \( H_2O \) trafficking is accompanied by electrolyte leakage and membrane damage. Thus, the involvement of a PIP in drought tolerance is not likely to be a direct consequence from its role in \( H_2O \) transportation. Presumably, water outflux from plant cells under an osmotic stress and enhancing role of a PIP, such as OsPIP2;2, induces physiological responses, including increases in proline and PA concentrations (Dong et al., 2004; Xu et al., 2019; Zhu et al., 2020), which turn to enhance drought resistance.

Based on these analyses, we propose a model that hypothesizes how OsPIP2;2 execute its functions toward \( H_2O \) transport and drought tolerance (Figure 11). In the model, OsPIP2;2 supports drought tolerance by physical and physiological regulatory mechanisms, which modulate the membrane integrity by controlling the molecular (\( H_2O \) and electrolyte) trafficking and by regulating the intracellular responses against drought stress, respectively (Figure 11). In the tested drought-tolerant constituents, OsABF1 is a basic leucine zipper transcription factor that functions in plant responses to biotic stresses through the abscisic acid (ABA) signaling pathway. Presumably, the OsPIP2;2-dependent drought tolerance is also subject to ABA signaling (Figure 11), which has been demonstrated to regulate plant tolerance to PEG-induced physiological stress (Dong et al., 2005). Furthermore, characterizing the possible role of OsPIP2;2 in \( H_2O \) transport will better explain the molecular basis of the membrane integrity. We recently proposed that \( H_2O \)-transporting AQPs are also potential channels for \( H_2O \) trafficking (Wang, Schoebel, et al., 2020). In Arabidopsis, AtPIP1;4 plays triple roles in \( CO_2, H_2O \) (Li et al., 2015), and \( H_2O \) transport across the PMs. \( H_2O \) is generated in the apoplast upon induction by pathogen infection, transported by AtPIP1;4 into the cytoplasm, and therefore participates in immunity signal transduction (Tian et al., 2016). If OsPIP2;2 has a role in \( H_2O \) transport, it may contribute to the membrane integrity by facilitating the stress-induced apoplastic \( H_2O \) into the cytoplasm and therefore reducing the oxidative pressure toward the cell membranes (Figure 11). Verification of these hypotheses will be the subject for further studies.

In conclusion, the physiological, molecular, and cytological performances of the WT, OsPIP2;2-silencing, and OsPIP2;2-overexpressing plants and protoplasts in response to the physiological drought stress demonstrate the critical role of OsPIP2;2 in \( H_2O \) transport and drought tolerance. This finding should stimulate further studies to characterize the functional relationship of OsPIP2;2 in \( H_2O \) transport and drought-tolerant responses. It is necessary to study the functional relationship between OsPIP2;1-mediated drought tolerance and ABA signaling. It is also necessary to study if OsPIP2;10 has a role in \( H_2O \) transport in relevance to plant defenses against biotic and abiotic stresses.

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**AUTHOR CONTRIBUTIONS**

J.B., X.W., L.Z., and H.D. designed the experiments. J.B., X.W., X.Y., X.C., Y.H., Z.W., and Y.M. performed the experiments and analyzed the data. X.W., L.Z., and H.D. wrote the article. LZ. and H.D. conceived the study. All authors approved the final manuscript.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.