

# The role of water channel proteins and nitric oxide signaling in rice seed germination

Hong-Yan Liu<sup>1</sup>, Xin Yu<sup>1</sup>, Da-Yong Cui<sup>1</sup>, Mei-Hao Sun<sup>1</sup>, Wei-Ning Sun<sup>1</sup>, Zhang-Cheng Tang<sup>1</sup>, Sang-Soo Kwak<sup>2</sup>, Wei-Ai Su<sup>1</sup>

<sup>1</sup>Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China; <sup>2</sup>Korea Research Institute of Bioscience and Biotechnology, Oun-dong 52, Yusong, Taejon 305-333, South Korea

Previous studies have demonstrated the possible role of several aquaporins in seed germination. But systematic investigation of the role of aquaporin family members in this process is lacking. Here, the developmental regulation of plasma membrane intrinsic protein (PIP) expression throughout germination and post-germination processes in rice embryos was analyzed. The expression patterns of the PIPs suggest these aquaporins play different roles in seed germination and seedling growth. Partial silencing of the water channel genes, *OsPIP1;1* and *OsPIP1;3*, reduced seed germination while over-expression of *OsPIP1;3* promoted seed germination under water-stress conditions. Moreover, spatial expression analysis indicates that *OsPIP1;3* is expressed predominantly in embryo during seed germination. Our data also revealed that the nitric oxide (NO) donors, sodium nitroprusside (SNP) and *S*-nitrosoglutathione (GSNO), promoted seed germination; furthermore, the NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide, inhibited germination and reduced the stimulative effects of SNP and GSNO on rice germination. Exogenous NO stimulated the transcription of *OsPIP1;1*, *OsPIP1;2*, *OsPIP1;3* and *OsPIP2;8* in germinating seeds. These results suggest that water channels play an important role in seed germination, acting, at least partly, in response to the NO signaling pathway.

**Keywords:** germination, nitric oxide, *Oryza sativa*, over-expression, silencing, water channel

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## Introduction

Seed germination begins with uptake of water by the dry seed and terminates with radicle emergence and elongation of the embryonic axis [1]. Water movement into living cells governs seed germination. Previous studies have shown that aquaporins control intra- and intercellular water flow in plant cells [2]. There are 33 aquaporin genes in rice, of which 11 are plasma membrane intrinsic protein genes (PIPs) [3, 4], and they can be divided into two groups, *PIP1* and *PIP2* as that in *Arabidopsis thaliana* [5] and *Zea mays*

[6]. Plant PIP2 proteins have high water channel activity in *Xenopus* oocytes, whereas PIP1 proteins are often inactive or have low activity [7]. Some PIP1 members may not be related to water movement across the membrane but they might act as transporters for small solutes or gases. To investigate genes involved in the control of *Medicago truncatula* germination, suppressive subtractive hybridization was used to clone several genes including AQP1, a  $\gamma$ -TIP member [8]. Gao *et al.* [9] have demonstrated that *BnPIP1* and the tonoplast intrinsic protein, *BnTIP2*, are involved in seed germination. In the present work, we investigated the role of PIPs in rice seed germination to further reveal the functions of the water channel family.

Increasing experimental data have demonstrated that the germination of seeds is controlled by a variety of positive and negative regulatory signals: GA, ABA, light, chilling, sugars and nitric oxide (NO) [10, 11]. NO is a gaseous free radical that diffuses readily through membranes. Previous

Correspondence: Wei-Ai Su

Tel: +86-21-54924247; Fax: +86-21-54924015

E-mail: zstressc@online.sh.cn

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studies have shown the presence of NO in plants and its functions in many biological processes, including seed germination, leaf extension, root development, respiration, plant defense and hypersensitive cell death [12–14]. However, NO exerts both beneficial and harmful effects on plants, depending on its concentrations in cells [15]. Several papers have reported that NO is an endogenous regulator of seed dormancy, illustrated by the NO donor compounds breaking dormancy of *Arabidopsis* seeds [16–18] and reducing dormancy of three barley cultivars [17]. Conversely, NO scavengers have been shown to strengthen dormancy of *Arabidopsis* and barley seeds [17, 18], and reduce the stimulative effects of NO donors on seed germination [10, 17]. Beligni and Lamattina [10] concluded that NO was involved in light-mediated signaling, such as light stimulation of seed germination, de-etiolation and inhibition of hypocotyl elongation. Kopyra and Gwóźdz [19] described that NO was a potent germination stimulator for the faster germination rate of lupin seeds. In addition, the endogenous NO concentration peak in homogenates from sorghum embryonic axes was reached at 24 to 30 h after imbibition, which is in coincidence with the initiation of germination [20].

Here, we report the expression, regulation and gene manipulation of PIPs during rice seed germination and early seedling growth. According to our data, we conclude that water channel proteins play an important role in the progress of seed germination, probably in part in response to the NO signaling pathway.

## Materials and Methods

### Plant materials

Rice (*Oryza sativa* L. Japonica cv. Zhonghua 11) was used for transformation of *SWPA2-OsPIP1;3* sense and antisense construct. The *OsPIP1;3* gene and its upstream sequence was amplified from rice genomic DNA with forward primer 5'-TTG CATCTAGAG CTC GGT TGA TTA TTT C-3' and reverse primer 5'-CAA GAG CTCTAGAAT GGA GGG GAA GGA G-3'. Restriction enzyme sites are underlined. The PCR fragment was digested with *Xba*I and inserted into the binary vector pCAMBIA 1301 between the *SWPA2* promoter and a nopaline synthetase terminator in a reverse orientation. *Agrobacterium tumefaciens* strain EHA105 was used to transform this resulting construct. The stress-inducible *SWPA2* promoter was strongly induced in response to environmental stresses [21, 22]. Transformation and regeneration of transgenic rice was performed according to the protocol as described previously [23].

### Seed germination

The *O. sativa* ssp *Japonica* cv. Zhonghua11 wild type, *SWPA2-OsPIP1;3* sense and antisense seeds ( $T_2$ ) were derived from parent plants that were grown at the same time and next to each other in the same green house. Seeds were harvested and dried before being kept in sealed jars. In each experiment, at least three Petri dishes of seeds were imbibed at 4 °C for 3 d before being transferred to 28 °C

for germination. Seeds were judged to be germinated when their radicles pierced the seed coat. For the seeds that were germinated in PEG, 15% PEG6000 was used to replace water. The imbibition and germination conditions remain the same.

To investigate the effect of NO on seed germination, the NO donors sodium nitroprusside (SNP) (100  $\mu$ M, Invitrogen), *S*-nitroso-glutathione (GSNO) (100  $\mu$ M, Sigma), the NO scavenger PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) (100  $\mu$ M, Invitrogen) and in combination with SNP or GSNO were used to imbibe Nipponbare seeds at 28 °C. To check the effect of cyanide on seed germination, potassium ferricyanide (100  $\mu$ M, Sigma) was also used to imbibe Nipponbare seeds at 28 °C. The Petri dishes were wrapped with parafilm during the experiment.

### RNA preparation

RNA was extracted from embryos or seedlings using Trizol RNA isolation reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Embryos were isolated from dry seeds (kept in 28 °C for 3 d), seeds imbibed for 3 d at 4 °C (0 h) and germinating seeds (incubated at 28 °C for 12, 24, 30, 36, 48 and 72 h). To determine the expression level of *OsPIP1;3* in antisense plants, total RNA was also extracted from 2-week-old  $T_2$  plants and wild-type plants. To determine *PIP1* transcripts in wild type, sense-transgenic and silenced-transgenic seeds, embryos were isolated from the seeds germinated for 36 h in the presence or absence of 15% PEG. To prevent RNA contamination from genomic DNA, total RNA was treated with RNase-free DNase II (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

### Real-time PCR

Real-time PCR quantification of RNA targets was performed in the Rotor-Gene 3000 real-time thermal cycling system (Corbett). A rice polyubiquitin gene (*UBQ*) [24] was used as a control in the real-time PCR reactions. A two-step RT-PCR procedure was performed in all experiments. First, total RNA samples (2  $\mu$ g per reaction) were reversely transcribed into cDNAs by AMV reverse transcriptase according to the manufacturer's instructions (TaKaRa, Japan). Then the cDNAs were used as templates in real-time PCR reactions with gene-specific primers and probes (designed and synthesized by GeneCore, Shanghai; Table 1). The amplification reactions were performed in a total volume of 25  $\mu$ l. The reaction mixture contained 2  $\mu$ l of cDNA, 0.5  $\mu$ l of the forward and reverse primers (10  $\mu$ M) along with 0.5  $\mu$ l of the TaqMan-MGB probe (5  $\mu$ M) corresponding to each primer and probe, 0.25  $\mu$ l of enzymes (TaKaRa), 0.75  $\mu$ l of dNTP (10 mM); 0.5  $\mu$ l of MgCl<sub>2</sub> (250 mM); 5  $\mu$ l of 5 $\times$  real-time PCR buffer and 15  $\mu$ l of PCR-grade sterile water. The Rotor-Gene 3000 cycler was programmed as follows: 4 min at 94 °C; 60 cycles of 30 s at 94 °C and 30 s at 60 °C. A control without the corresponding template DNA was included in every quantitative PCR assay for each primer and probe set. The fluorescence signal was carried out during the annealing step. All experiments were repeated three times. The real-time amplification data were analyzed using Rotor-Gene 3000 software (Corbett). The relative expression was evaluated using the comparative cycle threshold method taking *UBQ* as the reference gene.

### PCR

PCR strategy was used to identify the silenced-transgenic plant. To avoid the interference of endogenous *O*s*PIP1;3*, PCR was car-

**Table 1** Gene-specific primers and TaqMan-MGB probes used in the real-time PCR experiments

Name	cDNA GenBank Accession No.	Probe, forward and reverse primers
<i>OsPIP1;1</i>	AJ224327	FAM (5'-3') TGTAGTTGCCAGTGGCT FP (5'-3') TACATGGGCAATGGCGGT RP (5'-3') CAAGACCGTCACCCTTGGTG
<i>OsPIP1;2</i>	AK098849	FAM (5'-3') ACCGTCCTCACCGTCAT FP (5'-3') GGCCACCTTCCTCTTCCTCTAC RP (5'-3') GCGCACTTGGAGGTGGAGT
<i>OsPIP1;3</i>	AB016623	FAM (5'-3') TGTTCGAGGCGGAGGAG FP (5'-3') AAGGACTACCGGGAGCCG RP (5'-3') GGTAGAACGACCACGACGTCA
<i>OsPIP2;1</i>	AK 072519	FAM (5'-3') TCGGCCTCGTCAAGG FP (5'-3') AGTGCCTCGGCGCCAT RP (5'-3') CGTACCTGTTGAAGTAGGCGCT
<i>OsPIP2;2</i>	AK061782	FAM (5'-3') CTGATCAGGGCGGTGC FP (5'-3') TTCGGGCTGTTCCCTGGC RP (5'-3') GGCACCTGCGGATGATGTA
<i>OsPIP2;3</i>	AL662958 (DNA No.)	FAM (5'-3') AAGAGGAGCAAGCACGG FP (5'-3') TCTTCTCCGCCACCGACC RP (5'-3') AACACCGCGAACC CAATTG
<i>OsPIP2;4</i>	AK072632	FAM (5'-3') ACTCACATGTTCCCGTGT FP (5'-3') ACCGATCCCAAGCGCAA RP (5'-3') GCGAACCCGATTGGCAG
<i>OsPIP2;5</i>	AK107700	FAM (5'-3') CGACCATCCCCGTCAC FP (5'-3') CCGTGTTTCATGGTGACCT RP (5'-3') CCGGGTTGATGCCGGT
<i>OsPIP2;6</i>	AK061312	FAM (5'-3') ACACCGTCTTCTCCGC FP (5'-3') GAAATCATCGGCACCTTCGT RP (5'-3') GGGCATTGCGCTTTGG
<i>OsPIP2;7</i>	AK109439	FAM (5'-3') CTGTACCGTGCCCTC FP (5'-3') ACGAGCGAGCTGGGTAAGTG RP (5'-3') ATGAGCGTCGCCATGAATC
<i>OsPIP2;8</i>	AK109024	FAM (5'-3') CTGCTGTTGGTGTGCAT FP (5'-3') CATTGCGGAGTTCACAGCC RP (5'-3') CCGATCACGGTGCTCACA
<i>UBQ</i>	D12776	FAM (5'-3') CACCACAGCACACGC FP (5'-3') TTGTCCTGCGCCTCCGT RP (5'-3') GCATAGGTATAATGAAGTCCAATGC

ried out with the primers 5'-TTG CCA AAT CCT ATC ATA CAC-3' and 5'-CTG GGT TGG TCC GTT CAT-3', which were based on the sequence of promoter *SWPA2* and *OsPIP1;3*, respectively, at 94 °C for 4 min, followed by 30 cycles at 94 °C for 45 s, 56 °C for 1 min and 72 °C for 1 min, and finished by an extension at 72 °C for 10 min.

#### Plasma membrane protein extraction and western blot

Embryos were isolated from dry seeds and germinating seeds (12 and 24 h after germination). Embryo plasma membrane fractions were

prepared by the aqueous two-phase partitioning method as described previously [25]. Membrane protein concentration was measured according to Bradford [26]. Western blots were performed essentially by the standard method described by Sambrook *et al.* [27]. Proteins (20 µg) were separated by SDS-PAGE (12% acrylamide), transferred to nitrocellulose membranes and blocked with 5% skim milk in phosphate-buffered saline overnight. Rabbit serum raised against the N-terminal conserved peptide, KDYNEPPPAPLFEPGELSSWS, of PIP1 conjugated to bovine serum albumin was used as the primary

antibody (1:500) and anti-rabbit IgG (1:1000) conjugated to alkaline phosphatase as the secondary antibody. The signals were detected by the 5-bromo-4-chloro-3-indolylphosphate/nitro-blue tetrazolium (NBT/BICP) method.

#### *Histochemical staining of GUS activity*

To localize the *OsPIP1;3* expression in seeds during germination, rice seeds harboring the *OsPIP1;3* promoter::GUS fusions gene [28] were germinated for 24 h in water, split in half and stained for 2 h at 37 °C in GUS assay buffer (10 mM phosphate buffer (pH 7.0), 50 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 0.1% Triton X-100, 1 mg/ml 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucopyranoside (X-gluc)), destained with 70% ethanol and then photographed. To further detect GUS activities in embryos, the embryos detached from the seed samples were transferred into FAA solution (5% formaldehyde, 5% acetic acid, 63% ethanol and 27% H<sub>2</sub>O). Subsequently, the materials were vacuum-infiltrated for 30 min and then incubated at 4 °C overnight. The samples in FAA solution were dehydrated with graded ethanol series and then embedded into Epon812-resin. The samples were polymerized at 60 °C and cut into 5- $\mu$ m-thick sections. Sections were microscopically observed (Leica DMR, Germany) and photographed.

#### *Water uptake analysis*

The amount of absorbed water by the embryos, which were separated from wild type and silenced-transgenic seeds, was determined as the increase in weight of imbibed embryos with respect to the initial dry weight of the embryos. Prior to weighing, embryos were paper-blotted for 10 s.

#### *NO assay*

Seed embryos (0.5 g fresh weight) were homogenized in 5 ml of extraction buffer (40 mM HEPES, pH 7.2). The homogenate was filtered with gauze and then centrifuged (5000  $\times$  g, 10 min) at 4 °C. The supernatants were used for NO determination using the Griess reagent system (Promega).

## Results

#### *Expression patterns of PIPs during rice seed germination and post-germination*

Real-time PCR was performed to analyze the accumulation patterns of each of 11 *PIPs* in dry embryos and during the germination and post-germination phases at intervals from 0 to 72 h after germination (Figure 1A). Except for *OsPIP1;3*, *OsPIP2;4* and *OsPIP2;5*, other *OsPIPs* were expressed in dry seeds at different expression levels. The expression of *OsPIP1;1*, *OsPIP1;2*, *OsPIP2;7* and *OsPIP2;8* markedly decreased after imbibition at 4 °C for 3 d. After germination, expression of most *OsPIPs* started to increase within 24 h. Among these, *OsPIP1;1* and *OsPIP1;2* expression increased within 12 h, but *OsPIP2;2*, *OsPIP2;3* and *OsPIP2;6* were not induced markedly until 30 h after germination. Unlike most *PIPs*, whose expression peaked before radicle emergence and declined immediately afterwards, the levels of *OsPIP2;2* and *OsPIP2;6*

increased during germination and were maintained during the post-germination phase. The distinct *PIP* expression pattern suggests that specific isoforms of *PIPs* could play different roles in seed germination.

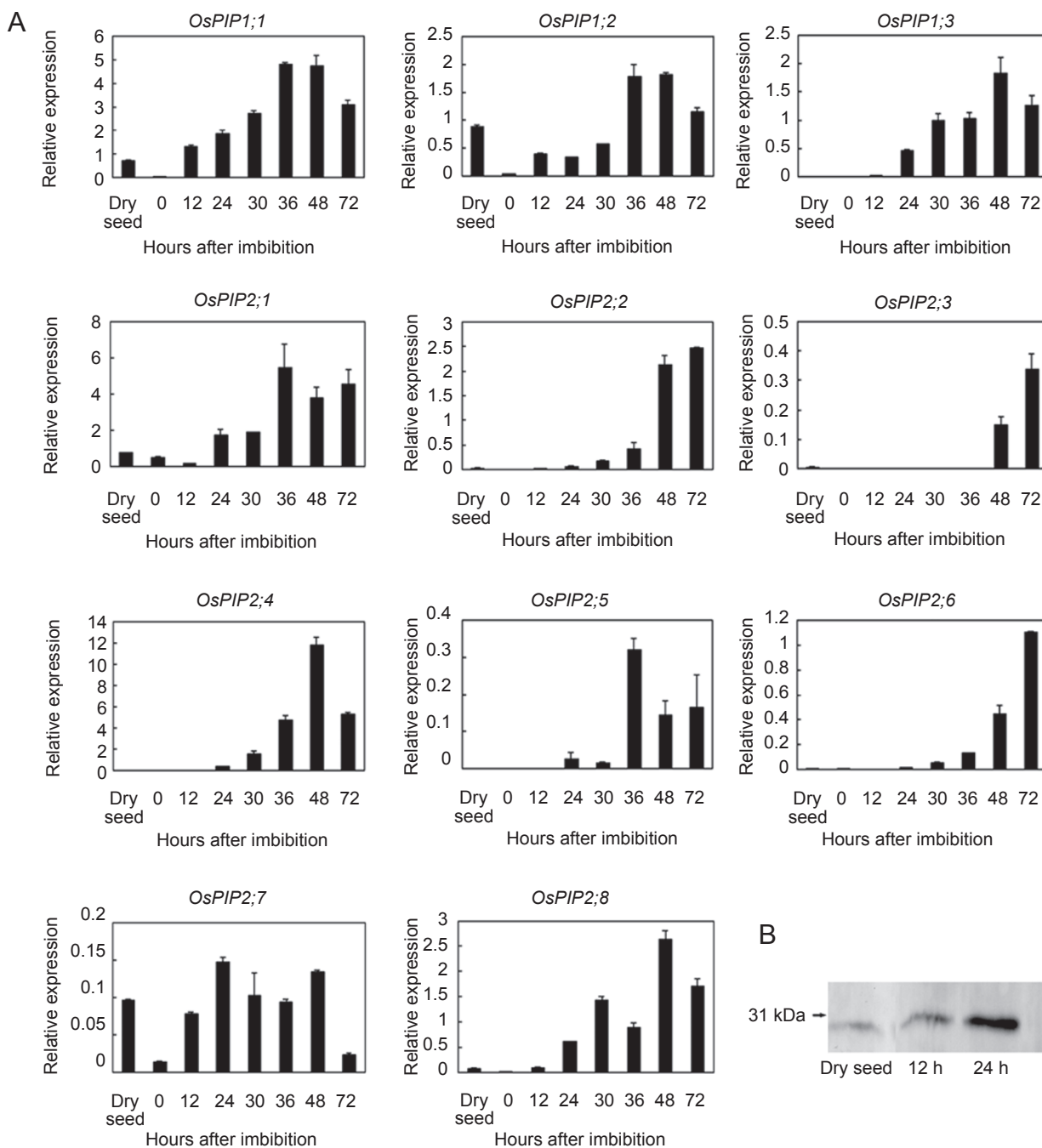
In addition, western blot was used to analyze the changes of *PIP1* protein abundance in embryos of seeds during germination. Figure 1B shows the results of a western blot analysis using purified antibody raised against a conserved N-terminal peptide of *PIPs* (Materials and Methods). According to the comparison of the sequences, the antibody should react mainly with *OsPIP1;1* and *OsPIP1;2*. *PIP1* protein abundance was stronger in the embryos after 24 h of germination than that in the embryos after 12 h of germination. In addition, *PIP1* proteins were expressed in dry embryos at very low levels (Figure 1B), indicating that the time of *PIP1* protein expression is later than that of *PIP1* mRNA expression. However, *PIP1* protein abundance increased during seed germination and exhibited a regulation pattern essentially similar to that of the transcript expression level of *PIP1* genes.

#### *Spatial expression of the OsPIP1;3 promoter-driven GUS*

The GUS activity was determined histochemically to analyze the pattern of GUS expression and infer the promoter activity of the endogenous water channel gene. The *OsPIP1;3::GUS* construct, harboring a 1.2 kb promoter, was transcribed in all the organs such as the root, stem, leaf, flower, immature embryo and the immature seed coat [28]. The GUS activity was distinctly high in the embryos of the 24-h-imbibed transgenic rice seeds in comparison to the seed coat and the endosperm (Figure 2A). In addition, a high level of GUS expression was also observed in the radicle (Figure 2B). The distribution pattern of GUS expression suggests that *OsPIP1;3* could be involved in the events of seed germination.

#### *Characterization of plants and seeds with altered expression of OsPIP1;3*

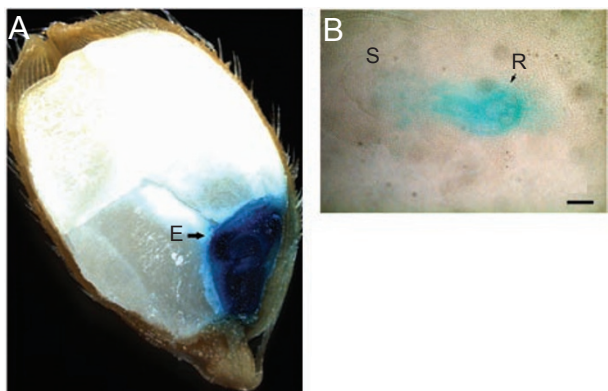
We previously described the role of *OsPIP1;3* overexpression (also known as RWC3) in drought avoidance in transgenic lowland rice [22]. To further explore water channel functions during seed germination, the expression of *OsPIP1;3* was suppressed using an antisense-silencing construct. The *OsPIP1;3* gene was cloned in an antisense orientation behind the *SWPA2* promoter [21] and transformed into wild-type rice. Eleven independent transgenic lines were selected based on their hygromycin-resistant phenotype. PCR was used to confirm the identity of transgenic plants. In order to avoid PCR detection of endogenous *OsPIP1;3*, we designed the forward primer and the reverse primer based on the sequence of the *SWPA2* promoter and



**Figure 1** PIP expression patterns in the rice embryos at various times throughout germination and post-germination. **(A)** PIP expression was determined by real-time PCR. Expression levels of PIP genes were normalized to levels of *UBQ*. Each data point represents the mean  $\pm$  S.E.M. of three independent experiments. **(B)** Western blot analysis of the PIP1 protein levels in the embryos of rice seeds. The rice seeds were imbibed in water for 12 h and 24 h. Twenty micrograms of plasma membrane protein was loaded.

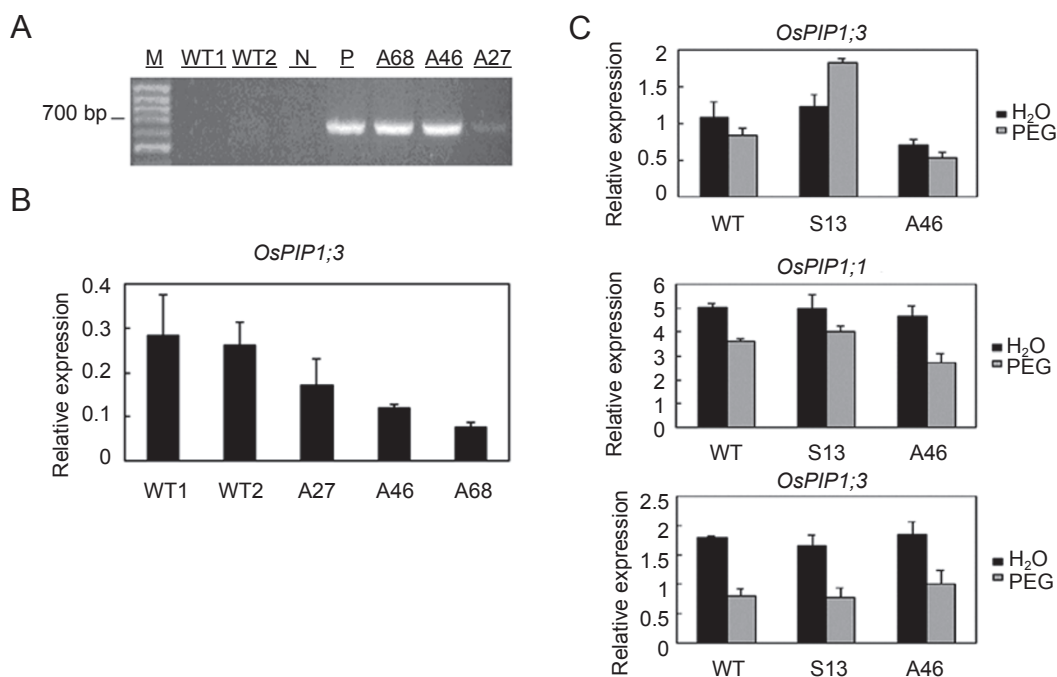
*OsPIP1;3*, respectively. As representative examples, Figure 3A shows that 700 bp PCR products were amplified specifically from genomic DNA in three lines of the T<sub>2</sub> transgenic

plants. Real-time PCR analysis revealed that three different T<sub>2</sub> *OsPIP1;3* antisense lines expressed *OsPIP1;3* at reduced levels, which range from 27% to 60% of the wild-type



**Figure 2** Tissue-specific expression analysis of the *GUS* reporter controlled by the *OsPIP1;3* gene promoter. **(A)** Localization of *OsPIP1;3::GUS* expression in the embryos of 24-h-imbibed seeds is shown. Seeds were split in half and then stained with *GUS* solution. **(B)** Strong *GUS* expression in the radicles of embryos is shown. The embryos were cut into 5- $\mu$ m-thick sections. Sections were microscopically observed (Leica DMR, Germany) and photographed. E: embryo; R: radicle; S: shoot. Scale bar = 150  $\mu$ m.

level at normal growth conditions (Figure 3B). Although *SWAP2* promoter is a stress-inducible promoter, it has a basal expression about one-fourth to the 35S promoter at normal growth conditions [21]. Therefore, the antisense plants have reduced expression levels of *OsPIP1;3* even at non-inducible conditions. The *OsPIP1;3* antisense lines A27, A46 and A68 were used in further experiments. We traced the expression pattern of *OsPIP1;3* in wild type, sense-transgenic and antisense-transgenic seeds treated with distilled water or water deficit mediated by PEG 6000 for 36 h. The sense-transgenic seeds had approximately the equivalent amount of *OsPIP1;3* mRNA under normal conditions, while they had an obviously increased *OsPIP1;3* expression compared with the wild-type seeds under the water-deficit condition (Figure 3C). The mRNA level of *OsPIP1;3* decreased in the antisense-transgenic seeds both under normal conditions and upon water deficit compared with the wild-type seeds, and it was lower under water-deficit conditions (Figure 3C). These results suggest



**Figure 3** Characterization of *OsPIP1;3* antisense-transgenic rice lines, sense-transgenic and antisense-transgenic seeds. **(A)** PCR analysis of the genomic DNA in transgenic plants. PCR was carried out with the primers 5'-TTG CCA AAT CCT ATC ATA CAC-3' and 5'-CTG GGT TGG TCC GTT CAT-3'. PCR products amplified from the genomic DNA of wild type (WT1 and WT2) and three lines of antisense-transgenic plants (A27, A46 and A68) are shown. Other lanes represent: plasmid PCAMBIA 1301-*SWPA2::OsPIP1;3* (P); DNA marker (M); water as template (N). **(B)** Real-time PCR showed that antisense-transgenic plants exhibited reduced expression of *OsPIP1;3*. The intensity of *OsPIP1;3* signal was normalized against *UBQ* signal. Data are the means  $\pm$  S.E.M. of three independent experiments. **(C)** Real-time PCR showed the expression of *OsPIP1;3*, *OsPIP1;1* and *OsPIP1;2* in wild type (WT), sense-transgenic (S13) and antisense-transgenic seeds (A46) imbibed in water or under water deficit induced by 15% PEG-6000 solutions. The intensity of these three PIP signals was normalized against *UBQ* signal. Data are the means  $\pm$  S.E.M. of three independent experiments.

that the stress-inducible *SWPA2* promoter could regulate the expression of *OsPIP1;3* gene in the seeds of transgenic rice under drought stress.

The amino-acid sequences are highly conserved among three members of the *OsPIP1* group with identities of more than 80%; therefore, we also examined the expression of *OsPIP1;1* and *OsPIP1;2* in the transgenic seeds. As shown in Figure 3C, both at normal growth condition and upon water deficit, the mRNA level of *OsPIP1;1* had no difference between sense-transgenic seeds and the wild type seeds; however, it was reduced in the antisense-transgenic seeds upon water deficit. The expression of *OsPIP1;2* transcript was unaffected both in sense-transgenic and in antisense-transgenic seeds (Figure 3C).

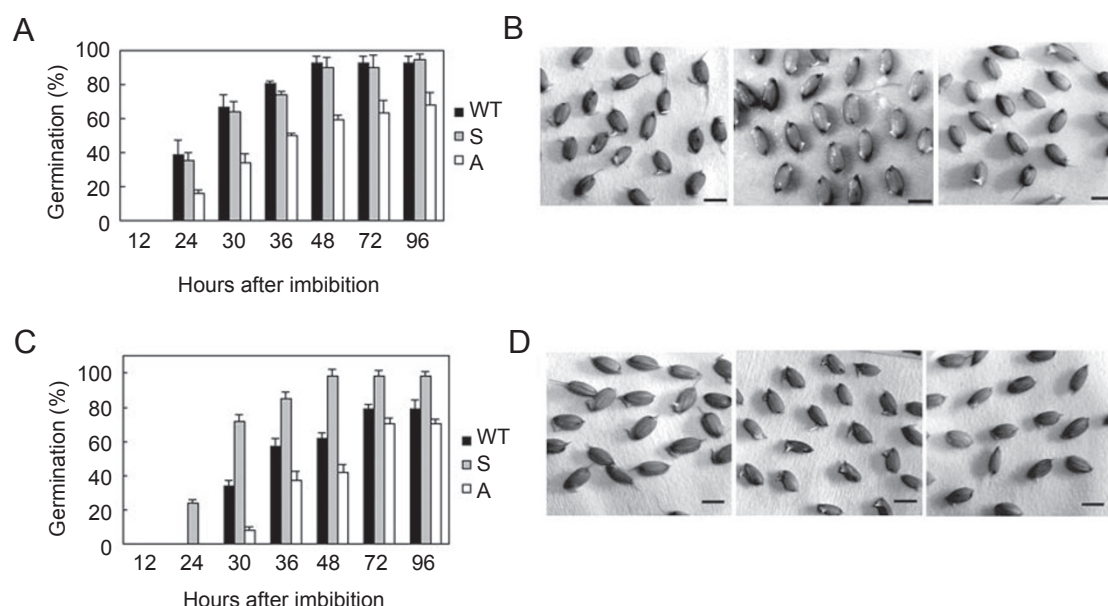
#### Effects of *OsPIPs* on seed germination

Figure 1A shows that *OsPIP1;3* expression was weak at 12 h after germination and started to increase at 24 h, peaked at 48 h, then declined at 72 h. The developmental regulation of *OsPIP1;3* in wild-type seeds is consistent with the progress of seed germination, which started at 24 h and terminated at 48 h (Figure 4A). We analyzed the germination of *OsPIP1;3* sense, antisense and wild-type rice seeds systematically. After 48 h of germination, 90% of wild-

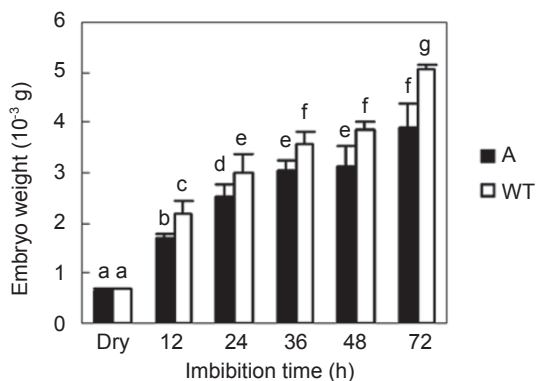
type seeds and 92% of sense-transgenic seeds germinated, but only 60% of antisense-transgenic seeds germinated (Figure 4A and 4B). Although 70% of the antisense seeds eventually germinated, this occurred as late as the fourth day after germination (Figure 4A). These results suggest that *OsPIP1;3* is required for seed germination in rice. In addition, *OsPIP1;1* may be another required component for rice seed germination, as its expression was also partially reduced in the *OsPIP1;3* antisense-transgenic seeds.

As shown in Figure 4C and 4D, wild type and antisense-transgenic seeds exhibited significantly reduced germination rate when germinated in 15% PEG, while seeds with over-expressed *OsPIP1;3* germinated normally under the same conditions. In addition, the final germination percentage from sense lines was higher than that of wild type (Figure 4C). The germination rate of the antisense lines was substantially inhibited under water-stress conditions with only 8% germination at 30 h after germination (Figure 4C). These observations suggested that increased water channel levels might provide seeds with additional ability to withstand water deficit. Taken together, these data further indicate that water channels play an important role in seed germination.

Because the rate of germination was significantly



**Figure 4** Phenotypic characterization of seeds from sense (S) and antisense (A) plants. **(A)** Time course of germination of WT, sense-transgenic and antisense-transgenic seeds in water. The percentage of germinated seeds corresponds to the mean of three experiments of 50 seeds. **(B)** Seeds of WT (left), sense (middle) and antisense-transgenic (right) lines corresponding to the 48 h time point in **(A)**. **(C)** Time course of germination of WT, sense and antisense seeds in the water containing 15% PEG. The percentage of germinated seeds corresponds to the mean of three experiments of 50 seeds. **(D)** Seeds of WT (left), sense (middle) and antisense (right) lines corresponding to the 48 h time point in **(C)**. Bar = 6 mm **(B)**, Bar = 5 mm **(D)**.



**Figure 5** Comparison of the capacity of water uptake in the wild type and antisense *OsPIP1;3* rice seed embryos at different stages of germination. At each time point, different letters above bars indicate significant differences at  $P < 0.05$  (0.01) as determined by one-way ANOVA followed by Turkey's test.

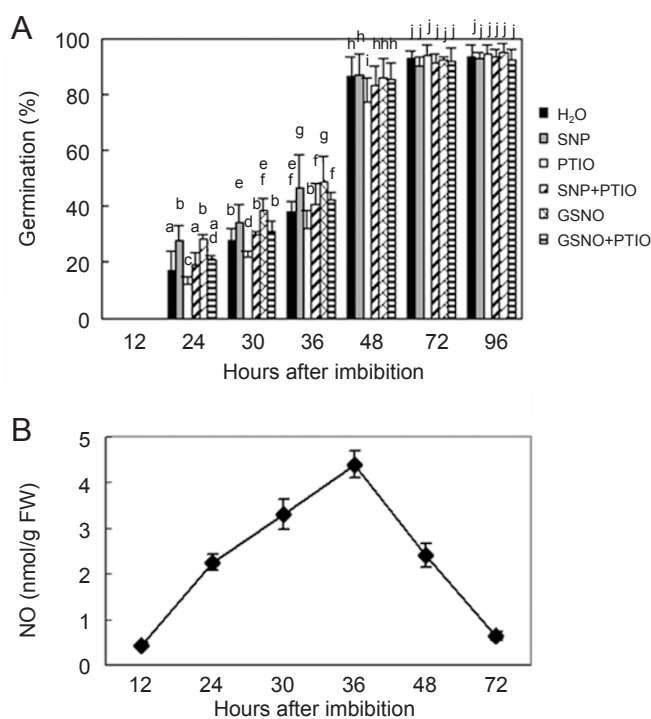
reduced in *OsPIP1;3* antisense-transgenic seeds, we investigated the effects of gene silencing on water uptake during seed germination. We measured water uptake from detached wild type and antisense-transgenic seed embryos. Throughout the duration of imbibition, wild-type seed embryos consistently took up more water than antisense-transgenic seed embryos as determined by embryo weight ( $P < 0.05$  at 24 and 36 h,  $P < 0.01$  at 12 and 48 h) (Figure 5). These results indicate that the reduced seed germination rate in antisense-transgenic plants resulted from the decreased availability of water uptake. The sense-seeds and the wild-type seeds showed no difference when they were germinated under near optimal conditions, and there was no difference in their water uptake (data not shown).

#### Function of NO on seed germination

We investigated the effects of the exogenous NO donors, SNP and GSNO, and the NO scavenger PTIO on rice seed germination to examine the role of NO in this process. SNP releases NO when illuminated [29] or when it reacts with biological tissues [30]. Murgia *et al.* [31] reported that different NO donors could have different or even opposite effects on plant metabolism; therefore, we used two NO donors GSNO and SNP. An increase in the seed germination percentage was detected when rice seeds were germinated under light in the presence of 100  $\mu$ M SNP or 100  $\mu$ M GSNO for 24, 30 or 36 h (Figure 6A). The results revealed that NO donors promote seed germination in the early stage of germination. Furthermore, the seed germination was delayed in 100  $\mu$ M PTIO (Figure 6A). In addition, when the NO scavenger PTIO was added to the solutions containing SNP or GSNO, the positive effects of SNP and

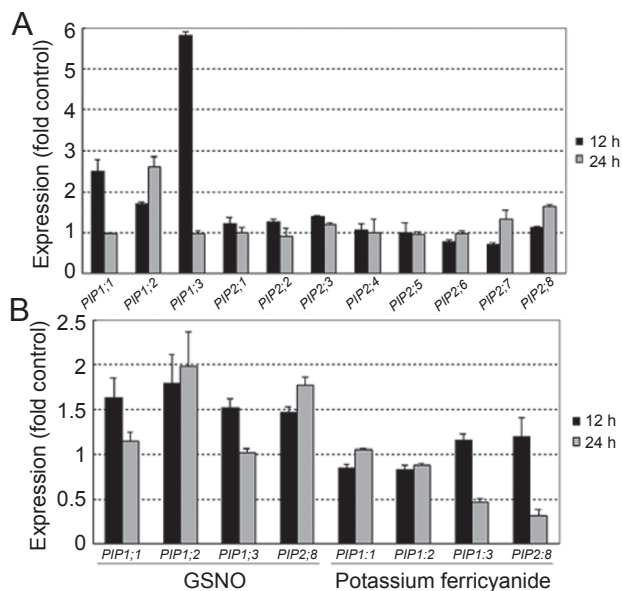
GSNO on seed germination were weakened (Figure 6A). It was reported recently that SNP, as well as potassium ferricyanide and potassium ferrocyanide, reduced dormancy of *Arabidopsis* seeds by generating cyanide [32]. To determine whether cyanide was the compound in SNP vapors that promoted rice seed germination, control experiments were carried out with potassium ferricyanide. There was no significant difference in the rate of germination and the final germination percentage between the seeds germinated in potassium ferricyanide and water (data not shown). These results suggest that exogenous NO can play a role in regulating rice seed germination. However, we could not rule out the possibility that SNP reduces dormancy of seeds by generating cyanide, because we did not directly investigate the effects of NO on seed dormancy in this study.

To document the effect of endogenous NO on seed



**Figure 6** Effects of NO on seed germination. (A) The NO donors, SNP and GSNO, stimulated seed germination and the NO scavenger PTIO inhibited seed germination. Seeds were imbibed with distilled water alone, or imbibed with 100  $\mu$ M SNP, 100  $\mu$ M GSNO, 100  $\mu$ M PTIO or 100  $\mu$ M SNP with 100  $\mu$ M PTIO, 100  $\mu$ M GSNO with 100  $\mu$ M PTIO. Values are the means  $\pm$  S.E.M. of three independent experiments. At each time point, different letters above bars indicate significant differences at  $P < 0.05$  as determined by one-way ANOVA followed by Turkey's test. (B) Nitrite concentration as an estimate for NO production in germinating seeds. Values are the means  $\pm$  S.E.M. of three independent experiments.





**Figure 7** The response of *PIP* expression to NO and cyanide. **(A)** SNP induced *OsPIP1;1*, *OsPIP1;2*, *OsPIP1;3* and *OsPIP2;8* expression in the embryos of germinating seeds. Seeds were imbibed with distilled water or 100  $\mu$ M SNP. **(B)** The effects of GSNO and potassium ferricyanide on *OsPIP1;1*, *OsPIP1;2*, *OsPIP1;3* and *OsPIP2;8* expression. The relative expression of *PIPs* in the treated embryos was described as fold control (treated/control ratio). Values are the means  $\pm$  S.D. of three independent experiments.

germination, we imbibed seeds in water for 12, 24, 30, 36, 48 and 72 h, and then analyzed NO concentration in embryos. As shown in Figure 6B, the content of NO in homogenates from embryos significantly increased between 12 and 36 h of imbibition, and sharply declined after 36 h of germination, coinciding with the initiation of an active germination.

*Effects of NO on PIP transcript levels in germinating seed*

Hormones such as ABA or GA<sub>3</sub> and a variety of environmental stimuli regulate the transcription of water channel genes [33]. NO/cGMP signal transduction has a crucial role in Ca<sup>2+</sup> homeostasis in the M3 muscarinic acetylcholine-stimulated increase in AQP5 levels in the apical plasma membrane of rat parotid glands [34]. NO plays a role in seed germination, yet little is understood about how it functions. The data presented here show that NO triggered certain *PIP* expression during seed germination. NO upregulated the expression of four of 11 rice *PIPs* (Figure 7A). As analyzed by real-time PCR, SNP caused a 2.6-fold increase in *OsPIP1;1* transcript levels

in germinating seeds after 12 h of treatment, and 1.7- and 2.6-fold increase in *OsPIP1;2* transcript levels after 12 and 24 h of treatment, respectively (Figure 7A). SNP also increased *OsPIP1;3* expression by 5.8-fold at 12 h after germination, but had no effect at 24 h after germination. GSNO also affected the expression levels of *PIP1* mRNA in seeds. As shown in Figure 7B, the expression of *OsPIP1;1* was increased to 1.6-fold at 12 h of imbibition. *OsPIP1;2* exhibited about 1.8- and 2.0-fold increase after 12 and 24 h of germination, meanwhile, *OsPIP1;3* expression at 12 h after germination was increased to 1.5-fold in treated embryos. Among the eight members of the rice *PIP2* sub-family, only *OsPIP2;8* was significantly induced by the application of exogenous NO. 1.6- and 1.8-fold increase in *OsPIP2;8* transcript levels were detected in germinating embryos after treatment with SNP and GSNO for 24 h compared with the control. Treatment with SNP did not result in an increase in transcript levels of any *PIPs* after 36 h of germination (data not shown). In order to determine if the effect of SNP on *PIP* expression is the result of NO or the result of cyanide, it is necessary to know the response of the *PIP* to cyanide. The response of *PIP* expression to the treatment of potassium ferricyanide solution is shown in Figure 7B. These data show that potassium ferricyanide at a concentration of 100  $\mu$ M did not result in enhancement of *OsPIP1* and *OsPIP2;8* expression; on the contrary, it reduced their expression at certain time points. Although the NO donors could promote germination of seeds from *OsPIP1;3* antisense lines, the final germination percentage of these seeds was still lower than that of the wild-type seeds (data not shown). On the basis of the above data, we conclude that the effects of NO on rice seed germination may be partly mediated through induction of water channels and therefore enhanced ability of water uptake.

**Discussion**

The presence of *PIP1* members in dry and germinating seeds has been noted before. The mRNA of *PsPIP1;1* or its possible homologs was detected in the dry and germinating seeds, suggesting that they could play a role in water absorption during seed imbibition [35]. It was also shown previously that the water channel proteins, *BnPIP1* and *BnTIP2*, play a role in seed germination [9]. The authors speculated that *BnPIP1* was involved in water transport required for the activation of enzymatic metabolism of storage nutrients in the early stages of canola seed germination, while *BnTIP2* expression was correlated with cell growth during radicle emergence. To gain insight into the role of water channel family in germination, we investigated the expression of *PIP1* and *PIP2* subfamilies in rice seed. The time course of *PIP* expression in embryos

showed that it was developmentally regulated. The expression of *OsPIP1;1*, *OsPIP1;2*, *OsPIP1;3*, *OsPIP2;1*, *OsPIP2;4*, *OsPIP2;5* and *OsPIP2;8* increased strongly in the embryo a few hours before radicle emergence. This expression profile indicated that they might be involved in the processes linked to seed germination. Other PIPs could also play an important role in the movement of water during seed germination, because *OsPIP2;2*, *OsPIP2;6* and *OsPIP2;7* existed in germinating seeds. In the embryos of dry seeds, except for *OsPIP1;3*, *OsPIP2;4* and *OsPIP2;5*, other *OsPIPs* were present at different expression levels. It is noteworthy that *OsPIP2;3* was not expressed until radicle emergence, followed by increased expression with seedling growth. These results suggest that *OsPIP2;3* does not contribute to seed germination but functions in plant growth. Both the PIP1 and PIP2 subfamilies might also be involved in the developmental process of the early phases of post-germination, because all were still present after radicle emergence.

Previous studies have revealed that water channels exist in almost all types of tissues [2, 28], and their crucial functions have been demonstrated in many plants [33, 36]. They comprise a relatively large gene family. For example, there are 35 water channel homologs in *Arabidopsis* [5] and 33 members in maize [6]. At least 33 water channel genes have been identified in the rice genome [3]. A series of papers have elucidated the function and regulation of PIP. These water channels are likely to play different roles in different cells and expected to be regulated differently. In the present experiment, we tried to clarify the function of water channel in seed germination by over-expressing and silencing *OsPIP1;3*. Our results indicated that the sense-transgenic rice seeds germinated faster than wild-type seeds upon water-deficit treatment. Yu *et al.* [37] have also demonstrated that over-expression of BnPIP1 in transgenic tobacco resulted in earlier seed germination upon drought condition than the untransformed control. Water channel proteins appear to be functionally sufficient when seeds germinate under normal conditions, since wild-type seeds have the ability to take up enough water for germination. Silencing water channel expression seems to reduce the ability of seeds to absorb water as a result of the water channel deficiency. However, PIP1 isoforms have often been found to have very low, or no, water permeability when expressed solely in *Xenopus* oocytes [22, 38-40]. Previously, Chaumont and his co-workers [41] have demonstrated that interactions between maize ZmPIP1;1 and ZmPIP1;2 or between ZmPIP1;2 and ZmPIP2 positively modulated their water channel activity. Hence, PIP1-members may function as water channels in seed germination via PIP interactions and these interactions enhance their water channel activity. There is another possibility that PIP1-members are not

directly involved in water transport, but are related to other physiological functions in seed germination.

Previous reports have demonstrated that water channels are regulated by many factors including developmental and environmental factors [42]. The transmembrane water flux via the regulation of aquaporin quantity and activity endows plants with a remarkable capacity to modulate water absorption, transport and compartmentation within tissues. In animal studies, the role of NO/cGMP signal transduction in the M3 muscarinic acetylcholine receptor-stimulated increase in AQP5 levels in the apical plasma membrane of rat parotid glands was reported [34]. Pretreatment of rat parotid tissue with the NO scavenger inhibited acetylcholine-induced increases in AQP5 in the apical plasma membrane. NO donors mimicked the effects of muscarinic acetylcholine receptor agonists. However, the possible role of NO signaling in changes of PIP levels in plants was unknown. In this study, we revealed the involvement of NO in regulation of PIP expression as observed in the response of germinating seeds to exogenous NO treatment. The phylogenetically divergent subgroups of water channels may reflect isoforms with different biochemical properties and may fulfill unique or diverse functions in plant development, and hence their patterns of regulation are expected to differ. Here, the three *PIP1* genes, *OsPIP1;1*, *OsPIP1;2* and *OsPIP1;3*, expressed in germinating rice seeds, were classified by phylogenetic analysis as belonging to the same subgroup [3]. They also exhibited similar developmental patterns of expression and were regulated similarly by exogenous NO. Most other members of the *PIP2* group did not respond to exogenous NO except *OsPIP2;8*; and it has a unique sequence different from the others in the family. This suggests that the regulation of individual water channels may be tied to their physiological functions in each organ.

Together, the data presented here indicate that the PIPs play an important role in seed germination. The reduced seed germination rate via *OsPIP1;3* silencing and the promotion of seed germination via *OsPIP1;3* over-expression under drought conditions demonstrated clearly that *OsPIP1;3*, as well as perhaps *OsPIP1;1*, is required for normal seed germination. In addition, our data also suggest that NO can promote rice seed germination and certain water channel proteins could function in this process as downstream effectors of the NO signaling pathway. Specific rice NO signaling mutants and other PIP transgenic plants are necessary to better understand NO-mediated signal transduction and its relationship with water channel proteins in plant growth and development.

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