Cell Research (2006)16: 277-286
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ORIGINAL ARTICLE

Expression and functional analysis of the rice plasma-membrane intrinsic protein gene family

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Plasma membrane intrinsic proteins (PIPs) are a subfamily of aquaporins that enable fast and controlled translocation of water across the membrane. In this study, we systematically identified and cloned ten *PIP* genes from rice. Based on the similarity of the amino acid sequences they encoded, these rice *PIP* genes were classified into two groups and designated as *OsPIP1-1* to *OsPIP1-3* and *OsPIP2-1* to *OsPIP2-7* following the nomenclature of *PIP* genes in maize. Quantitative RT-PCR analysis identified three root-specific and one leaf-specific *OsPIP* genes. Furthermore, the expression profile of each *OsPIP* gene in response to salt, drought and ABA treatment was examined in detail. Analysis on transgenic plants over-expressing of either *OsPIP1* (*OsPIP1-1*) or *OsPIP2* (*OsPIP2-2*) in wild-type *Arabidopsis*, showed enhanced tolerance to salt (100 mM of NaCl) and drought (200 mM of mannitol), but not to salt treatment of higher concentration (150 mM of NaCl). Taken together, these data suggest a distinct role of each *OsPIP* gene in response to different stresses, and should add a new layer to the understanding of the physiological function of rice *PIP* genes.

Cell Research (2006) **16:**277-286. doi:10.1038/sj.cr.7310035; published online 16 March 2006

***Keywords*:** aquaporins, PIP, quantitative RT-PCR, *Oryza sativa*, organ-specific

Introduction

Water is a fundamental, yet the most essential solvent in all organisms. Transmembrane water transport occurs by diffusion through the lipid bilayer and/or by increased membrane permeability due to the water channel proteins, aquaporins [1]. The presence of aquaporins, a class of major intrinsic proteins (MIPs), enables fast and controlled translocation of water across the membrane. Aquaporins have been found in bacteria, fungi, animals and plants. Plant aquaporins are classified into four major groups: the tonoplast intrinsic proteins (TIPs) localized to the vacuolar membranes, the plasma membrane intrinsic proteins (PIPs) localized to the plasma membranes, the NOD-26-like MIPs (NIPs), and the recently characterized small basic intrinsic proteins (SIPs) [2-5].

The structures of aquaporins have been intensively studied, revealing the functions of their conserved motifs and post-translational modification sites, in water transport [3-15]. The expression patterns of aquaporin genes in response to salt, drought, cold, submergence, light, and phytohormones have also been characterized in various plants [16-24]. Some aquaporins are tissue-specific, such as γ-*MIP* in shoots of rice [21], γ-*TIP* in seeds of *Brassica napus* [16] and *ZmTIP2-3* in roots of maize [25].

Aquaporins have been systematically identified and classified in *Arabidopsis* and maize. There were thirteen *PIP* genes among the identified thirty and thirty-one aquaporin genes in *Arabidopsis* and maize, respectively [13, 26]. The function and expression patterns of the *PIP* gene family have been intensively studied in *Arabidopsis*, *Brassicca*, and maize, especially in response to abiotic stress [7, 17,

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Received 4 Jul 2005; revised 30 Sep 2005; Accepted 17 Oct 2005, published online 16 March 2006

18, 26-29].

However, little is known about the aquaporin gene family in rice. Up to date, three *PIP* genes have been identified in rice, one of which was shown to have water transport activity in the *Xenopus* oocytes [30, 31]. The completion of rice genome sequence enables us to systematically identify the rice aquaporin genes and analyze their structure, biochemical property and physiological function.

In this paper, we identified and structurally analyzed 10 rice *PIP* genes (*OsPIPs*) that were classified into two subgroups (*OsPIP1* and *OsPIP2*). Utilizing the quantitative RT-PCR method, we analyzed the response to abiotic stress by exploring their expression profile under various treatments, including salinity, drought, and ABA. Moreover, to further investigate the function of rice PIP proteins, we examined the tolerance of transgenic *Arabidopsis* plants over-expressing the rice *PIP* genes to salinity and drought.

Materials and Methods

Plant material and stress treatment

A rice cultivar (*Oryza sativa* L. spp. *japonica* cv. Zhonghua 11) and *Arabidopsis thaliana* (Columbia ecotype) were used in this study. Rice seeds were surface-sterilized and germinated on N6 solid medium containing 3% sucrose, pH5.8. Rice seedlings of 3-d-old were transplanted into N6 liquid medium containing 3% sucrose, and grow at 25-28ºC under continuous light.

For salinity, drought and ABA treatments, 2-week-old rice seedlings were placed in the fresh N6 liquid medium containing 250 mM of NaCl (salinity stress), 10% PEG6000 (drought stress) and 50 μM of ABA [16, 31, 32], respectively. During the treatment, leaves and roots were collected separately at different time points and immediately frozen in liquid nitrogen. Eight individual seedlings were used for each time point and treatment, and the experiments were independently repeated three times.

RNA isolation and cDNA synthesis

Total RNAs of the leaves and roots of rice seedlings were isolated using the Plant RNeasy extraction kit (Qiagen, USA) and then digested with RNase-free DNaseI (Boeheringer Mannheim, Germany) according to the manufacture's instruction. RNA concentration was measured by spectrophotometer and RNA quality was examined in 1.2% formaldehyde agarose gel. Five microgram of total RNA was used to synthesize cDNA using SuperScript™ II RNase H$^-$ Reverse Transcriptase Kit (Invitrogen, USA) according to the manufacture's instruction.

Primer design and cDNA cloning

The sequences of primers for PCR amplification of full-length cDNAs of *OsPIP* genes and for quantitative RT-PCR are listed on Supplement 1. DNA sequence comparisons were made to ensure that each pair of primers is specific to the corresponding *OsPIP* genes. Amplified cDNAs were cloned into T-vector (Promega, USA) and sequenced.

Quantitative RT-PCR and quantification of transcript copy numbers of OsPIP *genes*

The quantitative RT-PCR was carried out in the DNA Engine OPTICON 2 Continuous Fluorescence Detector (MJ research, USA), using DyNAmo SYBR Green qPCR kit (Finnzymmes, Finland). PCR amplifications were performed in a total volume of 20 μl, containing 2 μl cDNA (1:10 diluted, corresponding to about 40 ng RNA), 100 nM of each gene specific primer and 10 μl 2 × SYBR (Finnzymmes, Finland). Each PCR reaction was performed in three or four parallel independent experiments, using the following conditions: 95 ºC 5 min; 40 cycles of 15 s at 94 ºC, 30 s at 55 ºC, 12 s at 72 ºC, and plate read. The data collection was carried out during the extension step. For a negative control, the PCR was performed without cDNA templates. The absolute and relative transcript copy number of *OsPIP* genes in each treated sample was calculated following the method described by Jang *et al.* [17], except that a rice actin gene (*OsACT1*) was used to calculate the correction factor instead of the *Arabidopsis* actin gene.

Plant transformation and root elongation analysis

OsPIP1-1 and *OsPIP2-2* cDNAs were cloned into pQGIE110, and transformed into the *Agrobacterium* GV3101. The cDNAs were then transformed into the wild-type *Arabidopsis* by floral dip method [33]. The total RNAs were extracted from transgenic seedlings as described above. The over-expression of *OsPIP1-1* and *OsPIP2-2* in transgenic plants was confirmed by RT-PCR.

Seeds of transgenic plants with *OsPIP1-1* (line 7 and line 10), *OsPIP2-2* (line 8 and line 19), and pQGIE110 vector were surface-sterilized and sown onto Murashige and Skoog (MS) solid medium [34] at 4 ºC for vernalization. After three days, the plates were placed vertically under normal growth condition in a growth chamber (Percival, USA). About 4-5 d later the healthy seedlings were transferred to fresh MS medium with or without 100 mM of NaCl, 150 mM of NaCl, and 200 mM of mannitol. The plates were then placed vertically in the growth chamber again, but with the seedlings up-side-down [35]. The root length of each seedling was measured everyday for 7 d and analyzed by Image J software (NIH, USA).

Results

Identification and sequence analysis of OsPIP*s in rice*

After a systematic search over the NCBI database, rice genome sequence databases of the Beijing Genomics Institute (http://rise.genomics.org.cn), and the International Genome Research Program Website in Japan (http://rgp.dna.affrc.go.jp) using the cDNA sequences of 13 maize *PIP* genes as queries, we identified 10 rice genes that encode the plasma membrane intrinsic proteins (*OsPIP*s). The cDNAs (coding region and 3'-UTR) of the 10 rice *PIP* genes were amplified by RT-PCR, cloned and sequenced. The 10 genes were divided into two groups based on the similarity of the amino acid sequence they encoded and the phylogenetic analysis (Figure 1A and 1B), and were designated as *OsPIP1-1* (*OsPIP1a*), *OsPIP1-2*, *OsPIP1-3* (*RWC-3*), *OsPIP2-1* (*OsPIP2a*), *OsPIP2-2*, *OsPIP2-3*, *OsPIP2-4*, *OsPIP2-5*, *OsPIP2-6,* and *OsPIP2-7* following the nomenclature of *PIP* genes in maize [31, 32].

The DNA sequences of six *OsPIP* genes (*i.e. OsPIP1-1*,

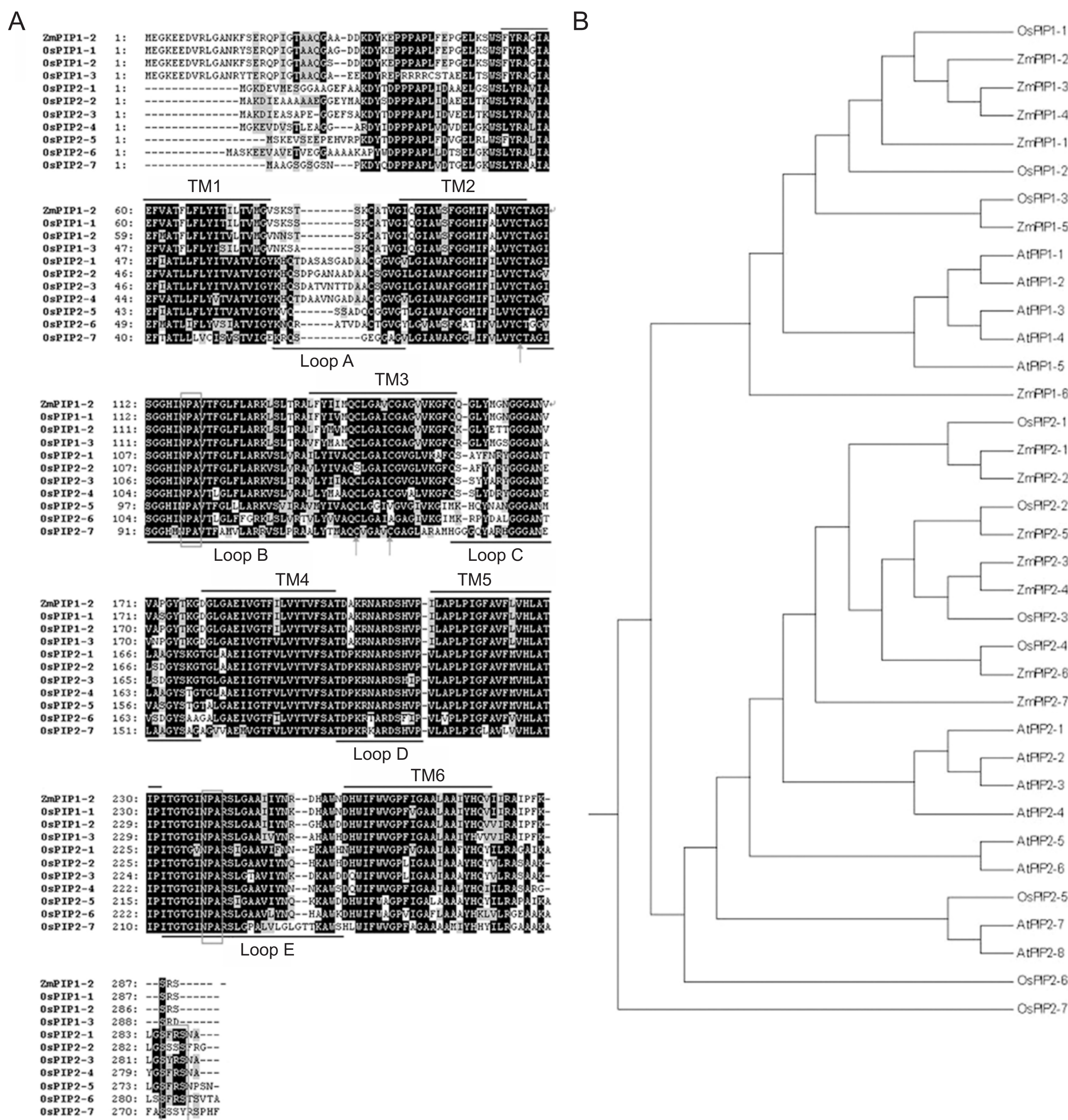

Figure 1 Deduced amino acid sequences of OsPIP proteins and their phylogenetic relationships. **(A)** Alignment and the deduced structure of the OsPIP proteins. Two conserved NPA motifs in loop B and loop E, and putative phosphorylation sites of *PIP2* at C-terminus are boxed. Putative mercury sensitive sites are indicated by arrows. Putative bilayer-spanning domains (TM 1-6) and the connecting loops (A-E) are indicated by solid line. **(B)** Phylogenetic tree of PIP proteins from rice (OsPIP), maize (ZmPIP) and *Arabidopsis* (AtPIP) based on the deduced amino acid sequences aligned by Clustal W.

OsPIP1-2, *OsPIP2-1*, *OsPIP2-2*, *OsPIP2-3,* and *OsPIP2-7*) were identical to those in the databases, while the sequences of *OsPIP1-3*, *OsPIP2-4*, *OsPIP2-5*, and *OsPIP2-6* were slightly different from those in the databases. The differences in DNA sequences did not alter their encoded amino acid sequences. The OsPIP proteins contained 280 to

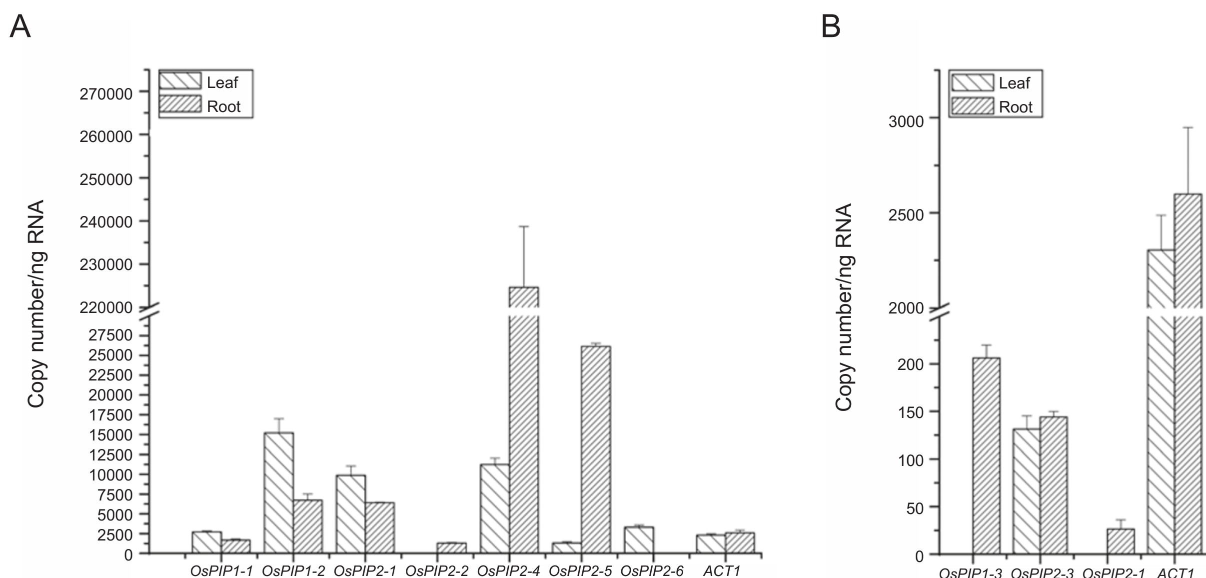

Figure 2 Absolute transcript copy numbers of each *OsPIP* gene in the leaves and roots of 2-week-old rice seedlings. **(A)** The genes with transcript copy numbers more than 10^3. **(B)** The genes with transcript copy numbers less than 210. Error bars: standard errors.

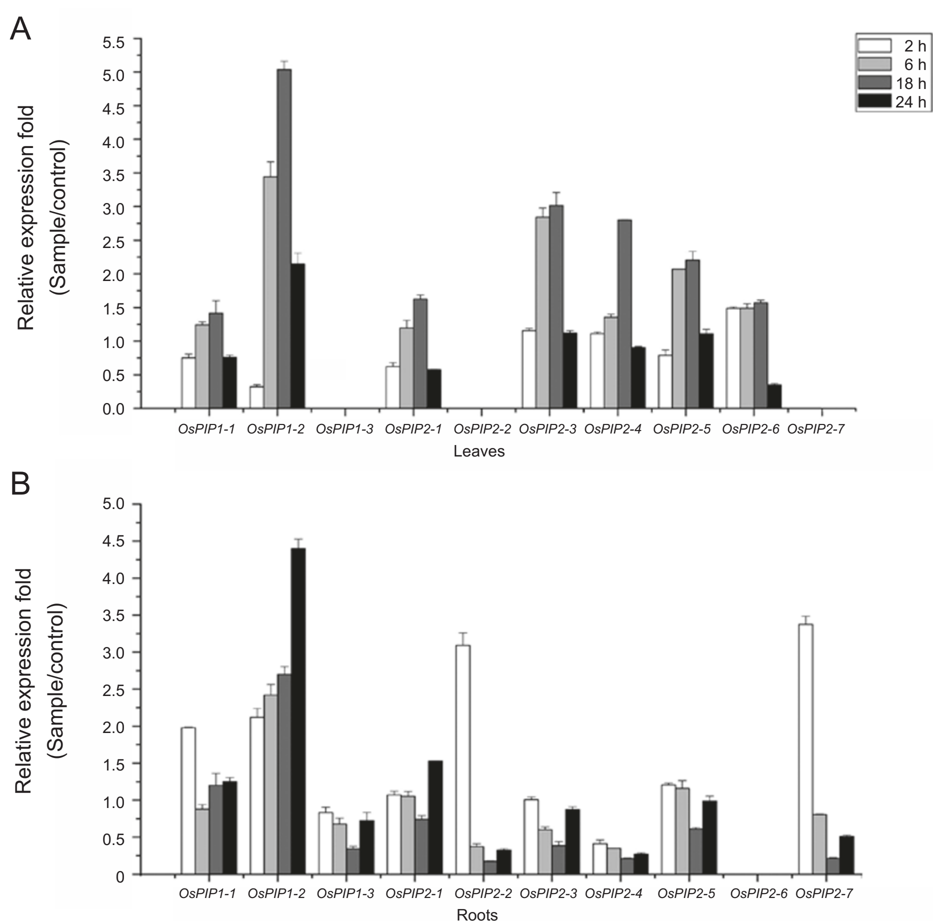

Figure 3 Relative expression levels of the *OsPIP* genes in response to ABA in the leaves and roots of rice seedlings. **(A)** Relative expression level of the *OsPIP* genes in the leaves. **(B)** Relative expression level of the *OsPIP* genes in the roots. The transcript levels of each *OsPIP* gene in the leaves and roots of treated seedlings were plotted as the relative expression fold of the non-treated seedlings (0 h) for 2, 6, 18 and 24 h. Error Bars: standard errors.

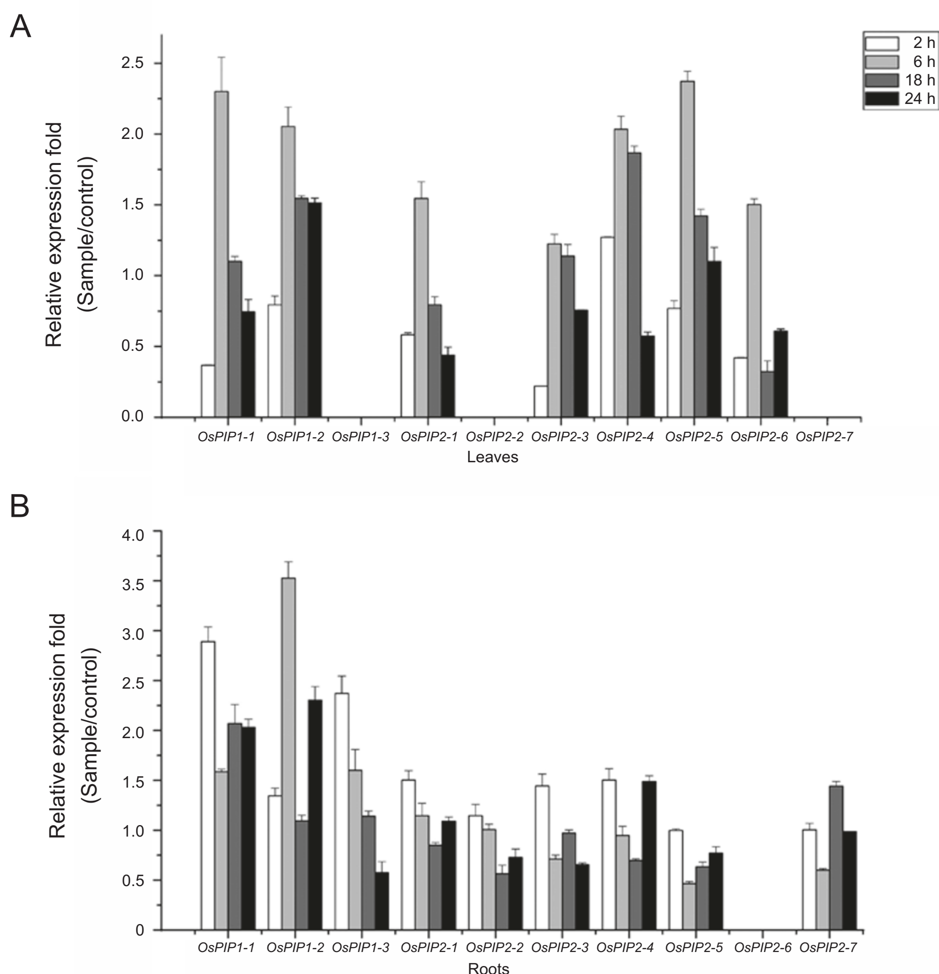

Figure 4 Relative expression levels of the *OsPIP* genes in response to NaCl in the leaves and roots of rice seedlings. **(A)** Relative expression level of the *OsPIP* genes in the leaves. **(B)** Relative expression level of the *OsPIP* genes in the roots. The transcript levels of each *OsPIP* gene in the leaves and roots of treated seedlings were plotted as the relative expression fold of the non-treated seedlings for 2, 6, 18 and 24 h. Error Bars: standard errors.

290 amino acid residues, and the sequence similarity among them ranged from 57% (between OsPIP1-3 and OsPIP2-7) to 92% (between OsPIP1-1 and OsPIP1-2). The 10 OsPIP proteins were found sharing six conserved transmembrane domains (TMs) and five connecting loops (Figure 1A) [30, 36, 37]. Moreover, the sequences around the NPA motifs (Asn-Pro-Ala) were highly conserved (Figure 1A).

Expression profile of the 10 OsPIP genes in rice seedlings

To further characterize the function of these *OsPIP* genes, we examined the transcript level of each *OsPIP* gene using quantitative RT-PCR method in the leaves and roots of 2-week-old rice seedlings. The results showed that all the *OsPIP* genes had transcript copy numbers smaller than 2.6×10^4 per nanogram of total RNA, except for *OsPIP2-4* whose copy number in root was ten folds larger(2.2×10^5). Based on the copy numbers, the rice *OsPIP* genes could roughly be divided into two groups, one with the copy number larger than 10^3 (Figure 2A) and the other with the copy number smaller than 210 (Figure 2B). *OsPIP2-7* had the lowest transcript level in roots, only 26 copies per nanogram of total RNA (Figure 2B). As an internal control, the transcript of the rice *OsACT1* gene in roots and leaves was between 2 000-3 000 copies per nanogram of total RNA.

Interestingly, the result also showed that the expression of three genes, *OsPIP1-3*, *OsPIP2-2* and *OsPIP2-7*, could be detected only in roots, whereas that of *OsPIP2-6* was found leaf-specific (Figure 2A and 2B), at this stage of growth. The expression of the other six genes were detected, although differentially, both in leaves and roots.

The transcript levels of *OsPIP1-1*, *OsPIP1-2* and *OsPIP2-1* were 1.5-2.3 times higher in leaves than in roots, whereas the expression levels of *OsPIP2-4* and *OsPIP2-5* were found 20 fold higher in roots than in leaves. *OsPIP2-3* was the only member whose expression was found no significant difference between the two organs (Figure 2), at this stage.

The expression of the OsPIP *genes in response to ABA*

In order to investigate the role of *OsPIP* genes in stress response, we examined the expression levels of the *OsPIP* genes in rice leaves and roots when treated with 50 μM of ABA at different time intervals, by quantitative RT-PCR. In response to this stimulus, all of the *OsPIP* genes shared a similar expression time course pattern in rice leaves, that is, the transcript level, although varied at 2 h, increased at 6 h, peaked at 18 h, but decreased at 24 h (Figure 3A).

The expression pattern of *OsPIP*s in roots varied significantly from that in leaves. As shown in Figure 3B, the expression of most *OsPIP* genes were not much altered or rather slightly depressed during 24 h ABA treatment. However, both *OsPIP2-2* and *OsPIP2-7* were immediately induced to 3-3.5 fold of the control at 2 h of the treatment, and then rapidly decreased to half of the control at 6 h (Figure 3B). Meanwhile, the expression of *OsPIP1-3*, *OsPIP2-3* and *OsPIP2-4* were much suppressed under the treatment at all indicated time points (Figure 3B).

The expression of the OsPIP *genes in response to salt and drought*

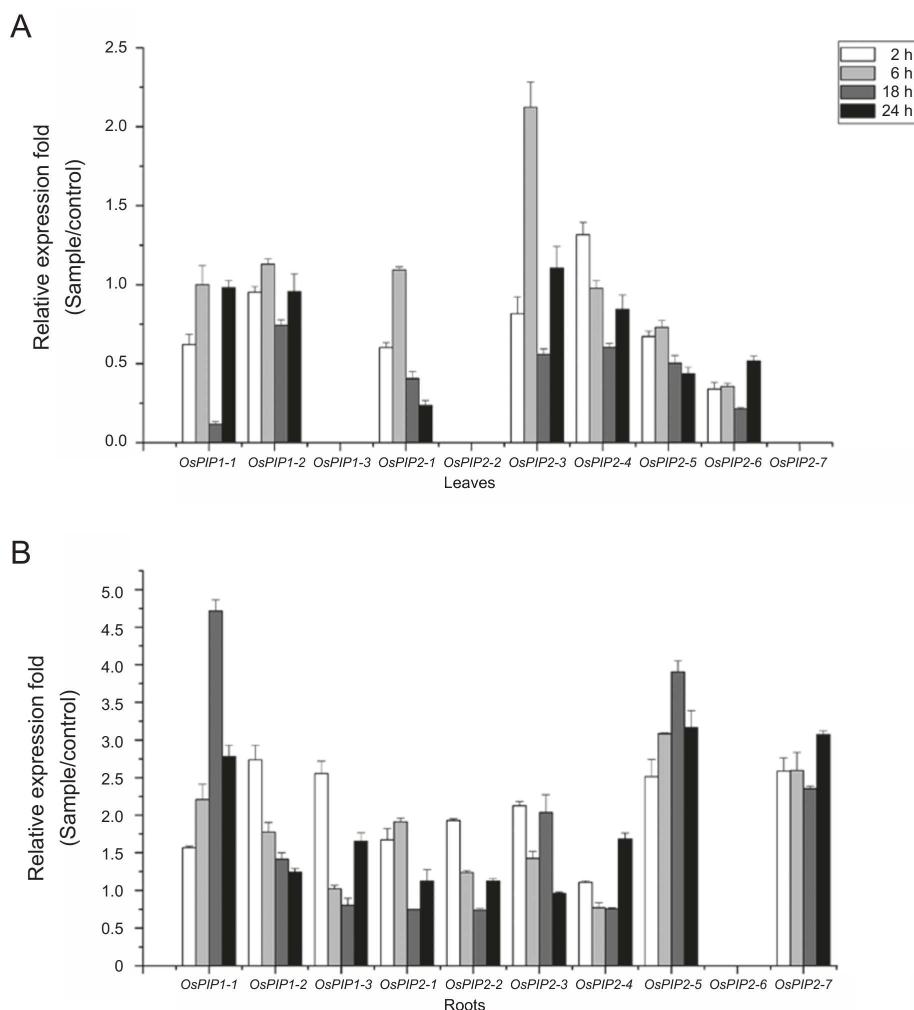

Figure 5 Relative expression levels of the 10 *OsPIP* genes in response to PEG6000 in the leaves and roots of rice seedlings. **(A)** Relative expression level of the 10 *OsPIP* genes in the leaves. **(B)** Relative expression level of the 10 *OsPIP* genes in the roots. The transcript levels of each *OsPIP* in the leaves and roots of treated seedlings were plotted as the relative expression fold of the non-treated seedlings for 2, 6, 18 and 24 h. Error Bars: standard errors.

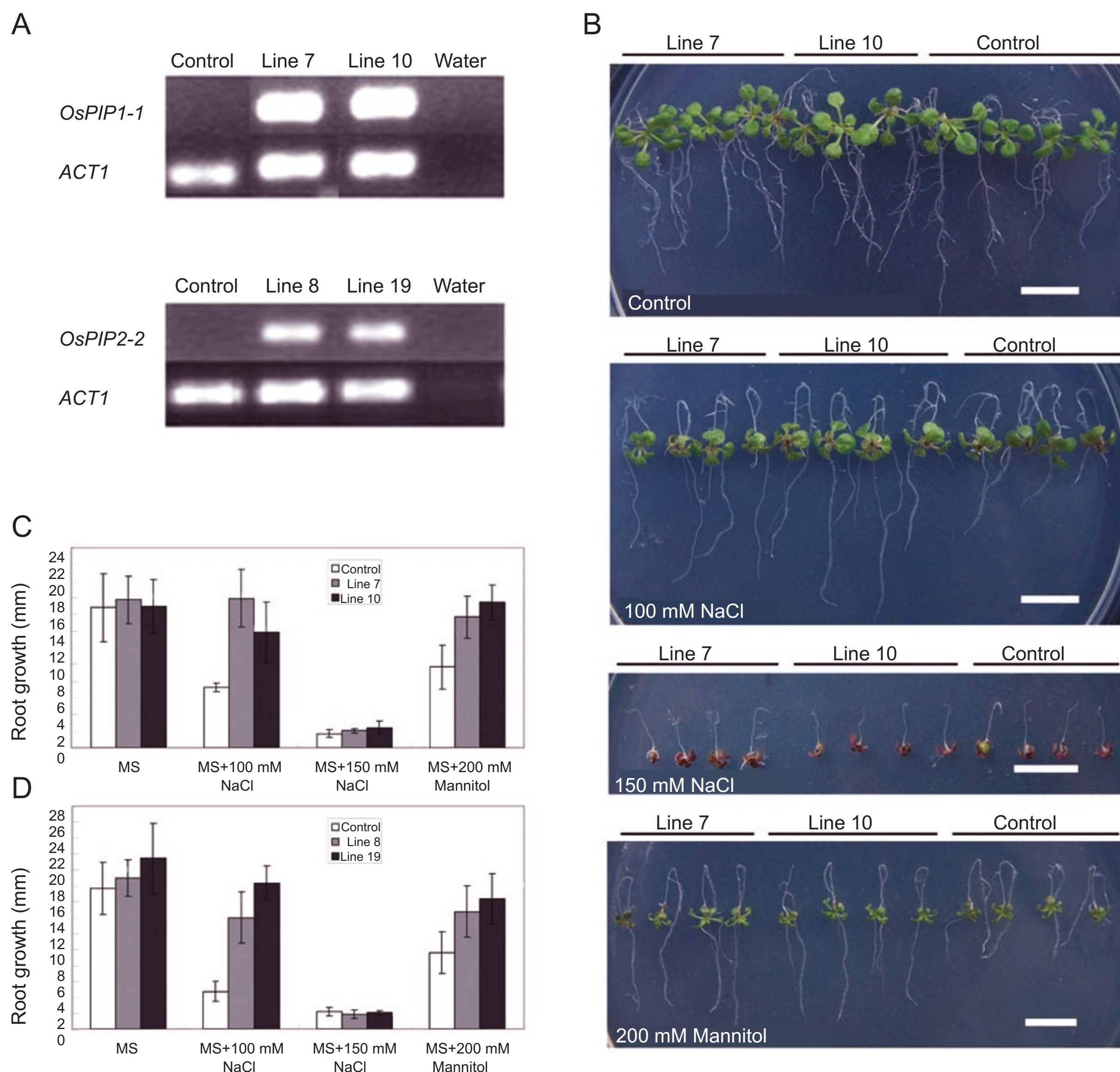

Figure 6 Analysis on the transgenic lines over expressing *OsPIP* genes. **(A)** Over-expression of *OsPIP1-1* and *OsPIP2-2* in transgenic *Arabidopsis* confirmed by RT-PCR. Up panel represents the expression of *OsPIP1-1* in transgenic *Arabidopsis* line 7 and line 10; low panel represents the expression of *OsPIP2-2* in transgenic *Arabidopsis* line 8 and line 19. Control: the transgenic plants with empty pQGIE110 vector. **(B)** Root-bending assay for examining the tolerance of salt and drought in transgenic plants over-expressing *OsPIP1-1*. The first panel: transgenic seedlings on MS medium without NaCl or mannitol; the second panel: transgenic seedlings on MS medium with 100 mM NaCl; the third panel: transgenic seedlings on MS medium with 150 mM NaCl; the fourth panel: transgenic seedlings on MS medium with 200 mM mannitol. Control: transgenic plants with empty pQGIE110 vector. Bars: 1 cm. **(C)** Statistic analysis of root length of transgenic plants over-expressing *OsPIP1-1* under MS medium, MS media supplemented with 100 mM of NaCl, 150 mM of NaCl, and 200 mM of mannitol for 7 d. Error bars: standard deviation. **(D)** Statistic analysis of root growth of transgenic plants over-expressing *OsPIP2-2* under fresh MS medium, MS media supplemented with 100 mM of NaCl, 150 mM of NaCl and 200 mM of mannitol for 7 d. Error bars: standard deviation.

Figure 4 shows the expression patterns of *OsPIP* genes in rice seedlings in response to 250 mM of NaCl. In leaves, the expression levels of most *OsPIP*s were first suppressed to a certain extent and then increased. The transcript level peaked at 6 h of the treatment (Figure 4A). In roots, the expression of all the *OsPIP2* genes were not altered or rather slightly suppressed during 24 h treatment (Figure 4B). It is worth noting that of all the *OsPIP1* genes increased at

certain time point of the treatment. For instance, *OsPIP1-3* was induced by 2.5-fold at 2 h of the treatment and then gradually declined; whereas the expression of *OsPIP1-1* increased by about two-fold constantly (Figure 4B).

Treatment with PEG6000 was adopted to mimic the drought stress [16, 32]. In leaves, *OsPIP2-3* was the only gene whose expression was induced by the treatment at 6 h. The expression of other genes such as *OsPIP1-2* and *OsPIP2-4* were not affected by the treatment, whereas the transcription level of three genes, *i.e. OsPIP2-1*, *OsPIP2-5*, and *OsPIP2-6*, were suppressed (Figure 5A). However, in roots, almost all the *OsPIP* genes, especially *OsPIP1-1*, *OsPIP2-5* and *OsPIP2-7*, were found induced during the treatment (Figure 5B), suggesting that *OsPIP* genes may play important roles in the response to drought in roots.

Over-expression of either OsPIP1 *or* OsPIP2 *could enhance the tolerance of transgenic* Arabidopsis *to salt and drought treatments*

To further characterize the possible function of *OsPIP* genes, we transformed an *OsPIP1* gene (*OsPIP1-1*) and an *OsPIP2* gene (*OsPIP2-2*) driven by a 35S promoter into *Arabidopsis thaliana* ecotype Columbia respectively and obtained transgenic lines. The expression of the transgenes were confirmed by RT-PCR (Figure 6A). Two over-expression lines for each transgenes, together with the line transformed with an empty vector as control, were examined for their tolerance to salt and drought treatments. When the *OsPIP1-1* over-expression lines were treated with 100 mM of NaCl, a salinity where the growth of the main roots in the control seedlings was inhibited, the lengths of the main roots in two transgenic lines (line 7 and line 10) were not significantly different from those of the plants grown on medium without salt (Figure 6B and 6C). However, when the concentration of NaCl was increased to 150 mM, the growth of both the transgenic and control lines were strongly inhibited (Figure 6B and 6C). Furthermore, when the transgenic lines were exposed to 200 mM of mannitol which was used to mimic drought stress in *Arabidopsis* [17], the growth of the main roots was not affected compared to the control line (Figure 6B and 6C). Interestingly, similar phenotypes had been observed for the *OsPIP2-2* over-expression transgenic lines (Figure 6D).

Discussion

Similar to the *Arabidopsis AtPIP* gene family [17], the rice *OsPIP* family exhibits significant differences in mRNA abundance among its members (Figure 2). The overall transcript level of *OsPIP2-4* was the highest, accounting for about 75% of the total *OsPIP* transcripts in the seedlings at two weeks after germination. In roots, *OsPIP2-4* even accounted for 80% of the total *OsPIP* transcripts. Therefore, *OsPIP2-4*, like *Arabidopsis AtPIP1-1* and *AtPIP1-2* [17], could be the predominant gene playing critical roles regulating water uptake and transport in rice seedlings, especially in roots. In leaves, *OsPIP1-2* accounted for about 35% of total *OsPIP* transcripts, which is more than that of *OsPIP2-4* (25%), suggesting an essential role of *OsPIP1-2* in water transport in rice leaves. No organ-specific expression pattern for *AtPIP* genes was reported in *Arabidopsis* [2, 17]. In this study, we found one leaf-specific *OsPIP* gene, *OsPIP2-6*, and three root-specific genes, *OsPIP1-3*, *OsPIP2-2*, and *OsPIP2-7* in rice seedlings (Figure 2). *ZmPIP1-5* and *ZmPIP2-5*, the maize homologues of *OsPIP1-3* and *OsPIP2-2* respectively (Figure 1B), were also detected only in roots [26, 37]. The organ-specific expression pattern of these *OsPIP* genes suggested that they could be involved in water uptake and transport in a particular area, like *ZmTIP1* in maize [7] and *BobTIP26-1* and *BobTIP26-2* in cauliflower [27].

The expression of other six *OsPIP* genes could be detected in both leaves and roots. However, the expression levels of *OsPIP1-1*, *OsPIP1-2* and *OsPIP2-1* were higher in leaves than in roots, whereas those of *OsPIP2-4* and *OsPIP2-5* were higher in roots than in leaves (Figure 2). Similar expression pattern was also reported in other plants. For instance, *ZmPIP1-5b*, a *PIP* gene in maize, was found abundant in roots but hardly detected in young and mature leaves [28]. This data may suggest that water homeostasis maintenance in rice leaves and roots may need the coordination of certain OsPIP proteins, or share a relatively redundant system.

Aquaporins play a significant role in recovery from water deficit in both prokaryotic and eukaryotic cells [1, 38]. Expression of aquaporin genes are regulated under water stress treatments in many plant species [17, 18, 20-22]. In order to understand the nature of aquaporin genes in rice, we analyzed the expression patterns of the ten *OsPIP* genes under the treatments of ABA, salinity and drought. The *OsPIP* genes were found showing different responses to different stress treatments. ABA and NaCl induced the expression of *OsPIP*s both in leaves and roots, whereas PEG6000-simulated drought stress mostly induced the expression of these genes in roots (Figure 3-5). Under the same stress, some *OsPIP* genes showed different inductivity in different organs. For instance, *OsPIP2-5* was induced in leaves but not in roots when treated with ABA (Figure 3), whereas *OsPIP1-1* was induced in roots but not in leaves when treated with PEG6000 (Figure 5). The different expression pattern may reflect the need for water uptake and transport in different tissues in response to different stresses. Meanwhile, the similar pattern shared by ABA and NaCl treatments suggests that some *OsPIP*s might be

coordinately orchestrated or redundant in the responses to these two stresses.

In *Arabidopsis*, all the *AtPIP* genes are either induced or suppressed both in roots and in aerial parts under ABA, salt, cold and drought stimuli. That is to say, the expression pattern of *AtPIP* genes in roots and the aerial parts of *Arabidopsis* are always similar in response to the same stress [17]. In rice, however, *OsPIP* genes expressed in a different, mostly opposite pattern in these two organs. For example, almost all the *OsPIP*s, especially *OsPIP1-1*, *OsPIP2-5* and *OsPIP2-7*, were induced in roots by PEG6000 treatment. However, in leaves, only *OsPIP2-3* was induced by this treatment, and the expression of other gene members was either not affected or even reduced (Figure 5). This may suggest different roles for *OsPIP* genes in different organs in response to the same stress. The difference between the expression pattern of *PIP* genes in the roots of *Arabidopsis* and rice might be related to their different root systems and habitats, i.e., *Arabidopsis* plants have taproots and live on land, while rice plants have fibrous roots and usually live in paddy field or wet land. Further studies on these genes are needed in order to reveal their biochemical and/or physiological functions.

The over-expression of either an *OsPIP1* or an *OsPIP2* gene enhanced the tolerance to the salt and drought treatments in *Arabidopsis* plants (Figure 6). However, this improvement only occurred when the concentration of NaCl was below 150 mM, or treated with 200 mM of mannitol. Different research groups reached different conclusions on the function of PIP proteins. It has previously been reported that OsPIP1-1 and OsPIP1-3 had ability to transport water in the *Xenopus* oocytes [30, 32], but other studies showed that only PIP2 proteins showed a rapid water transport activity, whereas PIP1s had no or a low water transport activity in the *Xenopus* oocytes [28, 39]. Our data indicated that the transgenic plants over-expressing *OsPIP1-1* or *OsPIP2-2* showed high tolerance to mild salt stress (Figure 6C and 6D), suggesting that *OsPIP* genes play important roles in regulating water homeostasis, and that both *OsPIP1-1* and *OsPIP2-2* could possibly have intrinsic water transport ability *in planta*, which needs to be further confirmed biochemically *in vivo*. In combination with the *Xenopus* system and further analyses of transgenic plants over-expressing individual *OsPIP* gene members, the function of the OsPIPs in water transport should be revealed.

Acknowledgments

This work was supported by the National Hi-Tech R&D Program of China (GN 2001AA222261 and 2002AA2Z1001), the State Key Basic Research and Development Plan (Project GN G1999011602 and G2000016202), the Excellent Young Teachers Program of Ministry of Education of China (to Li Jia Qu). The authors are grateful to Dr Tomohiko Tsuge in Kyoto University, Japan, Dr Fan Chen in the Institute of Genetics and Developmental Biology at the Chinese Academy of Sciences, Beijing, and Dr Hong Wei Xue in the Institute of Plant Physiology and Ecology at the Chinese Academy of Sciences, Shanghai, for their valuable suggestions and comments on the manuscript.

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Edited by Hong Wei Xue