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Identification of 33 Rice Aquaporin Genes and Analysis of Their Expression and Function

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Plant aquaporins form a large protein family including plasma membrane-type (PIPs) and tonoplast-type aquaporins (TIPs), and facilitate osmotic water transport across membranes as a key physiological function. We identified 33 genes for aquaporins in the genome sequence of rice (*Oryza sativa* L. cv. Nipponbare). We investigated their expression levels in leaf blades, roots and anthers of rice (cv. Akitakomachi) using semi-quantitative reverse transcription–PCR (RT–PCR). At both early tillering (21 d after germination) and panicle formation (56 d) stages, six genes, including *OsPIP2;4* and *OsPIP2;5*, were expressed predominantly in roots, while 14 genes, including *OsPIP2;7* and *OsTIP1;2*, were found in leaf blades. Eight genes, such as *OsPIP1;1* and *OsTIP4;1*, were evenly expressed in leaf blades, roots and anthers. Analysis by stopped-flow spectrophotometry revealed high water channel activity when OsPIP2;4 or OsPIP2;5 were expressed in yeast but not when OsPIP1;1 or OsPIP1;2 were expressed. Furthermore, the mRNA levels of *OsPIP2;4* and *OsPIP2;5* showed a clear diurnal fluctuation in roots; they showed a peak 3 h after the onset of light and dropped to a minimum 3 h after the onset of darkness. The mRNA levels of 10 genes including *OsPIP2;4* and *OsPIP2;5* markedly decreased in roots during chilling treatment and recovered after warming. The changes in mRNA levels during and after the chilling treatment were comparable with that of the bleeding sap volume. These results suggested the relationship between the root water uptake and mRNA levels of several aquaporins with high water channel activity, such as OsPIP2;4 and OsPIP2;5.

Keywords: Aquaporin — Gene expression — Rice — Water permeability.

Abbreviations: NIP, Nod26-like intrinsic protein; PIP, plasma membrane intrinsic protein; RT–PCR, reverse transcription–PCR; SIP, small and basic intrinsic protein; TIP, tonoplast intrinsic protein.

Introduction

Aquaporins are membrane proteins that facilitate water transport across the membranes in various microorganisms, animals and plants (King et al. 2004). Intensive research has revealed the involvement of aquaporins in plant growth and water relationships (see reviews by Maurel et al. 2002, Tyerman et al. 2002). Plant aquaporins are distributed in various plant tissues relating to water transport, cell differentiation and enlargement. Some aquaporins were also expressed in motor cells (Moshelion et al. 2002), guard cells (Kaldenhoff et al. 1995, Sarda et al. 1997) and reproductive organs (Dixit et al. 2001, O'Brien et al. 2002, Bots et al. 2005a, Bots et al. 2005b). Studies using mutant lines of aquaporins clearly demonstrated the involvement of aquaporins in plant–water relationships; for example, Ma et al. (2004) showed that reduction of TIP1;1 expression levels in *Arabidopsis thaliana* using the RNA interference method caused death of the plant, in the case of serious damage. In addition, gene expression and protein accumulation of aquaporins in response to various environmental stresses and hormones have been reported in several plants (Maurel et al. 2002, Tyerman et al. 2002). Furthermore, it has been reported recently that some types of plant aquaporin could transport not only water but also various small molecules such as glycerol, urea (Maurel et al. 2002, Gaspar et al. 2003, Liu et al. 2003), ammonia (Niemietz and Tyerman 2000, Loqué et al. 2005) and CO_2 (Uehlein et al. 2003, Hanba et al. 2004). These studies are developing our knowledge on the physiological meanings of aquaporins in plants.

Recent genome sequencing projects have revealed that aquaporins constitute a large gene family in plants; *A. thaliana* and *Zea mays* have 35 and 33 genes, respectively (Chaumont et al. 2001, Johanson et al. 2001). These aquaporins have been classified into four major subfamilies referred to as plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nod26-like intrinsic proteins (NIPs) and small and basic intrinsic proteins (SIPs). Although recent studies provided information on the physiological function of several of those aquaporin members, the number of aquaporins examined in detail is still limited.

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Table 1 List of rice aquaporin genes

Name	Genome sequence accession No.		cDNA clone accession No.	Notes
	Gene	Protein [a]		
OsPIP1;1	AP005108	BAD28398	AK061769	Identical to *OsPIP1a* (AJ224327), similar to *RWC1* (AB09665)
OsPIP1;2	(AL606687) [b]		AK098849	
OsPIP1;3	AP004026	BAD22920	AK102174	Similar to *RWC3* (AB029325)
OsPIP2;1	AP003802	BAC15868	AK072519	Similar to *OsPIP2a* (AF062393)
OsPIP2;2	AP006168	BAD23735	AK061782	
OsPIP2;3	AL662958	CAD41442		
OsPIP2;4	AP004668	BAC16113	AK072632	
OsPIP2;5	AP004668	BAC16116	(AK107700) [c]	
OsPIP2;6	AL731636	CAE05002	AK061312	
OsPIP2;7	AP006149	BAD46581	AK109439	
OsPIP2;8	AC092263	AAP44741	AK109024	
OsTIP1;1	AC090485	AAK98737	AK058322	Identical to *OsγTIP1* (D22534)
OsTIP1;2	AP003627	BAB63833	AK111747	Identical to *OsTIP1* (AB114829)
OsTIP2;1	AP005289	BAD25765	(AK064728) [c]	Identical to *OsTIP2* (AB114830)
OsTIP2;2	AP004784	BAD61899	AK099141	
OsTIP3;1	AC023240	AAG13544	AK111931	Identical to *OsTIP3* (AB114828)
OsTIP3;2	AL663019	CAE05657	AK108116	
OsTIP4;1	AC145396	AAS98488	AK060193	
OsTIP4;2	AP001550	BAA92993	AK099190	
OsTIP4;3	AP001550	BAA92991	(AK069192) [c]	
OsTIP5;1	AL663000		AK070602	
OsNIP1;1	AP004070	BAD27715	AK068806	Identical to *γMIP1* (D17443)
OsNIP1;2	AP003105	BAD73177		
OsNIP1;3	AC135918	AAV44140	(AK062320) [c]	
OsNIP1;4	AP003682	BAD53665		
OsNIP2;1	AP005297	BAD16128	AK069842	
OsNIP2;2	AP003569	BAD37471	AK112022	
OsNIP3;1	AC068924	AAG13499		
OsNIP3;2	AP005467	BAC99758		
OsNIP3;3	AP005467	BAC65382		
OsNIP4;1	AP003219	BAB61180	AK106825	
OsSIP1;1	AP003047	BAB32914	AK109424	
OsSIP2;1	AC119748		(AK071190) [c]	

[a] The protein accession numbers were obtained from the result of annotation analysis in the database.
[b] AL606687 encoded a gene which had a high homology to *OsPIP1;2* (see Results).
[c] cDNA clones in parentheses did not contain full-length genes.

We have been conducting a series of experiments to elucidate the physiological relevance of aquaporins in rice, which is an important major crop. Although several aquaporin genes have already been identified and characterized in rice (Liu et al. 1994, Malz and Sauter 1999, Li et al. 2000, Lian et al. 2004, Takahashi et al. 2004), a complete set of rice aquaporins is unclear. In the present study, we identified 33 genes for aquaporins in the rice genome sequence and investigated their expression profiles in rice plant organs. We also studied the expression profile in roots during a light/dark cycle and chilling treatment. Furthermore, we expressed typical members of rice PIPs in yeast cells and determined the water channel activity of the membrane accumulating these aquaporins by stopped-flow spectrophotometry. From these results, we discuss the function of aquaporins in rice roots.

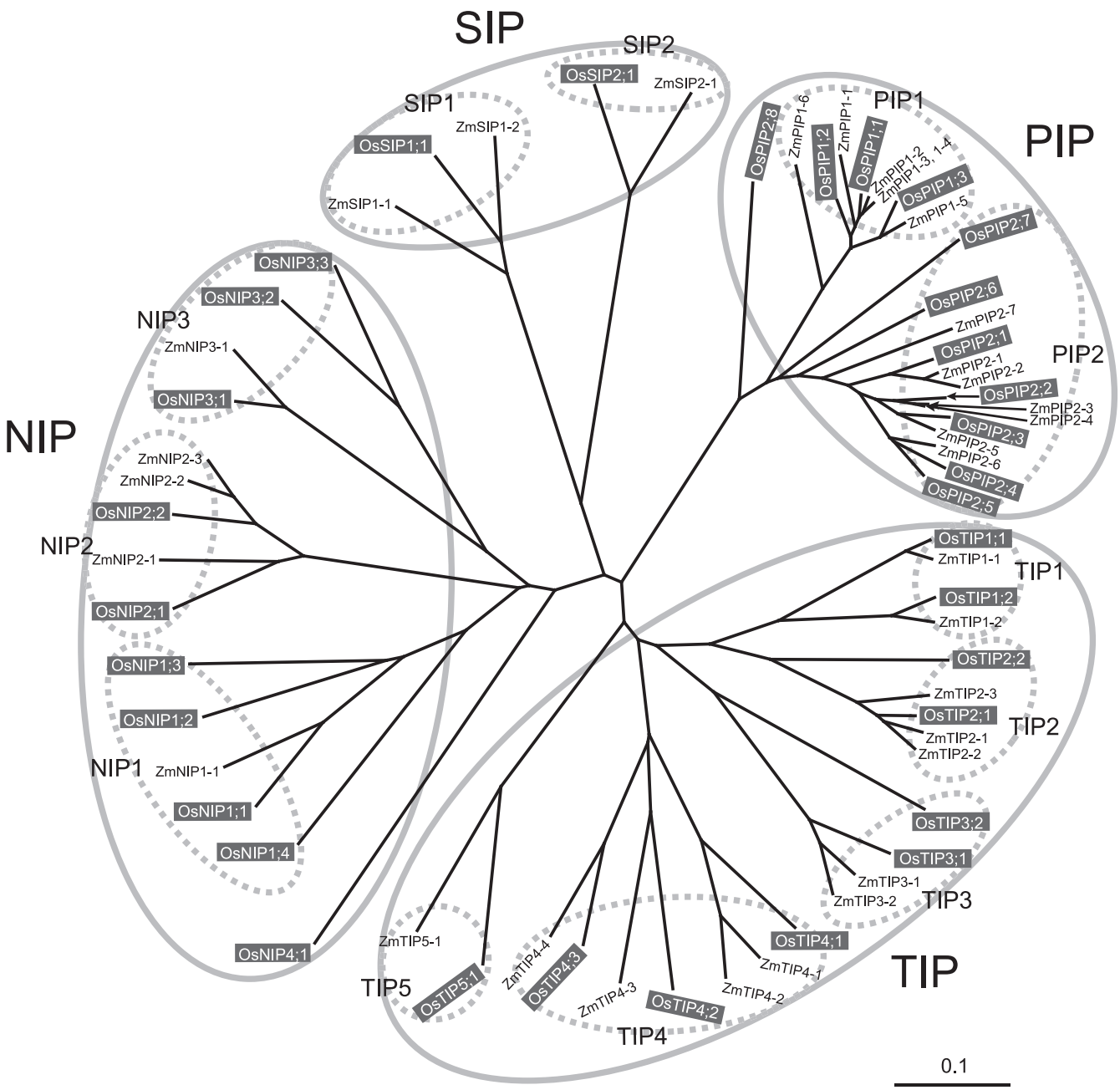

Fig. 1 Phylogenetic analysis of the deduced amino acid sequences of rice aquaporins with those of *Z. mays* aquaporins. The accession numbers of *Z. mays* aquaporins are listed in Chaumont et al. (2001). The scale bar of 0.10 is equal to 10% sequence divergence.

Results

Identification and nomenclature of rice aquaporin genes

We surveyed the genes for aquaporins in the genome database of rice (cv. Nipponbare). These genes were selected from a large number of the candidate sequences including both genome and cDNA sequences. The overlapping sequences were removed and finally 33 genes were selected, as shown in Table 1. Phylogenetic analysis of rice aquaporins was conducted with those of a monocotyledonous plant, *Z. mays* (Chaumont et al. 2001) as shown in Fig. 1. The aquaporins identified in rice were systematically named according to the phylogenetic relationship with the documented aquaporins in *A. thaliana* (Johanson et al. 2001) and *Z. mays* (Chaumont et al. 2001).

OsPIP1;2 was identified only from the cDNA sequence (*AK098849*, see Table 1, and *AK058323*, *AK065188* and *AK104736*). There was no genome sequence identical to those

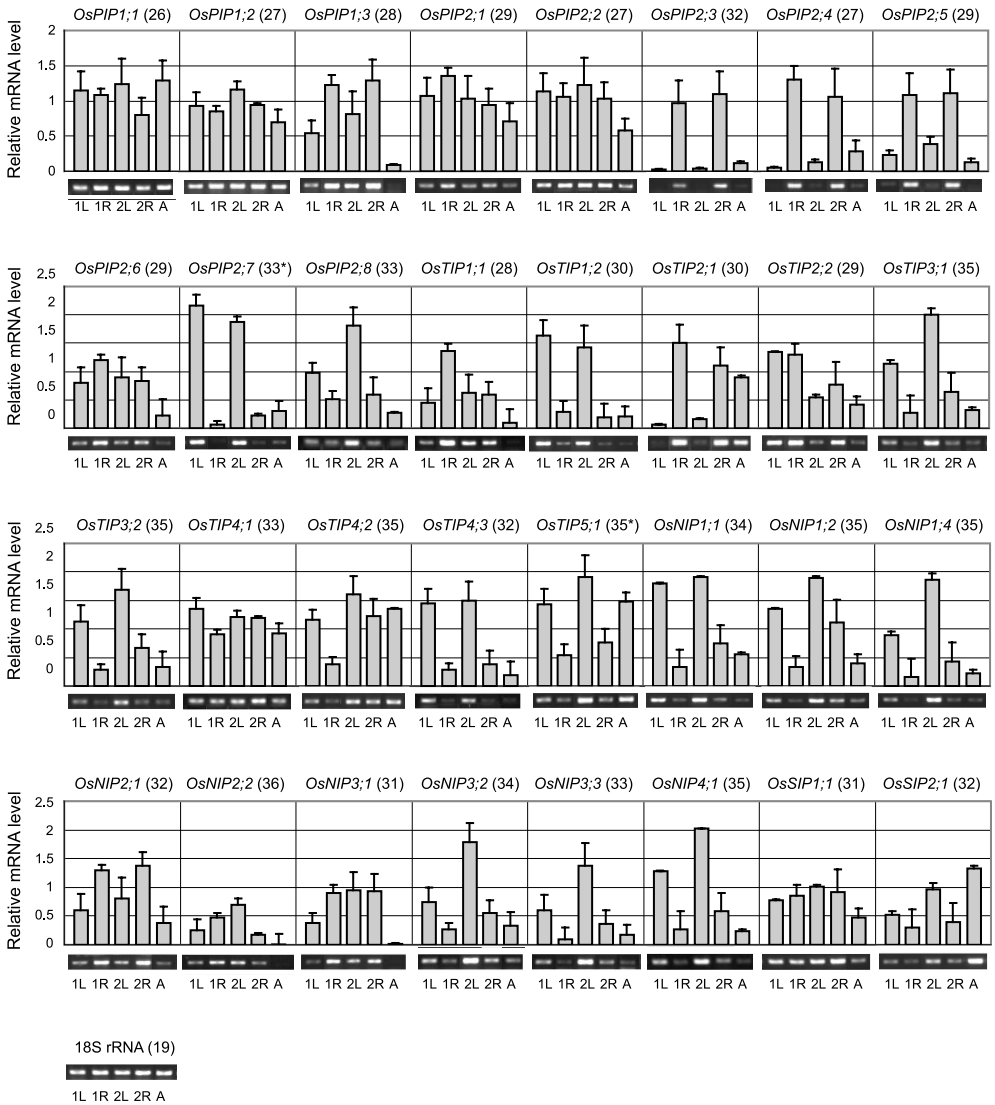

Fig. 2 Comparison of the expression of rice aquaporin genes in various organs. Total RNA fractions were prepared from leaf blades (L) and roots (R) of 21-day-old plants (1) and 56-day-old plants (2), and anthers (A) of 67-day-old plants as described in Materials and Methods. The levels of mRNAs were determined by semi-quantitative RT–PCR using specific primers as shown in Table 2 (Supplementary data) and normalized to that of 18S rRNA. Photographic images of gel electrophoresis are shown at the bottom of each graph. The data represent the means ± SE for three independent samples. Numbers in parentheses indicate the number of cycles needed to detect the PCR product. In the case of *OsPIP2;7* and *OsTIP5;1*, where marked by an asterisk, the annealing reaction was conducted at 61°C instead of 58°C (see Materials and Methods).

cDNAs. However, the genomic DNA *AL606687* contained a fairly similar sequence; it had a single base insertion and a few base substitutions when compared with *AK098849*. Thus, we named *AL606687* and *AK098849* as the same aquaporin gene *OsPIP1;2*.

Although several other genes encode the partial sequences of aquaporins (data not shown), they were considered to be non-functional pseudo-genes. This is mainly because they lacked some parts of characteristic sequences conserved in the aquaporin family, such as a transmembrane domain, when we analyzed their transmembrane topologies (data not shown).

Phylogenetic analysis of rice aquaporins

We classified the rice aquaporins into four subfamilies as same as those of *Z. mays* (Chaumont et al. 2001) and *A. thaliana* (Johanson et al. 2001) (Fig. 1). Rice had 11 PIPs, 10 TIPs, 10 NIPs and two SIPs. The OsPIP members had a relatively high sequence similarity of 58.5–92.7%, while the members of the OsTIP, OsNIP and OsSIP subfamilies had a somewhat variable sequence similarity. For example, the sequence similarity among the OsTIP members was 35.8–72.0%.

The OsPIP subfamily was divided into two groups, PIP1 and PIP2 (Fig. 1). OsPIP2;8 had a unique sequence and formed a long branch in the tree. However, we provisionally assigned

OsPIP2;8 as a member of the PIP2 group because its sequence was similar to that of OsPIP2 members but not OsPIP1 members.

The OsTIP subfamily consisted of five groups, OsTIP1, OsTIP2, OsTIP3, OsTIP4 and OsTIP5, which had two, two, two, three and one member, respectively. The OsNIP subfamily consisted of four groups, OsNIP1, OsNIP2, OsNIP3 and OsNIP4, which had four, two, three and one member, respectively. The OsSIP subfamily had only two members and their sequence similarity is low (33%).

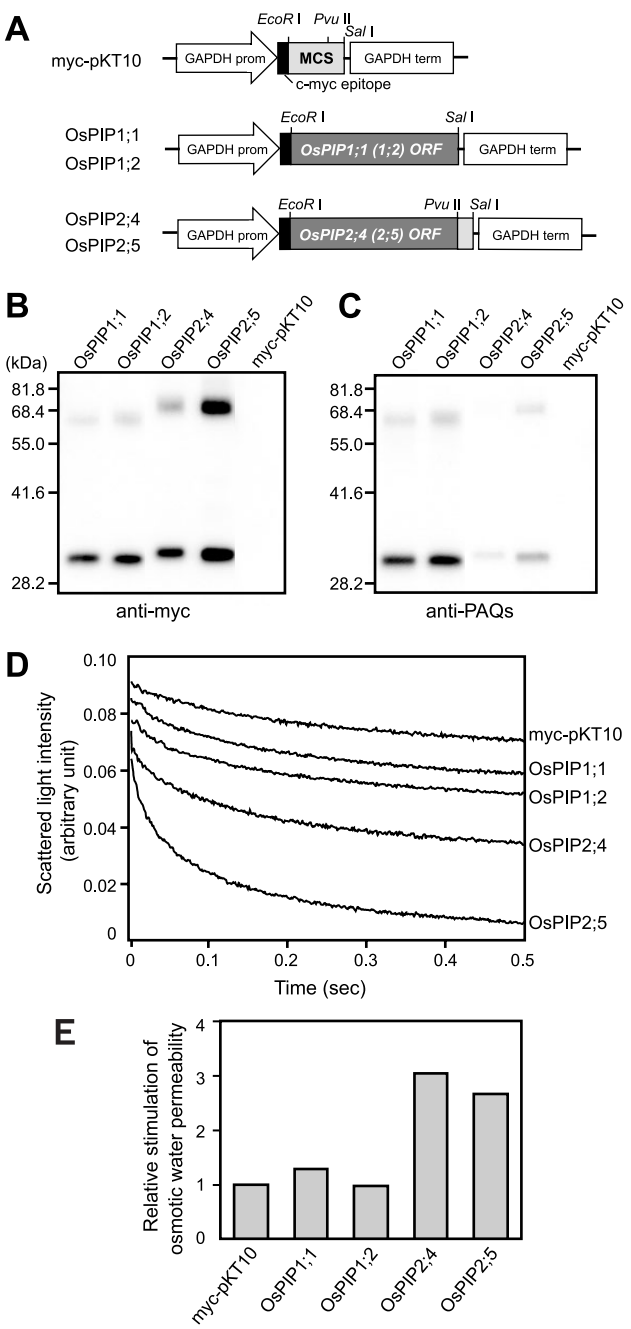

Fig. 3 Expression of OsPIP1;1, OsPIP1;2, OsPIP2;4 and OsPIP2;5 in *S. cerevisiae* and stopped-flow spectrophotometrical assay of the osmotic water permeability of yeast membrane vesicles. (A) Constructs for expression of rice aquaporin in yeast. cDNAs of the four OsPIPs were individually inserted into the multi-cloning site (MCS) in the interface region between the GAPDH promoter (GAPDH prom) and the terminator (GAPDH term) of the myc-pKT10 vector. Crude membranes were prepared from transformed yeast cells, and aliquots (1 μg) were immunoblotted with anti-myc (B) and anti-PIPs antibodies (C). The monomeric and dimeric forms were detected. The empty vector myc-pKT10 was examined as a negative control. (D) The osmotic water permeability of yeast membrane vesicles was measured by stopped-flow spectrophotometry. Each reaction curve shows the average trace of 7–12 independent determinations. (E) The relative fold stimulation of osmotic water permeabilities was calculated as the ratio of the initial rate constant of each aquaporin to that of the vector control on the basis of the expressed amount of protein (ratio to that of myc-OsPIP1;1).

Organ-specific expression of rice aquaporin genes

The mRNA levels of 32 aquaporin genes were quantified by reverse transcription–PCR (RT–PCR) and compared in some organs (Fig. 2). The expression profiles varied with aquaporins. The mRNA levels of *OsPIP1;3*, *OsPIP2;3*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP2;1* and *OsNIP2;1* were higher in roots than in leaf blades. In particular, *OsPIP2;3*, *OsPIP2;4* and *OsPIP2;5* were expressed predominantly in roots at both early tillering and panicle formation stages. In contrast, higher levels of the mRNA of 14 aquaporin genes (*OsPIP2;7*, *OsPIP2;8*, *OsTIP1;2*, *OsTIP3;1*, *OsTIP3;2*, *OsTIP4;2*, *OsTIP4;3*, *OsTIP5;1*, *OsNIP1;1*, *OsNIP1;2*, *OsNIP1;4*, *OsNIP3;2*, *OsNIP3;3* and *OsNIP4;1*) were detected in leaf blades than in roots. Several genes (*OsPIP1;1*, *OsPIP1;2*, *OsPIP2;1*, *OsPIP2;2*, *OsPIP2;6*, *OsTIP2;2*, *OsTIP4;1* and *OsSIP1;1*) were expressed almost equally in both roots and leaf blades.

The expression profile of some genes, such as *OsTIP1;1*, *OsNIP2;2*, *OsNIP3;1* and *OsSIP2;1*, differed with the stages, i.e. early tillering or panicle formation. For example, *OsTIP1;1* was highly expressed in roots at the early tillering stage; however, the level decreased to 43% at the panicle formation stage. Interestingly, the relative mRNA levels of *OsPIP1;1*, *OsTIP4;2*, *OsTIP5;1* and *OsSIP2;1* were also abundant in anthers in the heading stage.

Osmotic water permeabilities of OsPIP1;1, OsPIP1;2, OsPIP2;4 and OsPIP2;5

To measure the osmotic water transport activity of rice aquaporins by the yeast expression system, we expressed them in *Saccharomyces cerevisiae* strain BJ5458, which lacks vacuolar proteases, to avoid degradation of translation products. It has been confirmed that this strain lacks functional aquaporins (Suga and Maeshima 2004).

Rice aquaporins OsPIP1;1, OsPIP1;2, OsPIP2;4 and OsPIP2;5 were tagged with a c-myc epitope at the N-termini

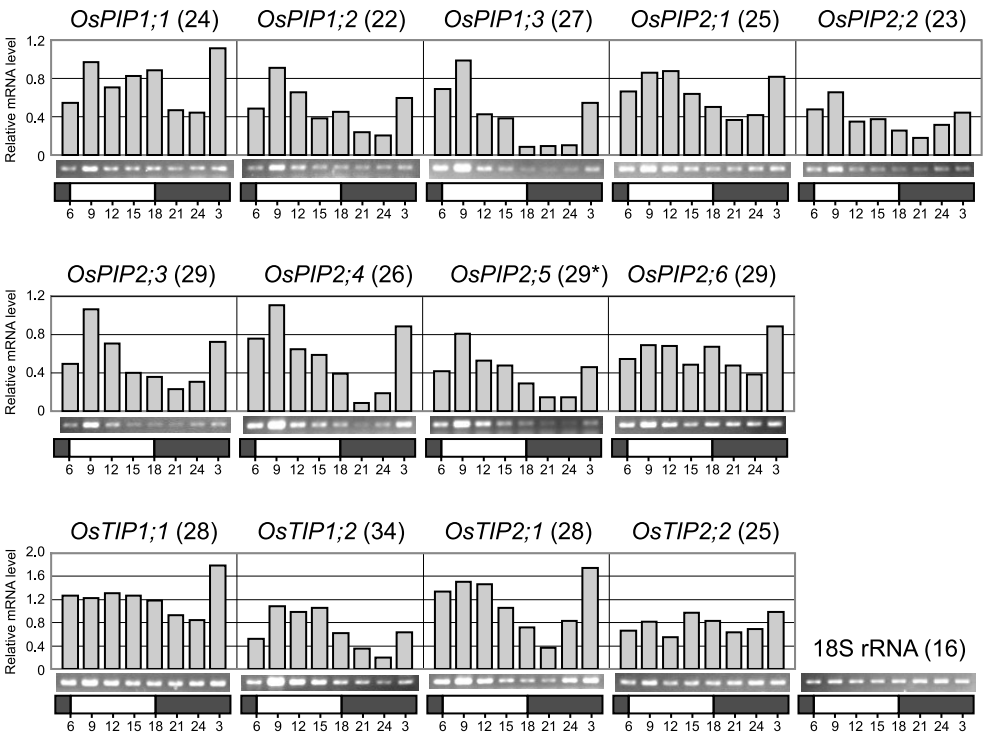

Fig. 4 Diurnal variations in the levels of aquaporin mRNA in rice roots. Rice plants were grown for 16 d under a cycle of 12 h light (white column)/12 h dark (gray column). The relative mRNA levels were quantified and calculated as described in Fig. 2. Numbers in parentheses indicate the number of PCR cycles. In the case of *OsPIP2;5*, where marked by asterisks, the annealing reaction was conducted at 61°C instead of 58°C.

(Fig. 3A). This c-myc epitope has been reported to have no effect on water channel activity of recombinant aquaporin (Suga and Maeshima 2004). Protein accumulation in the yeast membranes was detected at the calculated molecular sizes of monomers (31–32 kDa) and dimers (63–69 kDa) by immunobloting with an anti-myc antibody (Fig. 3B). The expressed rice aquaporins in yeast were also detected by the anti-PAQs antibody, which recognizes most PIP members in radish (Ohshima et al. 2001) (Fig. 3C). This antibody showed only qualitative information, because the immunoreactivity of the antibody depends on the amino acid sequences of aquaporin antigens. No immunostained band was observed in the membranes prepared from yeast cells expressing the vacant vector.

The osmotic water permeability of membrane vesicles was measured with a stopped-flow light scattering spectrophotometer. The swelling rate of vesicles in the hypotonic solution was monitored as a decrease in the scattered light intensity (Fig. 3D). The vacant vector showed a slow influx of water into the membrane vesicles and we thought that this was the basal activity of the yeast membrane preparation. Fig. 3E shows the relative stimulation of osmotic water permeability of yeast membranes on the basis of the expressed amount of aquaporin. Considering the protein expression levels, the myc-tagged OsPIP1;1 and OsPIP1;2 did not stimulate water permeability much compared with myc-OsPIP2;4 and OsPIP2;5.

Diurnal variations of aquaporin mRNA levels in roots

The mRNA levels in roots under 12 h light/12 h dark conditions were determined by RT–PCR. In this experiment, we selected several aquaporins that were expressed at relatively high levels in the roots to analyze the relationship between mRNA levels and the water uptake from the roots. Interestingly, the mRNA levels of *OsPIP1;2*, *OsPIP1;3*, *OsPIP2;3*, *OsPIP2;4*, *OsPIP2;5*, *OsTIP1;2* and *OsTIP2;1* showed a clear diurnal change with a large amplitude (Fig. 4). These aquaporins reached the maximum level in the daytime and the basal level at midnight. In particular, the mRNA levels of *OsPIP2;4* and *OsPIP2;5*, which had high water channel activity (Fig. 3D), showed a peak 3 h after the onset of light and dropped to a minimum 3 h after the onset of darkness.

Effect of chilling treatment on bleeding sap volume and the aquaporin mRNA levels in roots

The bleeding sap is an exudate of the xylem and its volume is highly correlated with root hydraulic conductance. The bleeding sap volume of rice plants decreased immediately after incubation at 4°C and then recovered 12 h after warming at 25°C with continuous light (Fig. 5A). In a similar manner, 4°C treatment caused a decrease in the transcript level of 10 aquaporin genes: *OsPIP1;1*, *OsPIP1;2*, *OsPIP2;1*, *OsPIP2;2*, *OsPIP2;3*, *OsPIP2;4*, *OsPIP2;5*, *OsPIP2;6*, *OsTIP1;1* and *OsTIP2;2* (Fig. 5B). *OsPIP1;2*, *OsPIP2;4* and *OsPIP2;5*

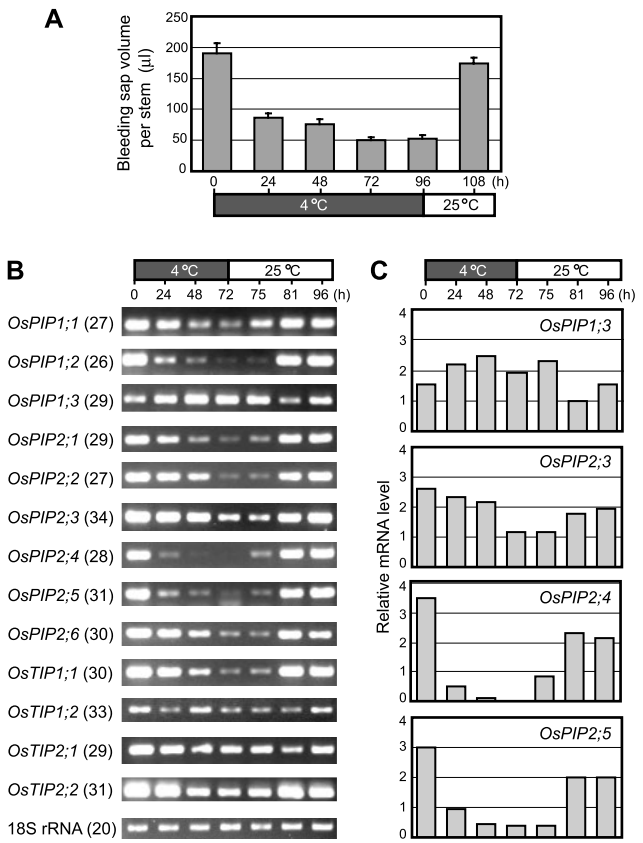

Fig. 5 Effect of chilling treatment on mRNA levels of aquaporins in the roots. (A) Sixteen-day-old rice plants grown under 12 h light/12 h dark conditions were placed in a chamber set at 4°C (gray column) without light under 100% relative humidity for 96 h and then moved to a chamber set at 25°C with continuous light (white column). The bleeding sap was collected into the cotton at 25°C in continuous light for 12 h. The absorbed water volume was calculated from the increase in weight of the cotton covered with parafilm. The data represent the means ± SE for 19–27 independent samples. (B) The mRNA levels were analyzed by semi-quantitative RT–PCR. The PCR products were separated on an agarose gel and photographed after ethidium bromide staining. Numbers in parentheses indicate the number of PCR cycles. (C) The relative mRNA levels of *OsPIP1;3*, *OsPIP2;3*, *OsPIP2;4* and *OsPIP2;5* were quantified from the images of RNA gel electrophoresis and normalized to that of 18S rRNA.

decreased markedly after 24 h at 4°C, while the mRNA levels of *OsPIP1;1*, *OsPIP2;1*, *OsPIP2;2*, *OsPIP2;3*, *OsPIP2;6*, *OsTIP1;1* and *OsTIP2;2* decreased slowly (Fig. 5B, C). The decreased mRNA levels of most aquaporins were recovered 9 h after warming (Fig. 5B, C). It is noteworthy that the *OsPIP1;3* mRNA level increased by 60% during the chilling treatment (Fig. 5C).

Discussion

Thirty-three rice aquaporin genes

We identified 33 rice aquaporin genes from the database obtained by the rice genome sequencing project (Table 1). The number of rice aquaporin genes was comparable with those in *Z. mays* and *A. thaliana* (33 and 35 genes, respectively). There were some differences between rice and other plant aquaporins. First, there were only 11 rice PIP members, which was less than those of *Z. mays* and *A. thaliana* (13 members in both) (Chaumont et al. 2001, Johanson et al. 2001). This is because rice had only three PIP1 members, while *Z. mays* and *A. thaliana* had six and five members, respectively. Secondly, rice had unique aquaporin members: *OsPIP2;7*, *OsPIP2;8* and *OsNIP4;1*. Although these three members showed low sequence identity to other plant aquaporins (Fig. 1), they had two sets of the common Asn-Pro-Ala motif and six transmembrane domains. These results suggest that *OsPIP2;7*, *OsPIP2;8* and *OsNIP4;1* might have their own characteristic functions in rice.

Several rice aquaporin genes have already been registered in the databases. Although we identified most of them in this study (Table 1), we could not identify three genes, *RWC1* (Li et al. 2000), *RWC3* (Lian et al. 2004) and *OsPIP2a* (Malz and Sauter 1999). This may be due to the difference in the cultivar of rice used for gene analysis. For example, *RWC1* identified in the japonica cultivar Josaeng Tongli (Wasetoitsu) (Li et al. 2000) was slightly different from *OsPIP1;1* in the cultivar Nipponbare. We considered that *RWC1* in Josaeng Tongli corresponds to *OsPIP1;1* in Nipponbare. Similarly, *RWC3* and *OsPIP2a* may correspond to *OsPIP1;3* and *OsPIP2;1*, respectively. On the other hand, *OsPIP1a* (Malz and Sauter 1999), *γTIP1* (Liu et al. 1994), *OsTIP1*, *OsTIP2*, *OsTIP3* (Takahashi et al. 2004) and *γMIP1* (Liu et al. 1994) were identical to the rice aquaporin genes identified in this study (Table 1), although some of them were cloned from other cultivars. These results indicated that there could be minor differences in some aquaporin sequences among rice cultivars.

Organ specificity of rice aquaporins

The expression profile of aquaporin genes in different organs and growth stages should provide information on their physiological roles. Most genes showed clear organ specificity, which was maintained over a long period of growth from early tillering to panicle formation stages (Fig. 2). This tendency was seen in other developmental stages, such as the maximum tillering and heading stage (data not shown). Therefore, the organ specificity may be tightly related to the physiological function in each organ.

The transcript levels of *OsTIP3;1*, *OsTIP3;2*, *OsTIP4;2* and *OsTIP5;1* and most *OsNIP* genes were estimated to be extremely low judging from the number of cycles of RT–PCR needed to detect those mRNAs (Fig. 2). In addition, no cDNA clones corresponding to *OsPIP2;3*, *OsNIP1;2*, *OsNIP1;4*, *OsNIP3;1*, *OsNIP3;2* and *OsNIP3;3* were identified in the

database (Table 1). These results indicate that the expression of these genes might be extremely low or limited to specific tissues in rice plants.

OsPIP2;4 and OsPIP2;5 have high water channel activity

By the yeast heterologous expression system and stopped-flow spectrophotometric analysis, we found that OsPIP2;4 and OsPIP2;5 had significant osmotic water channel activity (Fig. 3E). In contrast, OsPIP1;1 and OsPIP1;2 showed no significant activity. Judging from the high expression of *OsPIP2;4* and *OsPIP2;5* in roots (Fig. 2), both OsPIP2 members might play a crucial role as water channels in roots.

The low and high water channel activities in PIP1 and PIP2 members, respectively, were reported for other plants (Chaumont et al. 2000, Moshelion et al. 2002, Fetter et al. 2004, Suga and Maeshima 2004). Suga and Maeshima (2004) demonstrated that the valine residue in loop E of radish PIP2 members is essential for the water channel activity. Radish PIP1 members have an isoleucine residue at the corresponding site. Similarly, isoleucine and valine residues were conserved in all members of the OsPIP1 and OsPIP2 group, respectively, except for OsPIP2;8. We should examine whether the members of OsPIP1 group facilitate the transport of other substrates and whether their water channel function is regulated by post-translational modification.

Response to chilling treatment and diurnal variation of rice aquaporins

One of the aims of the present work is to examine the involvement of rice aquaporins in water uptake from the roots under low temperature conditions. Therefore, we analyzed the relationship between bleeding sap volume, which is highly correlated with root hydraulic conductivity, and mRNA levels of rice aquaporins during chilling treatment. The change in the bleeding sap volume during and after chilling treatment was closely correlated with changes in the expression of aquaporin genes, especially genes for functional water channels, such as OsPIP2;4 and OsPIP2;5 (Fig. 5A, B, C). The down-regulation of PIP gene expression in roots was also reported for *Z. mays* (Aroca et al. 2005) and for rice (Li et al. 2000). On the other hand, Aroca et al. (2005) reported that the protein levels of *Z. mays* PIP members increased during chilling treatment. Therefore, further studies, such as analysis at the protein level, should be conducted to understand the physiological function of OsPIP2;4 and OsPIP2,5 in water uptake during chilling treatment.

We also found a diurnal variation in the mRNA levels of rice aquaporin in roots. *OsPIP2;4* and *OsPIP2;5* mRNA levels in roots varied diurnally with a large amplitude (Fig. 4). The diurnal changes in the mRNAs and proteins have also been reported for aquaporins of other plants such as *Lotus japonicus* (Henzler et al. 1999), *Hordeum vulgare* (Katsuhara et al. 2003) and *Z. mays* (Lopez et al. 2003, Lopez et al. 2004). Lopez et al. (2003) revealed that the protein levels of *Z. mays* ZmPIP2 members, but not ZmPIP1s, in roots were correlated closely with the diurnal variation in root water flux. ZmPIP2-1 and ZmPIP2-5 showed high water channel activities (Lopez et al. 2003), while ZmPIP1 members had low water channel activities (Chaumont et al. 2000, Gaspar et al. 2003). Therefore, Lopez et al. (2003) concluded that ZmPIP2 members might contribute to diurnal water transport in roots. These observations in conjunction with the present results underline the importance of members of the PIP2 group for diurnal water movement in plant roots.

Materials and Methods

Identification and phylogenetic analysis of rice aquaporin genes

Aquaporin genes from the genome sequence (*O. sativa* L. cv. Nipponbare) were identified by BLAST searches on the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) and the Rice Genome Research Program (RGP; http://rgp.dna.affrc.go.jp/) based on the sequence similarity with aquaporins of *A. thaliana* (Johanson et al. 2001) and *Z. mays* (Chaumont et al. 2001). The phylogenetic analysis was conducted for their deduced amino acid sequences using the Clustal W program (Thompson et al. 1994) and the results were displayed using the TreeView program (Page 1996). The transmembrane topology of the rice aquaporins was predicted by the ConPred II method (Arai et al. 2004, http://bioinfo.si.hirosaki-u.ac.jp/~ConPred2/).

Plant materials and growth conditions

Rice (cv. Akitakomachi) seeds were germinated in the dark for 3 d at 25°C and grown in a growth chamber under 12 h light/12 h dark (light period; 450 μmol s^{-1} m^{-2}) and at day/night temperatures of 25/20°C at a relative humidity of 75%. Plants were grown in tap water for the first 5 d, and then were supplied continuously with fresh culture solution at a slow rate. The culture solution contained 10 ppm nitrogen (NH_4NO_3), phosphorus (NaH_2PO_4), potassium (K_2SO_4), calcium ($CaCl_2$), magnesium ($MgSO_4$), 0.4 ppm iron (Fe(III)-EDTA) and 0.1 ppm manganese ($MnCl_2$), pH 5.0. Leaf blades and roots were harvested from 21-day-old plants (early tillering stage) and 56-day-old plants (panicle formation stage). Leaf blades were harvested from all tillers of 21-day-old plants and from the upper three leaves of 56-day-old plants. Roots were collected from the apical half of the roots. Anthers were harvested from 67-day-old plants (heading stage, a few days before anthesis). For analysis of diurnal change in the levels of aquaporin mRNAs, total RNA fractions were obtained from 16-day-old plants.

Chilling treatment of plants and measurements of bleeding sap volume

Sixteen-day-old rice plants cultivated under 12 h light/12 h dark conditions were placed in a chamber set at 4°C without light under 100% relative humidity for 96 h to avoid the stresses of drought and light. Then plants were moved to a chamber set at 25°C with continuous light. The bleeding sap was collected into the cotton for 12 h from the stem cut off 3–4 cm above the soil surface at 25°C in continuous light to avoid the effect of diurnal variation of water uptake from roots. The bleeding sap volume was calculated from the increase in the weight of the cotton covered with parafilm. For analysis of aquaporin mRNAs, plants were chilled for 72 h and then moved to the continuous light chamber at 25°C.

RNA extraction and semi-quantitative RT–PCR

Tissues of rice plants were frozen in liquid nitrogen and ground in a mortar with a pestle. RNA was extracted from frozen powder of

the tissue with the RNeasy Plant Mini kit (Qiagen K.K., Tokyo, Japan). For RT–PCR, the first strand cDNA was synthesized using ReverTra Ace (Toyobo Co., Ltd., Osaka, Japan). PCR was performed using AmpliTaq GOLD (ABI, Foster City, CA, USA) and the primers listed in Table 2 (Supplementary data). All primers were designed based on the sequences of the 3′-untranslated regions in each aquaporin gene and to have similar T_m values (59.3 ± 1.2°C). The PCR conditions used were 94°C for 30 s, 58°C for 30 s and 72°C for 1 min. In the case of some aquaporins, the annealing temperature was set at 61°C to prevent the amplification of non-specific PCR products. The reaction was repeated for 16–36 cycles to obtain an appropriate amount of DNA. The conditions and cycle numbers were determined to avoid the saturation of DNA amplification. The obtained DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide. The signal intensity of the stained bands was photographed by a charge-coupled device (CCD) camera and analyzed by the NIH Image program (http://rsb.info.nih.gov/nih-image). The fact that there was no contamination of genomic DNA in the cDNA samples was confirmed by PCR using the primer sets listed in Table 2 (Supplementary data).

Expression of rice aquaporin genes in yeast

*Eco*RI–*Sal*I or *Eco*RI–*Pvu*II fragments of rice aquaporin cDNA (*OsPIP1;1* and *OsPIP1;2*, *Eco*RI–*Sal*I; *OsPIP2;4* and *OsPIP2;5*, *Eco*RI–*Pvu*II) were amplified by RT–PCR with gene-specific primers (Table 2, Supplementary data) and LA Taq (TAKARA SHUZO Co., LTD., Kyoto, Japan) using total RNA of the rice roots as a template. Reverse primers included an additional 3′-non-coding region (8–17 bases), because the open reading frames of rice aquaporins were quite similar to each other. The obtained fragments were inserted into the yeast expression vector myc-pKT10 (Tanaka et al. 1990, Suga and Maeshima 2004) (see Fig. 3A). This vector includes a c-myc epitope sequence at the down-stream region of the GAPDH promoter. After confirming the DNA sequences, the obtained plasmid was introduced into *S. cerevisiae* strain BJ5458, which is deficient in major vacuolar proteinases and functional aquaporins (Suga and Maeshima 2004).

Transformed yeast, which was selected using URA3 (orotidine-5′-phosphate decarboxylase), was grown in AHCW/Glc plates [0.17% yeast nitrogen base without amino acid, 0.5% ammonium sulfate (Difco), 1% casamino acid, 0.002% adenine sulfate, 0.002% tryptophan, 50 mM potassium phosphate, pH 5.5, 2% glucose and 2% agar] as described previously (Nakanishi et al. 2001, Suga and Maeshima 2004). Accumulation of the transformed aquaporins was confirmed by immunoblotting using anti-myc (9E10) (Nacalai Tesque Inc., Osaka, Japan) and anti-PAQs antibodies, which recognize most isoforms of PIP1s and PIP2s of radish (Ohshima et al. 2001). The expression level of each aquaporin protein was calculated from the signal intensity of the stained bands by anti-myc antibody (Fig. 3C) using the NIH Image program.

Determination of the osmotic water permeability of membranes

The osmotic water permeability of membranes was measured by a stopped-flow spectrophotometer (model SX18MV, Applied Photophysics, Surrey, UK) as described previously (Ohshima et al. 2001, Suga and Maeshima 2004). Yeast membrane vesicles (0.5 mg ml^{-1}) containing each rice aquaporin in a 0.45 M mannitol solution were quickly mixed with an equal volume of 0.1 M mannitol solution. The membrane suspension medium contained 0.45 M mannitol, 90 mM KCl, 1 mM EDTA and 20 mM Tris–HCl, pH 7.2. The light-scattering assay was carried out at 10°C for 7–12 times and the average was calculated for each membrane sample. The initial rate constants were calculated from the lines between 0 and 10 ms. The relative fold stimulation of osmotic water permeabilities was determined by the ratio of the initial rate constant of each aquaporin to that of vector control on the basis of the expressed amount of protein (ratio to that of myc-OsPIP1;1).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oupjournals.org.

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References

Arai, M., Mitsuke, H., Ikeda, M., Xia, J.-X., Kikuchi, T., Satake, M. and Shimizu, T. (2004) ConPred II: a consensus prediction method for obtaining transmembrane topology models with high reliability. *Nucleic Acids Res.* 32: W390–W393.

Aroca, R., Amodeo, G., Fernández-Illescas, S., Herman, E.M., Chaumont, F. and Chrispeels, M.J. (2005) The role of aquaporins and membrane damage in chilling and hydrogen peroxide induced changes in the hydraulic conductance of maize roots. *Plant Physiol.* 137: 341–353.

Bots, M., Feron, R., Uehlein, N., Weterings, K., Kaldenhoff, R. and Mariani, T. (2005a) PIP1 and PIP2 aquaporins are differentially expressed during tobacco anther and stigma development. *J. Exp. Bot.* 56: 113–121.

Bots, M., Vergeldt, F., Wolters-Arts, M., Weterings, K., Van As, H. and Mariani, C. (2005b) Aquaporins of the PIP2 class are required for efficient anther dehiscence in tobacco. *Plant Physiol.* 137: 1049–1056.

Chaumont, F., Barrieu, F., Jung, R. and Chrispeels, M.J. (2000) Plasma membrane intrinsic proteins from maize cluster in two sequence subgroups with differential aquaporin activity. *Plant Physiol.* 122: 1025–1034.

Chaumont, F., Barrieu, F., Wojcik, E., Chrispeels, M.J. and Jung, R. (2001) Aquaporins constitute a large and highly divergent protein family in maize. *Plant Physiol.* 125: 1206–1215.

Dixit, R., Rizzo, C., Nasrallah, M. and Nasrallah, J. (2001) The *Brassica MIP-MOD* gene encodes a functional water channel that is expressed in the stigma epidermis. *Plant Mol. Biol.* 45: 51–62.

Fetter, K., Wider, V.V., Moshelion, M. and Chaumont, F. (2004) Interactions between plasma membrane aquaporins modulate their water channel activity. *Plant Cell* 16: 215–228.

Gaspar, M., Bousser, A., Sissoëff, I., Roche, O., Hoarau, J. and Mahé, A. (2003) Cloning and characterization of *ZmPIP1-5b*, an aquaporin transporting water and urea. *Plant Sci.* 165: 21–31.

Hanba, Y.T., Shibasaka, M., Hayashi, Y., Hayakawa, T., Kasamo, K., Terashima, I. and Katsuhara, M. (2004) Overexpression of the barley aquaporin HvPIP2; 1 increases internal CO_2 conductance and CO_2 assimilation in the leaves of transgenic rice plants. *Plant Cell Physiol.* 45: 521–529.

Henzler, T., Waterhouse, R.N., Smyth, A.J., Carvajal, M., Cooke, D.T., Schäffner, A.R., Stuedle, E. and Clarkson, D.T. (1999) Diurnal variations in hydraulic conductivity and root pressure can be correlated with the expression of putative aquaporins in the roots of *Lotus japonicus*. *Planta* 210: 50–60.

Johanson, U., Karlsson, M., Johansson, I., Gustavsson, S., Sjövall, S. Fraysse, L., Weig, A.R. and Kjellbom, P. (2001) The complete set of genes encoding major intrinsic proteins in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. *Plant Physiol.* 126: 1358–1369.

Kaldenhoff, R., Kölling, A., Meyers, J., Karmann, U., Ruppel, G. and Richter, G. (1995) The blue light-responsive *AthH2* gene of *Arabidopsis thaliana* is primarily expressed in expanding as well as in differentiating cells and encodes a putative channel protein of the plasmalemma. *Plant J.* 7: 87–95.

Katsuhara, M., Koshio, K., Shibasaka, M. and Kasamo, K. (2003) Expression of an aquaporin at night in relation to the growth and root water permeability in barley seedlings. *Soil Sci. Plant Nutr.* 49: 883–888.

King, L.S., Kozono, D. and Agre, P. (2004) From structure to disease: the evolving tale of aquaporin biology. *Natl. Rev. Mol. Cell Biol.* 5: 687–698.

Li, L.-G., Li, S.-F., Tao, Y. and Kitagawa, Y. (2000) Molecular cloning of a novel water channel from rice: its products expression in *Xenopus* oocytes and involvement in chilling tolerance. *Plant Sci.* 154: 43–51.

Lian, H.-L., Yu, X., Ye, Q., Ding, X.-S., Kitagawa, Y., Kwak, S.-S., Su, W.-A. and Tang, Z.-C. (2004) The role of aquaporin RWC3 in drought avoidance in rice. *Plant Cell Physiol.* 45: 481–489.

Liu, L.-H., Ludewig, U., Gassert, B., Frommer, W.B. and von Wirén, N. (2003) Urea transport by nitrogen-regulated tonoplast intrinsic proteins in Arabidopsis. *Plant Physiol.* 133: 1220–1228.

Liu, Q., Umeda, M. and Uchiyama, H. (1994) Isolation and expression analysis of two rice genes encoding the major intrinsic protein. *Plant Mol. Biol.* 26: 2003–2007.

Lopez, F., Bousser, A., Sissoëff, I., Gaspar, M., Lachaise, B., Hoarau, J. and Mahé, A. (2003) Diurnal regulation of water transport and aquaporin gene expression in maize roots: contribution of PIP2 proteins. *Plant Cell Physiol.* 44: 1384–1395.

Lopez, F., Bousser, A., Sissoëff, I., Hoarau, J. and Mahé, A. (2004) Characterization in maize of ZmTIP2-3, a root-specific tonoplast intrinsic protein exhibiting aquaporin activity. *J. Exp. Bot.* 55: 539–541.

Loqué, D., Ludewig, U., Yuan, L. and von Wirén, N. (2005) Tonoplast intrinsic proteins AtTIP2;1 and AtTIP2;3 facilitate NH_3 transport into the vacuole. *Plant Physiol.* 137: 671–680.

Ma, S., Quist, T.M., Ulanov, A., Joly, R. and Bohnert, H.J. (2004) Loss of TIP1; 1 aquaporin in *Arabidopsis* leads to cell and plant death. *Plant J.* 40: 845–859.

Malz, S. and Sauter, M. (1999) Expression of two PIP genes in rapidly growing internodes of rice is not primarily controlled by meristem activity or cell expansion. *Plant Mol. Biol.* 40: 985–995.

Maurel, C., Javot, H., Lauvergeat, V., Gerbeau, P., Tournaire, C., Santoni, V. and Heyes, J. (2002) Molecular physiology of aquaporins in plants. *Int. Rev. Cytol.* 215: 105–148.

Moshelion, M., Becker, D., Biela, A., Uehlein, N., Hedrich, R., Otto, B., Levi, H., Moran, N. and Kaldenhoff, R. (2002) Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. *Plant Cell* 14: 727–739.

Nakanishi, Y., Saijo, T., Wada, Y. and Maeshima, M. (2001) Mutagenic analysis of functional residues in putative substrate-binding site and acidic domains of vacuolar H^+-pyrophosphatase. *J. Biol. Chem.* 276: 7654–7660.

Niemietz, C.M. and Tyerman, S.D. (2000) Channel-mediated permeation of ammonia gas through the peribacteroid membrane of soybean nodules. *FEBS Lett.* 465: 110–114.

O'Brien, M., Bertrand, C. and Matton, D.P. (2002) Characterization of a fertilization-induced and developmentally regulated plasma-membrane aquaporin expressed in reproductive tissues, in the wild potato *Solanum chacoense* Bitt. *Planta* 215: 485–493.

Ohshima, Y., Iwasaki, I., Suga, S., Murakami, M., Inoue, K. and Maeshima, M. (2001) Low aquaporin content and low osmotic water permeability of the plasma and vacuolar membranes of a CAM plant *Graptopetalum paraguayense*: comparison with radish. *Plant Cell Physiol.* 42: 1119–1129.

Page, R.D.M. (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *CABIOS* 12: 357–358.

Sarda, X., Tousch, D., Ferrare, K., Legrand, E., Dupuis, J.M., Casse-Delbart, F. and Lamaze, T. (1997) Two TIP-like genes encoding aquaporins are expressed in sunflower guard cells. *Plant J.* 12: 1103–1111.

Suga, S. and Maeshima, M. (2004) Water channel activity of radish plasma membrane aquaporins heterologously expressed in yeast and their modification by site-directed mutagenesis. *Plant Cell Physiol.* 45: 823–830.

Takahashi, H., Rai, M., Kitagawa, T., Morita, S., Masumura, T. and Tanaka, K. (2004) Differential localization of tonoplast intrinsic proteins on the membrane of protein body type II and aleurone grain in rice seeds. *Biosci. Biotechnol. Biochem.* 68: 1728–1736.

Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K. and Toh-e, A. (1990) IRA2, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian ras GTPase-activating protein. *Mol. Cell. Biol.* 10: 4303–4313.

Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22: 4673–4680.

Tyerman, S.D., Niemietz, C.M. and Bramley, H. (2002) Plant aquaporins: multifunctional water and solute channels with expanding roles. *Plant Cell Environ.* 25: 173–194.

Uehlein, N., Lovisolo, C., Siefritz, F. and Kaldenhoff, R. (2003) The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. *Nature* 425: 734–737.

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