Aquaporins are water channel proteins that facilitate passage of water and other small neutral molecules across biological membranes. There are usually a large number of members of this family in higher plants, which exhibit various physiological functions and are regulated in a time-specific and particular mode. We have previously shown that a rice gene, OsPIP2;7, was generally up-regulated in roots but down-regulated in shoots at the early stage of chilling stress. Here, OsPIP2;7 was cloned and proved to be an aquaporin with high activity in Xenopus oocytes. OsPIP2;7 was localized mainly in mesophyll cells of leaves. In roots it was detected in the vascular tissues, epidermis cells and exodermis cells at the elongation zone, as well as in the epidermis cells, exodermis cells and root hair at the maturation zone. Yeast cells overexpressing OsPIP2;7 showed a higher survival rate after freeze–thaw stress. Furthermore, OsPIP2;7 enhanced the transpiration rate and tolerance to low temperature when overexpressed in rice. These results indicated that OsPIP2;7 was involved in rapid water transport and maintenance of the water balance in cells, and ultimately improves the tolerance of yeast and rice to low temperature stress.

Keywords: In situ hybridization expression — Low temperature stress — Rice — Water channel activity — Yeast. 

Abbreviations: MIP, major intrinsic protein; NIP, nodulin 26-like intrinsic protein; PIP, plasma membrane intrinsic protein; SIP, small and basic intrinsic protein; TIP, tonoplast intrinsic protein.

Introduction

Maintenance of water balance is crucial for all living organisms. In plants, water is absorbed across the root tissues from the soil and transported to the aerial parts. There are apoplastic and symplastic pathways for water transport in plants. In the symplastic pathway, most water molecules have to cross a number of cellular membranes (Quigley et al. 2002). It has been proved that aquaporins take part in the rapid transport of water across the cell membrane.

Aquaporins form a large family of membrane channels that facilitate the permeation of water and small neutral solutes across the cellular membranes in most living organisms (Agre 1998). According to their localization and structural characteristics, plant aquaporins were classified into four subfamilies: the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the nodulin 26-like intrinsic proteins (NIPs) and the small basic intrinsic proteins (SIPs). The PIPs were further classified into PIP1 and PIP2 subgroups. Numerous studies have shown that aquaporins are related to various physiological processes, such as osmoregulation, leaflet movement, CO₂ assimilation, stomatal closure and opening, and absorption of mineral elements (Gerbeau et al. 1999, Dordas et al. 2000, Hanba et al. 2004, Siefrizt et al. 2004, Ma et al. 2006).

Low temperature may induce an imbalance between water uptake and transpiration of plants. The relationship between aquaporins and chilling stress has been reported (Li et al. 2000, Melkonian et al. 2004, Aroca et al. 2005, Sakurai et al. 2005). The response of all PIP genes to chilling stress was investigated in rice, showing that most PIP genes were remarkably depressed both in shoots and in roots during chilling treatment. However, OsPIP2;5 and OsPIP2;8 in shoots, and OsPIP2;3 and OsPIP2;7 in roots were enhanced during the early stage of chilling. It was suggested that the induction of OsPIP2;3 and OsPIP2;7 in roots might compensate the severe chilling stress-induced water stress (Yu et al. 2006).

In this study, the OsPIP2;7 gene was isolated and it was shown that it encodes a functional water channel protein with high osmotic permeability. Its spatial expression in rice was then investigated using in situ hybridization. Yeast cells overexpressing OsPIP2;7 showed a higher survival rate after freeze–thaw treatment. Furthermore, we observed that transgenic rice overexpressing OsPIP2;7 had higher transpiration and its resistance to chilling was improved, supporting a role for OsPIP2;7 in plant chilling resistance.

*Corresponding author: E-mail, zstressc@online.sh.cn; Fax, +86-21-54924015.
Results

OsPIP2;7 belongs to PIP2 group with a special expression pattern

PIP is an important subfamily of plant aquaporins and can be further divided into PIP1 and PIP2 groups. OsPIP2;7 belongs to the PIP2 group on the basis of sequence homology (Sakurai et al. 2005). The homology of OsPIP2;7 is higher or lower than 60% to the OsPIP2s or OsPIP1s at the amino acid level, respectively (Fig. 1).

Our data showed that OsPIP2;7 is expressed with specific patterns at different growth stages. Its expression level in leaves increased along with rice growth and was much higher than that in roots at all the growth stages investigated. At the reproductive growth stage, the

![Phylogenetic tree of all rice PIPs (PIP1 and PIP2).](image)

Fig. 1 Phylogenetic tree of all rice PIPs (PIP1 and PIP2). The phylogenetic tree was constructed using the Clustal W and MEGA 3.1 programs on the basis of the deduced amino acid sequences of PIPs from the rice genome database (Sakurai et al. 2006).

OsPIP2;7 transcripts could be detected in stems, glumes and flowers (Fig. 2).

OsPIP2;7 is an aquaporin with high osmotic permeability

It was shown that OsPIP2;7 was a functional aquaporin with high water channel activity when expressed in Xenopus oocytes (Fig. 3). The osmotic water permeability ($P_f$) was calculated from the rate of cell volume change in the presence of an osmotic gradient. The $P_f$ value of OsPIP2;7-expressing oocytes was $2.74 \times 10^{-2}$ cm s$^{-1}$

![Water permeability of OsPIP2;7.](image)

Fig. 3 Water permeability of OsPIP2;7. (A) Initial swelling rates of Xenopus laevis oocytes injected with water and cRNA of OsPIP2;7. The rate of oocyte swelling upon immersion in hypo-osmotic medium is plotted as $V/V_0$ vs. time, where $V$ is the volume at a given time point and $V_0$ is the initial volume. (B) Osmotic water permeability coefficient ($P_f$) of oocytes injected with water and cRNA of OsPIP2;7. The $P_f$ values were calculated from the initial rate of oocyte swelling. Data are given as the mean ± SD (n > 10).
(Fig. 3A), which was approximately 13 folds to the value of water-injected oocytes (Fig. 3B).

**Analysis of OsPIP2;7 expression by in situ hybridization**

We performed in situ hybridization using digoxigenin-labeled antisense RNA as probe to investigate the detailed spatial expression pattern. In leaves, OsPIP2;7 transcripts were expressed mainly in mesophyll cells, but not in the vascular cells and the epidermal cells (Fig. 4B). In roots, OsPIP2;7 was highly expressed in epidermal cells and endodermal cells around the stele of the meristematic zone and elongation zone (Fig. 4C). In the mature region of roots, the hybridization signals were detected mainly in the epidermal cells and cortex cells (Fig. 4D, E). These results implied that OsPIP2;7 was related to water transport in the mesophyll cells of leaves and contributed to water uptake in roots.

**OsPIP2;7 improved the tolerance of yeast cells to freezing**

Because OsPIP2;7 responds to chilling stress by repressing its expression in shoots, but inducing its expression in roots at the early stage of stress treatment (Yu et al. 2006), it prompted us to investigate further the function of OsPIP2;7 in low temperature, including chilling and freezing stress tolerance. We firstly overexpressed OsPIP2;7 in yeast. Under normal growth conditions, the yeast cells expressing OsPIP2;7 grew indistinguishably from those transformed with an empty plasmid (control). The control yeast cells had a lower survival rate after the freeze–thaw stress. However, the survival rate was significantly improved by overexpressing OsPIP2;7. The survival rate was increased from 0.201 to 1.639% and from 0.004 to 0.326% after one or two freeze–thaw cycles, respectively (Fig. 5).

**OsPIP2;7 was highly expressed in transgenic rice**

We also developed OsPIP2;7 transgenic rice to estimate the contribution of OsPIP2;7 to chilling stress tolerance. Overexpression of OsPIP2;7 in transgenic rice seedlings was confirmed using real-time PCR. Compared with the control, the expression of OsPIP2;7 in two independent transgenic lines (OE1 and OE2) was approximately 10- or 100-fold higher in leaves or roots, respectively (Fig. 6).

**Effects of OsPIP2;7 overexpression on the transcript levels of endogenous PIP genes in rice**

It has been reported that constitutive overexpression of a specific aquaporin gene in Arabidopsis plants may disturb the natural expression patterns of endogenous aquaporin
genes, and it was presumed that this phenomenon might explain the different responses of aquaporin transgenic plants to various abiotic stresses (Jang et al. 2007a, Jang et al. 2007b). We investigated whether the transcript levels of the other PIP genes are modulated by overexpression of OsPIP2;7 in the transgenic plants. The data showed that expression of OsPIP genes in leaves and roots was regulated in different ways by the overexpression of OsPIP2;7 under normal conditions. In leaves, the expression level of OsPIP2;4 and OsPIP2;8 was 3- to 8-fold higher than the control plants, and the other OsPIP genes decreased slightly (Fig. 7A). The expression level of OsPIP2;3 could not be detected in leaves as in control plants. In roots, OsPIP2;1, OsPIP2;2 and OsPIP2;8 were increased; however, OsPIP1;1, OsPIP1;2, OsPIP2;3, OsPIP2;4 and OsPIP2;5 were decreased under normal conditions (Fig. 7B).

The responses of OsPIP genes to chilling were also disturbed by overexpression of OsPIP2;7. In leaves, the expression of OsPIP1;1, OsPIP1;2, OsPIP2;1, OsPIP2;4, OsPIP2;5 and OsPIP2;6 under chilling stress was decreased compared with the control (Fig. 8A). In roots, the expression of OsPIP2;1, OsPIP2;6 and OsPIP2;8 was increased; however, the expression of OsPIP2;5 was decreased (Fig. 8B).
The response of rice overexpressing OsPIP2;7 to chilling stress

Under normal growth conditions, there was no difference in growth rate between the OsPIP2;7-overexpressing plants and control plants (Fig. 9A), but the moisture loss of the OsPIP2;7-overexpressing plants was notably higher than that of control plants (Fig. 9B). Under chilling conditions, both the OsPIP2;7-overexpressing and control plants grew slowly (Fig. 9C); however, the moisture loss under the chilling conditions was higher than that in normal conditions (Fig. 9C). Moreover, the moisture loss of the OsPIP2;7-overexpressing plants was higher than that of control plants (Figure 9D). During chilling stress at 7°C for 10 d, the leaves of both overexpressing and control rice were slightly wilted, suggesting that the rice plants are sensitive to chilling treatment. The relative electrical conductivity (REC), a key parameter of stress injury, of the OsPIP2;7-overexpressing rice was lower than that of the control plants (Fig. 10), suggesting that overexpression of OsPIP2;7 increased the tolerance of rice to chilling stress.

Discussion

Low temperature, including freezing and chilling, is one of the major stresses that limit the productivity and geographical distribution of many crops. Low temperature suppresses water transport and causes water deficit in leaves as a result of imbalance between water transport and transpiration (Sanders and Markhart, 2001). Plant aquaporins are believed to play an important role in maintaining water homeostasis not only under normal growth conditions but also under various stress conditions. Our previous study showed that the expression of a rice aquaporin gene OsPIP2;7 was regulated in opposite ways in shoot and root upon chilling stress (Yu et al. 2006), suggesting its possible role in facilitating water uptake and...
controlling water loss from transpiration upon chilling-induced water deficit.

OsPIP2;7 is a functional aquaporin with high water transport activity (Fig. 3). Plant PIP aquaporins were divided into PIP1 and PIP2 on the basis of their sequence. PIP2s have higher osmotic permeability, while PIP1s are inactive or have low activity (Fetter et al. 2004). OsPIP2;7 belongs to the PIP2 group (Fig. 1) and our results showed that it has high water channel activity (Fig. 3). Several PIPs have been investigated in rice: OsPIP1;1, OsPIP1;2 and OsPIP1;3 showed low osmotic permeability; OsPIP2;1, OsPIP2;3, OsPIP2;4, OsPIP2;5 and OsPIP2;7 had relatively high osmotic permeability (Li et al. 2000, Lian et al. 2004, Sakurai et al. 2005, Sakurai et al. 2008). There might be two reasons to explain the difference: one could be the different mechanisms used by PIP1s and PIP2s to regulate their localization on the plasma membrane. PIP2s could locate on the plasma membrane independently, whereas PIP1s required the assistance of other proteins (such as PIP2s) to locate correctly (Zelazny et al. 2007). The second reason could be that there is a molecular gating mechanism for plant PIPs (Tornroth-Horsefield et al. 2006). The basic structure of PIP1s and PIP2s might lead to a difference in steric structures and, further, a difference in conformation between PIP2s and PIP1s.

Aquaporins are expressed with a temporal- and spatial-specific pattern in plants. In agreement with the recent report that OsPIP1s and OsPIP2;1 accumulated in mesophyll cells (Sakurai et al. 2008), OsPIP2;7 transcripts were localized mainly in the mesophyll cells of leaf blades (Fig. 4). OsPIP2;7, which has high water transport activity, might play crucial roles together with other PIP members in maintenance of water potential in mesophyll cells.

It was reported that the freezing tolerance of yeast cells was determined by cellular dehydration and intracellular ice crystal formation (Tanghe et al. 2004), and water channels played important roles in osmotic adjustment of yeast cells under low temperature conditions (Soveral et al. 2006). To support this, the tolerance of yeast cells to freezing was improved by overexpressing OsPIP2;7 (Fig. 5). We speculated that OsPIP2;7 was involved in the rapid water transport from the intracellular to the extracellular space of yeast cells, with its high water transport activity, which might help to reduce intracellular ice crystal formation and the injury to the yeast cell membrane.

Water transport was sharply suppressed during the chilling stress conditions (Fennell and Markhart 1998, Aroca et al. 2001, Sakurai et al. 2005). Consequently water deficit was encountered by plants as a result of the imbalance between water transport and transpiration in leaves (Sanders and Markhart 2000). It has been suggested that aquaporins were required for water flow during cold stress (Sakr et al. 2003), and experiments proved that the transgenic plants overexpressing PIP1;4 or PIP2;5 could transport more water, which was observed as a shorter half-time of the water exchange (T_w1/2) in cortical cells and higher sap flow (Jang et al. 2007). In our experiments, OsPIP2;7 was highly expressed both in leaves and in roots of the transgenic plants (Fig. 6). The ratio between the cumulative transpiration and the increase of fresh weight in the OsPIP2;7-overexpressing plants was higher than that of the control plants under both normal conditions and chilling conditions (Fig. 9). We speculated that the transgenic plant with overexpression of OsPIP2;7 both in roots and in leaf mesophyll cells enhanced the ability for water uptake and rapid transport from roots to the aerial parts, which was reflected as a cumulative transpiration increase under normal conditions (Fig. 9B). Consistent with the findings that PIP1;4 or PIP2;5 overexpression resulted in enhanced water uptake upon cold stress (Jang et al. 2007), OsPIP2;7 overexpression in rice also improved the water transport and the cumulative transpiration was increased (Fig. 9D). In addition, the leaf REC, a key parameter of chilling injury, was found to be lower in the transgenic rice when compared with the controls (Fig. 10).

The complex expression pattern of different aquaporins under various stress conditions implied that maintenance of a reasonable water status required both increased water transport via aquaporins in some cells and tissues and reduced water transport via aquaporins in others.
Characterization of OsPIP2;7

(Jang et al. 2004, Guo et al. 2005, Zhu et al. 2005). No visible phenotype between the OsPIP2;7-overexpressing and control plants during chilling stress could be due to an enhanced water uptake by roots and meanwhile an increased water loss via leaves. Expression and accumulation of aquaporins were regulated in a spatial- and temporal-specific manner (Figs. 2, 4). In the transgenic plants OsPIP2;7 was constitutively overexpressed and it influenced the expression of other PIP genes (Figs. 7, 8). Our experiments supported the view that constitutive overexpression of a specific aquaporin in a given plant may disturb the natural expression patterns of endogenous aquaporin genes, which in turn influences responses of transgenic plants to various abiotic stresses (Jang et al. 2007). In addition, the functions of aquaporins are necessary but not sufficient to resist chilling injury (Aroca et al. 2005). We speculated that in the transgenic rice, OsPIP2;7 was advantageous to water transport and maintenance of the water balance, but could not avoid or repair the membrane damage caused during the chilling conditions.

Materials and Methods

Water permeability analysis

OsPIP2;7 was amplified by PCR from the rice cDNA, and then cloned into the pXBG-evl vector, a pSP64T-derived pBluescript type vector into which the 5'- and 3'-untranslated sequences of the Xenopus β-globin gene was inserted. The capped RNA (cRNA) transcript of OsPIP2;7 was synthesized in vitro using the mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (Ambion, Austin, TX, USA). The oocyte preparation, cRNA injection and osmotic water permeability assay were performed as described by Li et al. (2000). Each oocyte was injected with 46 ng of cRNA or 46 nl of sterile water as control. After incubation for 3 d at 18°C in Barth’s buffer, oocyte swelling was checked after shifting to 1/3 Barth’s buffer, and the P2 was calculated according to the initial oocyte swelling rate.

In situ hybridization

Tissues from 4-week-old rice were prepared as previously described (Furukawa et al. 2006). To prepare a gene-specific probe, the 5’ terminus of OsPIP2;7 (296 bp) was obtained by PCR with the following primers: forward, 5’-atggctcaagaggaagctg-3’; and reverse, 5’-cattgtagcagaggaataat-3’. Riboprobes for in situ hybridization were labeled with digoxigenin-11-UTP using a DIG RNA labeling kit (Roche, Mannheim, Germany) according to the manufacturer’s recommendation. In situ hybridization was performed as previously described (Furukawa et al. 2006).

Heterologous expression in yeast

OsPIP2;7 were inserted into the vector pYX212. Yeast transformation was carried out using the Li-acetate transformation method (Gietz and Schiestl 1995). The Saccharomyces cerevisiae strain used in this study was W303-1A. For the freeze tolerance test, the yeast cells expressing OsPIP2;7 and the control cells expressing the empty vector were frozen in liquid N2 and then thawed at room temperature. The freeze–thaw treatment was done once or twice. Then the frozen yeast cells or the non-frozen controls in liquid SD medium were diluted and 100 μl of cells were plated on SD agar plates. The plates were incubated at 30°C until colonies appeared (2–4 d). The survival rate = (the colonies of the frozen yeast cells)/(the non-frozen controls) × 100%. All the experiments were repeated at least three times.

Plant transformation

OsPIP2;7 was cloned into the BglII and SpeI sites of the binary vector pCAMBIA1302. Agrobacterium tumefaciens strain EHA105 was transformed with the resulting construct. The vector control plants were transformed with the empty pCAMBIA1302. Transformation and regeneration of transgenic rice were performed according to the protocol provided by Liu et al. (1998).

Low temperature treatment and electrolyte leakage test

Rice seedlings were cultured hydroponically in nutrient solution, and 2-week-old rice seedlings were used in this study. The chilling treatment was carried out by exposing the plants to cool air at 7 ± 1°C for 10 d. Low irradiance (200 μmol m⁻² s⁻¹ PAR) was used to avoid photo-oxidation damage during chilling. The electrolyte leakage test was performed as reported by Yu et al. (2006). Briefly, washed leaves were cut into 1 cm slices and put into a test tube containing 5 ml of deionized water. The leaf samples were immersed and vibrated occasionally at 25°C for 2 h, and then the electrical conductivity of the solution (C1) was measured. After boiling the samples for 10 min, the conductivity (C2) was measured again after the solution was cooled to room temperature. The REC was calculated as follows: REC (%) = C1/C2 × 100.

Measurement of the growth increase and moisture loss

The 2-week-old transgenic plants were planted in 150 ml conical flasks with fresh nutrient solution. The fresh weight was measured before and after the treatment. The increase in fresh weight in 10 d was measured. The total weight of the conical flask including liquid medium and rice seedlings was measured and the moisture loss was replenished every 5 d. The cumulative transpiration on the basis of the fresh weight increase was calculated as moisture loss.

Real-time PCR

Total RNA extraction from shoots and roots, cDNA synthesis and real-time PCR were conducted as described by Yu et al. (2006). Briefly, total RNA was extracted using RNArose reagent (Watson, China) and the residual genomic DNA was removed by a DNA-free Kit (Ambion). The cDNA was synthesized using oligo(dT)₁₈ primer and Rever-Tra Ace M-MLV RTase (Toyobo, Osaka, Japan) in a total volume of 20 μl. The primers and TaqMan-MGB probes were the same as described by Yu et al. (2006). Ubiquitin (NCBI accession No. D12629) was used as the internal control, which was constitutively expressed in roots and shoots under chilling conditions.

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