

Water relations and an expression analysis of plasma membrane intrinsic proteins in sensitive and tolerant rice during chilling and recovery

Xin Yu¹, Yan Hui Peng¹, Min Hua Zhang¹, Yan Jun Shao^{1,2}, Wei Ai Su¹, Zhang Cheng Tang¹

¹Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai, 200032, China; ²Life Science College, Hebei Agriculture University, 38 Ling-Yu-Si street, Baoding, Hebei, 071001, China

A symptom of chilling injury is development of water deficit in shoots, resulting from an imbalance of water transport and transpiration. In this work, two rice varieties (*Oryza sativa* L. var. Wasetoitsu and Somewake) seedlings were chilled at 7 °C, followed by recovery at 28 °C. Based on the growth phenotype and electrolyte leakage tests, Somewake was shown to be a chilling-tolerant variety, and Wasetoitsu a chilling-sensitive one. The chilling stress reduced markedly the relative water content (RWC) of leaves, accumulative transpiration and osmotic root hydraulic conductivity (L_p) in both varieties. But when returned to 28 °C, the water relation balance of Somewake recovered better. The mRNA expression profile of all the 11 plasma membrane intrinsic proteins (PIPs), a subgroup of aquaporins, was subsequently determined by real-time reverse transcription (RT)-PCR with TaqMan-minor groove binder (MGB) probes derived from rice var. Nipponbare during chilling treatment and recovery. Most of the *PIP* genes was down-regulated at the low temperature, and recovered at the warm temperature. The relative expression of some *PIPs* in both Somewake and Wasetoitsu decreased in parallel during the chilling. However during the recovery, the relative expression of *OsPIP1;1*, *OsPIP2;1*, *OsPIP2;7* in shoots and *OsPIP1;1*, *OsPIP2;1* in roots were significantly higher in Somewake than Wasetoitsu. This supports the role of PIPs in re-establishing water balance after chilling conditions. We discuss the diversified roles played by members of the aquaporin PIP subfamily in plant chilling tolerance depending on aquaporin isoforms, plant tissue and the stage of chilling duration.

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Introduction

Chilling to non-freezing temperature is detrimental to plant growth and development, and consequently decreases the productivity of crops. Tremendous efforts over several decades have been made to understand the mechanisms underlying plant responses to chilling [1]. Many reports have emphasized the changes in water relations because water

homeostasis is essential for plant survival under this stress condition, and a significant symptom of chilling injury is shoot water deficit resulting from imbalance between water transport and transpiration [2]. It has been well documented that root water transport is sharply suppressed by exposure to low temperature in a number of species, including maize [3-6], rice [7], bean [8], spinach [9], aspen [10], cucumber and figleaf gourd [11, 12]. Some studies have also demonstrated that a decrease in transpiration follows the reduction in root water flow [5, 10].

The concept that water flux across membranes is facilitated by aquaporins provides a new insight into plant water relations [13-16]. The relationship between aquaporins and plant chilling tolerance is an intriguing question with great

Correspondence: Wei Ai Su
Tel: 86-21-54924245; Fax: 86-21-54924015;
E-mail: zstressc@online.sh.cn
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agricultural significance. The composite water transport model proposes that water channels play a role in cell-to-cell water flow under adverse environmental conditions when low transpiration and xylem tension occurs [17, 18]. Aquaporins have previously been reported to be involved in chilling responses [4, 19] but only recently have comprehensive analysis of aquaporin expression at the mRNA level in response to low temperature been done. Techniques used were Northern blot, semi-quantitative reverse transcription (RT)-PCR and real-time RT-PCR using SYBR green dye in species such as *Arabidopsis* [20], maize [6] and rice [7]. Comparative analysis between cucumber (chilling sensitive) and figleaf gourd (chilling resistant) indicated that the decrease in cell hydraulic conductivity (L_p) at low temperature was associated with the open/close state of water channels [11, 12]. However, in chilling-sensitive maize, the decrease in root L_p upon chilling was due to oxidative damage to the membranes rather than a decrease in aquaporin amount or a change in phosphorylation status [6]. The roles of aquaporins in plant chilling tolerance remain elusive.

In order to gain insight into the integrated roles of aquaporins in response to stress, it is necessary to investigate the overall expression of the aquaporin family under stress conditions. Aquaporins belong to a large and highly conserved family of major intrinsic proteins (MIP) [21, 22]. For instance, 35 and 31 members of MIPs have been predicted or identified in *Arabidopsis* [23] and maize [24], respectively. MIPs can be classified into 4 subfamilies including plasma membrane intrinsic protein (PIP), tonoplast intrinsic protein (TIP), NOD26-like intrinsic protein (NIP) and small basic intrinsic protein (SIP). The recently developed technique of real-time RT-PCR using TaqMan- minor groove binder (MGB) probe, which has high specificity and sensitivity [25], provides a powerful tool for quantitatively investigating the overall expression of gene families with many members sharing high homology, such as the plant MIP family.

In this paper, we focused our efforts on the roles of rice PIPs in chilling tolerance. Rice seedlings were chilled at low temperature (7 °C), followed by recovery at 28 °C. The water relations of two rice varieties, Somewake and Wasetoitsu that are chilling tolerant and sensitive, respectively were initially compared during chilling and recovery. In order to investigate the integrated expression of the gene family encoding PIP in rice by real-time RT-PCR, genes encoding for PIPs in var. Nipponbare were surveyed. Sakurai *et al.* [7] recently reported the identification of 33 rice aquaporin genes (11 PIPs, 10 TIPs, 10 NIPs and 2 SIPs) by analysis of the rice genome. Hence, throughout this paper, we have used the aquaporin names as proposed by Sakurai *et al.* [7]. We selected 4 PIP genes including 3 coding for

functional water channel proteins (*OsPIP1;1*, *OsPIP2;1* and *OsPIP2;7*) and a root-specific one (*OsPIP2;3*) for comparing the transcript expression level of Somewake and Wasetoitsu during chilling and recovery. We attempted to unravel the roles of PIP aquaporins in plant chilling tolerance by matching the differences in water relations of the two varieties with the PIPs expression during chilling treatment.

Materials and Methods

Plant materials and growth condition

We were able to use information from the recently completed rice genome sequencing project based on *Oryza sativa* L. var. Nipponbare to investigate mRNA expression of all 11 PIPs in response to chilling. Two rice varieties were used, Wasetoitsu, a chilling sensitive variety developed in the Republic of Korea, and Somewake, a chilling tolerant variety developed in Japan. Seeds were germinated for 2-3 d at 28 °C in the dark on filter paper soaked with distilled water. Afterwards, the seedlings were grown hydroponically in nutrient solution [26] at photon flux density of 350-400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, 60-80% relative humidity, 12 h/12 h day-night cycle at 28 °C in a phytotron.

Low temperature treatment and recovery

Thirty-five-day-old rice seedlings were moved from the phytotron to a growth chamber (28 °C air temperature, 60-70% relative humidity and light density of 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR with 12 h photoperiod) for 2 d before the chilling treatment. The 7 °C chilling treatment was carried out by exposure to cool air and started 1 h after the beginning of illumination. The temperature was returned to 28 °C after 24 h of the chilling treatment and the plants was allowed to recover for 24 h. Low irradiance (200 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR) was used to avoid photo-oxidation damage during chilling and to focus our study on low temperature effects on water relations *per se*.

Electrolyte leakage test

Washed leaves (0.2 g) were cut into 1 cm slices and put in 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 using a conductivity meter. After boiling the samples for 10 min, their conductivity (C2) was measured again after the solution was cooled to room temperature. The relative electrical conductivity (REC) was calculated as follows:

$$\text{REC (\%)} = \text{C1/C2} \times 100$$

Relative water content (RWC) measurement

Rice leaves were collected and immediately weighed to determine their fresh weight (FW). Leaves were then transferred to sealed plates and hydrated between two pieces of filter paper, which were drenched in deionized water. After 10 h of hydration at 4 °C, the leaf surface was blotted dry, and the turgid weight (TW) recorded. Leaf samples were then oven dried at 80 °C for 24 h and the dry weight (DW) determined. The RWC was calculated via the following equation:

$$\text{RWC(\%)} = \frac{\text{FW-DW}}{\text{TW-DW}} \times 100$$

Accumulative transpiration

Accumulative transpiration was measured as described in a previous report [27]. The weight of tubes and liquid medium in which rice seedlings grew was measured and the moisture lost was replenished every 3 h. Moisture loss per hour on the basis of leaf dry weight was described as the accumulative transpiration.

Osmotic root hydraulic conductivity (L_p) measurement

The osmotic root L_p was measured essentially based on the method described by Miyamoto *et al.* [28] and Martinez-Ballesta *et al.* [29]. In brief, seedling shoots were excised with a razor blade 10–20 mm from the base, and the exuded sap was collected with a micropipette and the flow rate (J_v) on a root dry mass basis measured over the next 90 min. The osmotic potential of bath medium (Π_b) and sap (Π_s) exuded from individual plants were measured by a Vapor Pressure Osmometer (Wescor, USA). The osmotic root L_p was calculated from the following equation:

$$L_p = J_v / [\sigma \cdot (\Pi_b - \Pi_s)]$$

In this equation, σ was the root reflection coefficient for nutrient salts in the xylem and was estimated to be $\sigma = 0.4$ [28].

Extraction of RNA and first strand cDNA synthesis

Total RNA was extracted from roots and shoots using RNAase reagent (Watson, China). The residual genomic DNA was removed by DNA-free™ Kit (Ambion, USA). The concentration of RNA was accurately quantified by spectrophotometric measurement. The cDNA was synthesized using oligo (dT)₁₈ primer and ReverTra Ace M-MLV RTase (Toyobo, Japan) in a total volume of 20 μ L according to the manufacturer recommendation. The cDNA mixtures were diluted to 120 μ L with sterile ddH₂O and 2.5 μ L were used as template for real-time PCR in a total volume of 20 μ L.

Primers and TaqMan-MGB probes

The primers and TaqMan-MGB probes were designed using Primer Express 2.0 software (Applied Biosystems-Perkin-Elmer, USA) based on the sequences of rice *PIP* genes from Nipponbare. Ubiquitin (UBI) (NCBI accession number D12629) was used as the internal control. The sequence of PCR primers and TaqMan-MGB probes are shown in Table 1. All the primers and probes were synthesized by Shanghai GeneCore Company (Shanghai, China). The

Table 1 Primers and TaqMan-MGB probes for real-time RT-PCR

Name	Synonymy	Access	Probe	Primer
<i>OsPIP1;1</i>	<i>RWC1/PIP1a</i>	AK061769	TGTAGTTGCCAGTGGCT	F TACATGGGCAATGGCGGT R CAAGACCGTCACCCTTGGTG
<i>OsPIP1;2</i>		AK098849	ACCGTCCTCACCGTCAT	F GGCCACCTTCTCTTCTTTAC R GCGCACTTGGAGGTGGAGT
<i>OsPIP1;3</i>	<i>RWC3</i>	AK102174	CTCCTCCACCTCGAACA	F AAGGACTACCGGGAGCCG R GGTAGAACGACCACGACGTCA
<i>OsPIP2;1</i>	<i>PIP2a</i>	AK072519	TCGGCCTCGTCAAGG	F AGTGCCTCGGCGCCAT R CGTACCTGTTGAAGTAGGCGCT
<i>OsPIP2;2</i>		AK061782	CTGATCAGGGCGGTGC ^a	F TTCGGGCTGTTCTCTGGC R GGCCTGCGCGATGATGTA
<i>OsPIP2;3</i>		AL662958 ^b	AAGAGGAGCAAGCACGG	F TCTTCTCCGCCACCGACC R AACACCGCGAACCCAATTG
<i>OsPIP2;4</i>		AK072632	ACTCACATGTTCCCGTGTT	F ACCGATCCCAAGCGCAA R GCGAACC CGATTGGCAG
<i>OsPIP2;5</i>		AK107700	CGACCATCCCCGTCAC ^a	F CCGTGTTTCATGGTGCACCT R CCGGGTTGATGCCGGT
<i>OsPIP2;6</i>		AK061312	ACACCGTCTTCTCCGC ^a	F GAAATCATCGGCACCTTCGT R GGGCATTGCGCTTTGG
<i>OsPIP2;7</i>		AK109439	CTGTACCGTGCCCTC	F ACGAGCGAGCTGGGTAAGTG R ATGAGCGTCGCCATGAACTC
<i>OsPIP2;8</i>		AK109024	CTGCTGTTGGTGTGCAT	F CATTGCGGAGTTCACAGCC R CCGATCACGGTGCTCACA
<i>UBI</i>		D12629	CACCACAGCACACGC	F TTGTCCTGCGCCTCCGT R GGCATAGGTATAATGAAGTCCAATGC

^a These probes were designed to span the introns of their genomic sequences.

^b AL662958 was genome sequence access number, and others were cDNA clone access number.

probes were labeled with the fluorescent reporter dye 6-carboxy-fluorescein (FAM) on the 5' end, and with the fluorescent quencher dye 6-carboxytetramethylrhodamine (TAMRA) and MGB on the 3' end. They formed extremely stable duplexes with single-stranded DNA targets, allowing shorter probes to be designed. All PCR primers were designed to produce PCR products of about 70 bp in length.

Real-time PCR

TaqMan-MGB real-time PCR was performed in Rotor-Gene 3000 real-time thermal cycling system (Corbett Research, Australia) using Real-time PCR Master Mix (Toyobo, Japan). The reaction mixture (20 μ L) contained 2.5 μ L of diluted cDNA, 4 pmol of each primer, 4 pmol TaqMan-MGB probe and appropriate amounts of other components as recommended by the manufacturer (Toyobo, Japan). The real-time PCR was programmed for 5 min at 95 $^{\circ}$ C; 50 cycles of 20 s at 94 $^{\circ}$ C and 30 s at 60 $^{\circ}$ C. The data were collected and analyzed with the Rotor Gene 3000 software (Corbett Research, Australia). Each sample was quantified in triplicate. For the control reactions, either no sample was added or RNA alone was added without reverse transcriptase to test if the RNA sample contained contaminating genomic DNA. The housekeeping gene *UBI* was used as the internal standard to normalize target genes [30]. To generate standard curves for the *PIPs* and *UBI*, a range of ten-fold dilutions of the conventional RT-PCR products corresponding to *PIP* and *UBI* were tested in the same conditions as the experimental samples.

Plasma membrane protein extraction and Western blot

Root plasma membrane fractions were prepared by the aqueous two-phase partitioning method as describe by Ohshima *et al.* [31]. Membrane protein concentration was measured according to Bradford [32]. Western blots were done essentially by the standard method described by Sambrook *et al.* [33]. Protein samples (20 μ L) were separated by SDS-PAGE [12% (w/w) acrylamide], transferred

to nitrocellulose membranes and blocked with 5% (w/v) skim milk in phosphate-buffered saline for 1 h. Rabbit serum raised against the N-terminal conserved peptide, KDY NEP PPA PLF EPG ELS SWS, of PIP conjugated to bovine serum albumin (BSA) was used as the primary antibody (1:500) and anti-rabbit IgG (1:1,000) conjugated to alkaline phosphatase as the secondary antibody. The signals were visualized by 5-bromo-4-chloro-3-indolyphosphate/nitro-blue tetrazolium (NBT/BICP) substrates.

Results

Wasetoitsu and Somewake were different in chilling tolerance

Wasetoitsu and Somewake rice varieties were used. After 6-9 h treatment at 7 $^{\circ}$ C, Wasetoitsu showed obvious symptoms of chilling-induced water deficit, such as wilting, leaf rolling and drooping. However with Somewake, no obvious phenotypic change was observed except for slight leaf rolling. As a representative example, Figure 1A shows the different phenotypes of these varieties chilled at 7 $^{\circ}$ C for 24 h. After 1 h of recovery at 28 $^{\circ}$ C, the leaves of Somewake had expanded completely yet leaf tips in Wasetoitsu were observed to be dry even after 24 h of the recovery (data not shown). The leaf REC, a key parameter of chilling injury, was increased after 6 h at low temperature in both varieties, but the leaf REC of Wasetoitsu was approximately 3 times higher than that of Somewake after 9 h of chilling (Figure 1B). These data confirmed that Wasetoitsu was a less chilling tolerant variety, which was consistent with a previous evaluation by Kitagawa and Yoshizaki [34].

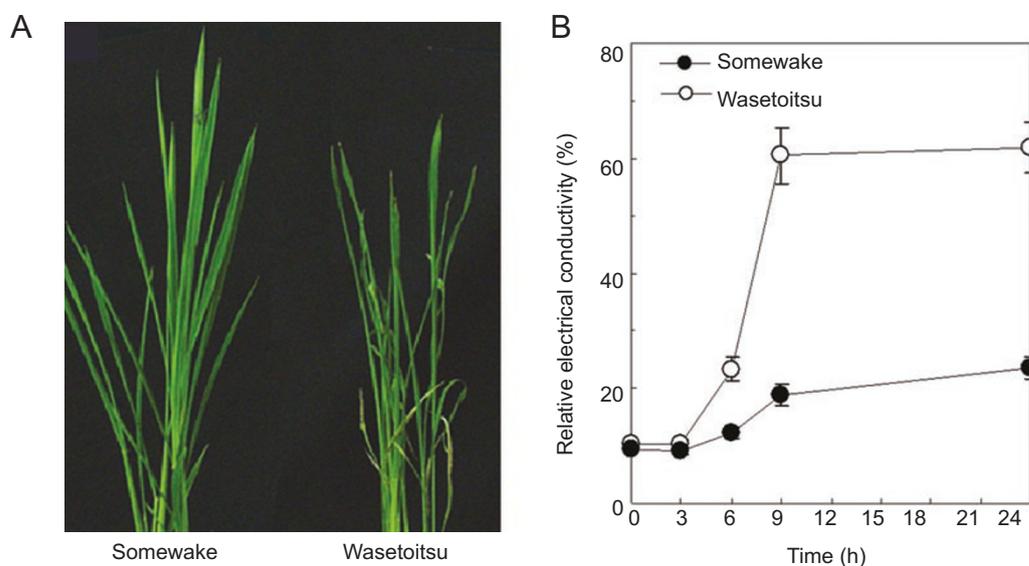


Figure 1 Different phenotypes (A) and leaf relative electrical conductivity (REC) (B) of two rice varieties differing in chilling sensitivity (Somewake, tolerant and Wasetoitsu, sensitive) when chilled at 7 $^{\circ}$ C for 24 h. Young leaves of Wasetoitsu were rolled, and some old leaves wilted and drooped, while no visible response was observed in Somewake (A). The data are means \pm S.D. for 5 independent samples (B).

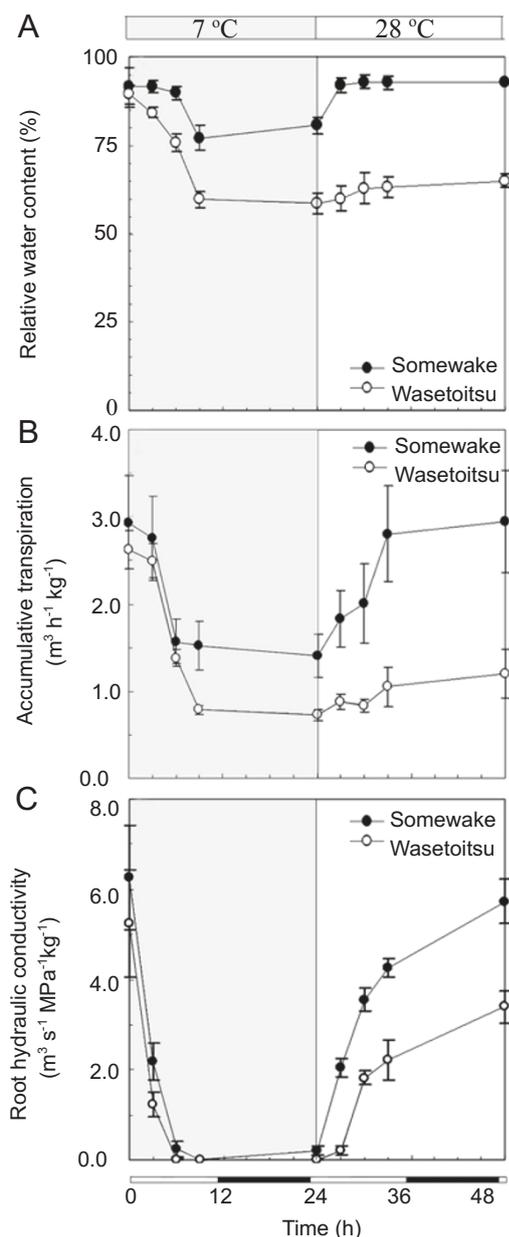


Figure 2 Relative water content (RWC) of leaves (A), accumulative transpiration (B) and root osmotic hydraulic conductivity (C) in the rice varieties Somewake and Wasetoitsu, at low temperature (7 °C) (grey column) and after recovery at 28 °C (white column). Plants were grown in a 12 h light (white rectangle) / 12 h dark (black rectangle) cycle. The data are means \pm S.D. for 5 independent samples.

Water relations in Wasetoitsu and Somewake during chilling and recovery

During the chilling and recovery, the root RWC of both varieties remained unchanged (data not shown). However, during the low temperature treatment, leaf RWC gradually declined, and Wasetoitsu had a remarkably lower leaf RWC

than the Somewake (Figure 2A). The two varieties were different in extent of improvement to leaf water status during the recovery. After a recovery period of 3 h, leaf RWC of Somewake returned to the pre-chilling level. Yet after a 24 h recovery period, the leaf RWC of Wasetoitsu was only 72.5% of the pre-treatment level (Figure 2A). Accumulative transpiration decreased during chilling, with a minimum, after 24 h of chilling, of approximately 48% and 30% of initial values for Somewake and Wasetoitsu, respectively (Figure 2B). Osmotic root Lp dropped sharply and immediately after the chilling treatment in both varieties. After 9 h chilling treatment, osmotic root Lp was too low to be detected in either variety, and only Somewake showed very slight recovery of Lp 24 h after warming (Figure 2C). Osmotic root Lp decreased more quickly and to a larger extent than accumulative transpiration (Figure 2B and 2C), suggesting the imbalance between water transport in root and water evaporation in aerial parts. This water imbalance probably resulted in the low RWC in leaves (Figure 2A). Somewake returned to more normal levels of accumulative transpiration and osmotic root Lp than Wasetoitsu, consistent with the higher leaf RWC. For instance, after 24 h of recovery, the accumulative transpiration and Lp of Somewake had returned to the initial values, yet for Somewake they were only 46% and 65% of initial values, respectively.

Profile of PIP gene expression in rice (var. Nipponbare) under non-stressed and chilling conditions

The ratio of *PIP/UBI* was used to determine transcript levels of rice *PIPs*. The transcript levels of the 11 *PIP* genes, varied considerably and there were also differences between shoots and roots under non-stressed conditions (Figure 3). The *OsPIP1;1* in roots was transcribed at an extremely high level of 1.43, much higher than the range of 10^{-2} ~1 for *OsPIP1;1* (in shoots), *OsPIP1;2*, *OsPIP2;1*, *OsPIP2;2*, and *OsPIP2;3* (in roots), *OsPIP2;4* (in roots), *OsPIP2;6* and *OsPIP2;7* (in shoots), which had intermediate transcript levels. A group with low expression were *OsPIP1;3* and *OsPIP2;4* (in shoots), *OsPIP2;5* and *OsPIP2;7* (in roots), and *OsPIP2;8* with transcript levels ranging from 10^{-5} ~ 10^{-2} . *OsPIP2;3* was only expressed in roots and barely detected in shoots, therefore, *OsPIP2;3* in shoots was omitted in Figure 3 and Figure 4.

Rice *PIPs* in control plants had large fluctuations in expression during a light/dark cycle (data not shown) confirming a previous report [7]. Thus we compared the changes in expression of all 11 *PIPs* in concurrent control plants without chilling treatment to the chilled plants both during chilling and after recovery. The relative expression level was defined as the ratio of treatment/concurrent control. As shown in Figure 3, the chilling treatment resulted in a sharp decrease in relative expression of most *PIPs* in both shoots

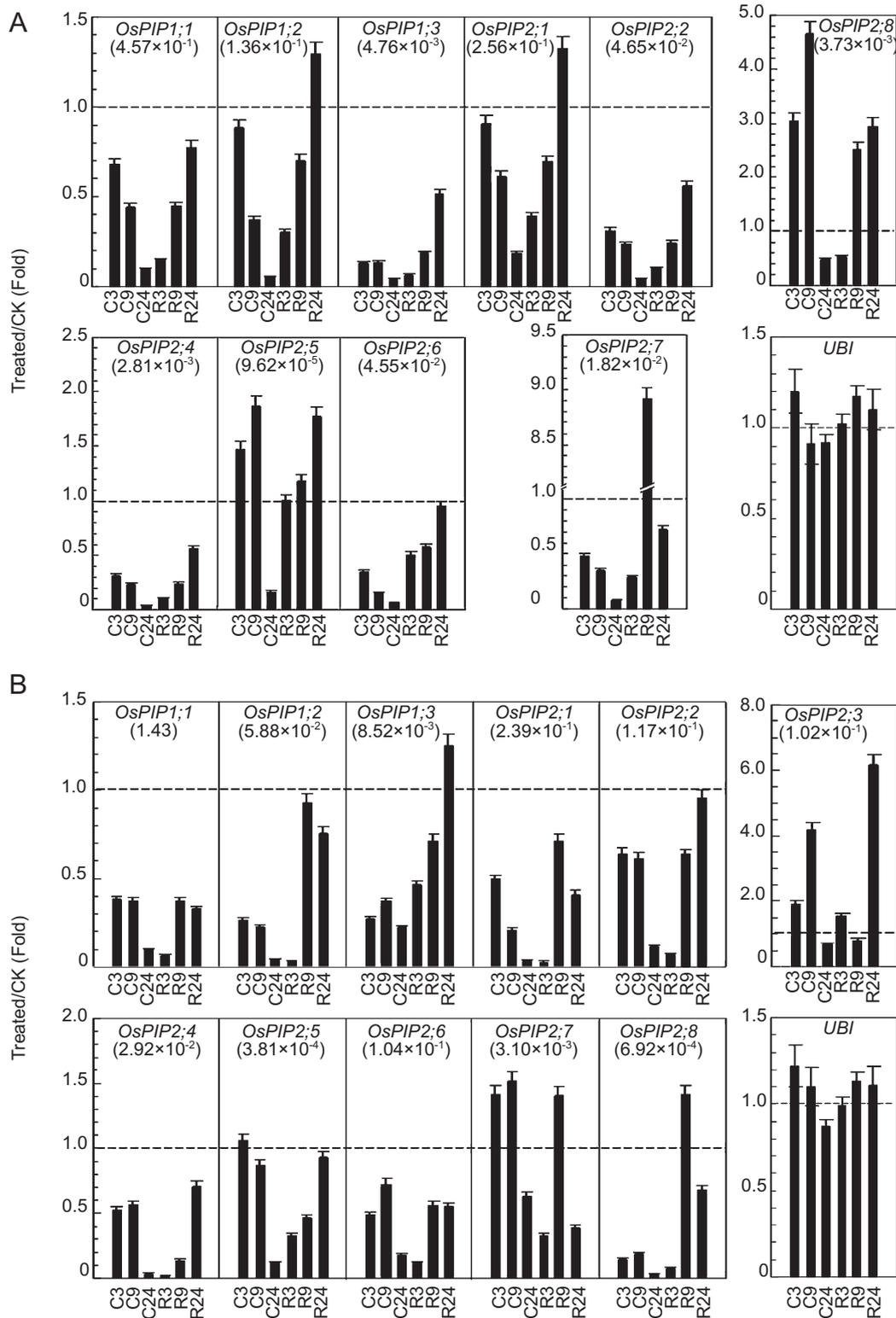


Figure 3 Expression profile of the 11 *PIP* genes in rice var. Nipponbare chilled at 7 °C for 3 (C3), 9 (C9) and 24 h (C24) and after recovery at 28 °C for 3 (R3), 9 (R9) and 24 h (R24). To allow for fluctuation of *PIP* expression under normal conditions, the relative expression of *PIPs* in the shoots (**A**) and roots (**B**) are described as Treated/Control ratio (fold). The *PIP* expression in chill-treated samples was taken at the same time point in time as the non-stressed control samples. Numbers in parentheses are the average values for each *PIP* transcript levels (*PIP*/*UBI*) at 12 time points evenly distributed in a 24 h cycle under non-stressed condition. The relative expression of *UBI* described by Treated/Control (fold) is also shown as a reference. Values are means \pm S.D. of 3 replicates.

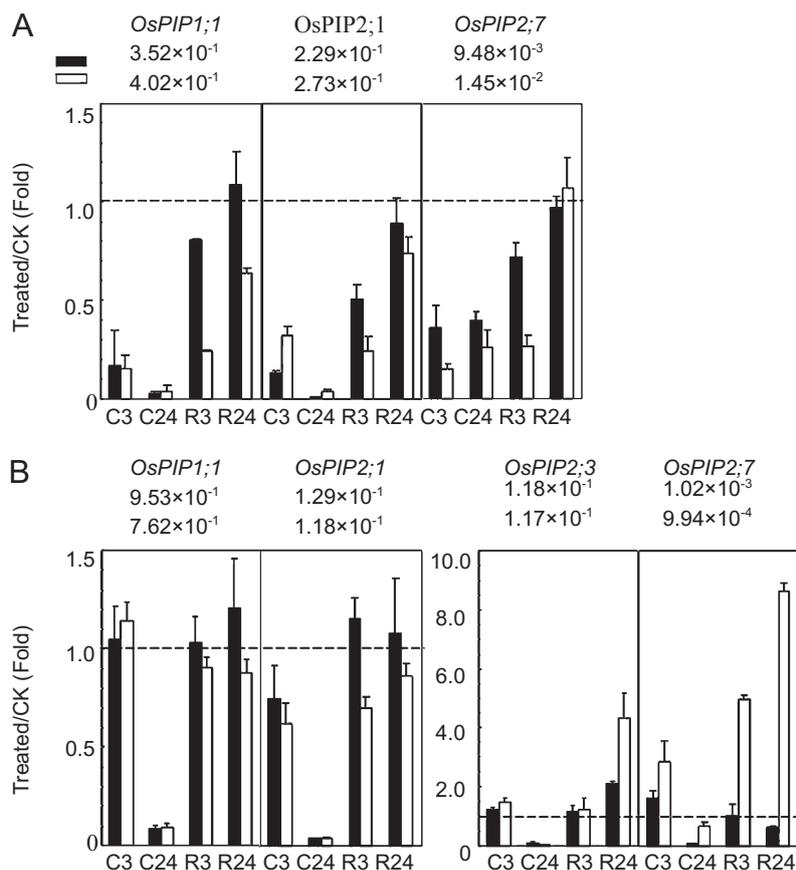


Figure 4 Expression of *PIP* genes in Somewake (black column) and Wasetoitsu (white column). The plants were chilled at 7 °C for 3 (C3) and 24 h (C24) then allowed to recover at 28 °C for 3 (R3) and 24 h (R24). To allow for fluctuation of *PIP* expression under normal conditions, the relative expression of *PIPs* in the shoots (**A**) and roots (**B**) were compared to the concurrent controls. Values are means \pm S.D. of 3 replicates. The average values for each *PIP* transcript levels compared to *UBI* (*PIP/UBI*) at the time point corresponding to C3, C24, R3 and R24 under non-stressed condition are also shown. Black bars, Somewake; white bars, Wasetoitsu.

and roots, followed by a recovery upon return to optimum temperature. The exceptions to this general pattern were *OsPIP2;5* and *OsPIP2;8* in shoots, *OsPIP2;3* and *OsPIP2;7* in roots, which were enhanced during the early stage of chilling, but decreased after 24 h of chilling. During the recovery, it was noted that expression of *OsPIP2;8* in shoots and *OsPIP2;3* in roots increased as much as 3 and 6 fold, respectively. This might reflect overcompensation for the sharp drop in water transport resulting from the chilling treatment. Most *PIPs* had similar expression patterns during the chilling and recovery, yet distinct features were exhibited among different *PIP* isoforms and plant tissues (shoots or roots) when the timing and scope of changes was considered in detail.

The PIP gene expression in Wasetoitsu and Somewake during chilling and recovery

In order to gain insight into the role of *PIPs* in chilling

tolerance, the expression of *OsPIP1;1*, *OsPIP2;1*, *OsPIP2;3* and *OsPIP2;7* by Somewake and Wasetoitsu was compared during chilling and recovery. In both Somewake and Wasetoitsu, the relative expression of *OsPIP1;1*, *OsPIP2;1*, *OsPIP2;3* and *OsPIP2;7* in shoots and *OsPIP1;1* and *OsPIP2;1* in roots decreased significantly when the plants were chilled for 24 h, but increased when returned to 28 °C. *OsPIP2;3* and *OsPIP2;7* were induced early in the chilling period but their expression was reduced after 24 h of chilling. During the recovery, Somewake had larger increases in relative expression of *OsPIP1;1*, *OsPIP2;1* and *OsPIP2;7* in shoots, and *OsPIP1;1* and *OsPIP2;1* in roots than Wasetoitsu. Interestingly, the relative expression of *OsPIP2;3* and *OsPIP2;7* in roots increased 4 and 8 fold, respectively in Wasetoitsu after 24 h of recovery, whereas this surprising overexpression of *PIPs* was not observed in Somewake (Figure 4).

In addition, Western blots were used to analyze the

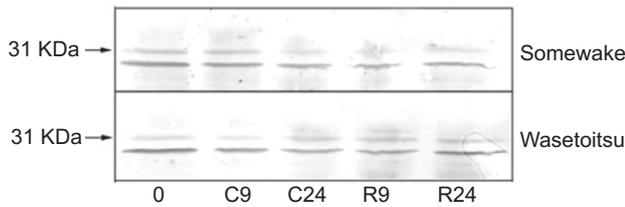


Figure 5 Western blot analysis of the PIP1 protein expression in roots of Wasetoitsu and Somewake. The plants were chilled at 7 °C for 9 (C9) and 24 h (C24) and then allowed to recover at 28 °C for 9 (R9) and 24 h (R24). Plasma membrane protein (20 µg) was loaded in each lane. Rabbit serum raised against N-terminal conservative peptide (KDY NEP PPA PLF EPG ELS SWS) of PIP conjugated to BSA was used as the primary antibody (1:500) and anti-rabbit IgG (1:1,000) conjugated to alkaline phosphatase was used as the secondary antibody. The signals were visualized by NBT/BICP substrates.

changes of PIP protein abundance in roots of Somewake and Wasetoitsu during chilling and recovery. Figure 5 shows the results of a Western blot analysis using purified antibody raised against a conserved N-terminal peptide of PIPs (See Material and Methods). Two bands of approximately 30 kDa reacted with the PIP antibody. Both were considered to be PIP proteins but probably differed in their posttranslational modification [35]. PIP protein abundance slightly decreased during chilling and recovered to approximately the initial value after warming to 28 °C in both varieties. No difference of PIP protein abundance in roots of Somewake and Wasetoitsu was observed.

Discussion

In this research, the effects of chilling on water relations in two rice varieties differing in chilling tolerance were analyzed. The chilling-tolerant variety, Somewake had slightly impaired water status and osmotic root L_p during chilling and recovery. We determined the integrated expression profile of the PIP gene family in the rice var. Nipponbare. Moreover, in order to investigate the relationship between PIP aquaporins and plant chilling tolerance, we compared the expression of 4 PIPs (3 functional water channel proteins and a root-specific PIP) in Somewake and Wasetoitsu, a chilling sensitive variety during chilling and recovery.

A large number of reports have centered on root water uptake of plants subjected to chilling stress. In this study, chilling led to a dramatic decrease in osmotic root L_p in Somewake and Wasetoitsu (Figure 2C), an observation that paralleled observations in maize [3, 6], spinach [9], bean [8], cucumber and figleaf gourd [12]. The water imbalance was probably triggered by the sharp drop in root water uptake at the onset of chilling, whereas decreased transpira-

tion occurred later (Figure 2B). The chilling-induced water deficit was reflected in low leaf RWC (Figure 2A).

In figleaf gourd [12] and chilling tolerant maize [3, 4], long-term chilling treatment resulted in an even larger root L_p than unchilled control plants. This overcompensation in root water transport may be a type of cold acclimation in chilling tolerant plants. No overcompensation in osmotic root L_p occurred in Somewake and Wasetoitsu, but a very slight increase in L_p was observed in Somewake after 24 h of chilling (Figure 2C). Insufficient duration (24 h) of low temperature possibly explains this inconsistency with the other species, as the overcompensation in figleaf gourd occurred only after 6 d of 8 °C [12].

Aquaporins, which facilitate water flux across membranes, belong to a highly conserved MIP family, and play an essential role in maintaining water homeostasis under conditions that require adjustments in water flux. At the mRNA level, decreased expression of individual *PIP* genes during chilling was observed in rice [19, 7], maize [6] and *Arabidopsis* [20]. We determined the expression of all the 11 *PIP* genes by real-time RT-PCR using TaqMan-MGB probes, and found that chilling reduced the expression of most *PIP* genes in our experiments (Figure 3), a result consistent with a previous report [7]. However, our data showed wider changes in expression of *PIPs*, explained by the more sensitive method and detailed time scale we used to detect gene expression. The decreased relative expression of 4 *PIP* genes (Figure 4) and the low PIP protein content (Figure 5) were in line with the reduction in osmotic root L_p in Somewake and Wasetoitsu during chilling (Figure 2C). Water channel proteins are known to allow bi-directional water movement along the water potential gradient. The result suggested that lower aquaporin expression and water transport facilitates achieving appropriate water status at low temperature.

Some of our results indicated that water channel activity was not controlled exclusively by PIP expression level or protein amount, and additional mechanisms were involved in this process. Results supporting this include the fact that the relative expression of some *PIP* genes was not always parallel to the changes in root L_p ; that Western blots showed little difference between PIP protein abundance in Wasetoitsu and Somewake during the recovery (Figure 5), even though L_p were clearly different (Figure 2C); and that, the changes in total PIP protein abundance were not always consistent with the transcript expression level of individual *PIP* isoforms as described previously [36]. This idea is also supported by the results in Figure 5 that suggest posttranscriptional actions regulate PIP protein. A very recent review by Luu and Maurel [35] summarized possible mechanisms that may regulate water channel activities under various stress conditions including aquaporin

subcellular localization, phosphorylation, cytosolic pH, reactive oxygen, *etc.* How plants integrate these mechanisms in response to chilling is a challenge for future research.

Several reports suggested that aquaporins were necessary for chilling responses [4, 6], but the roles of aquaporins in chilling tolerance was still elusive. *PIP* genes did not exhibit a uniform expression pattern (Figure 3 and Figure 4), suggesting that PIPs did not respond in a simple uniform way but have more complex roles in influencing plant water balance during chilling. In support of this, the relative expression of *OsPIP1;1*, *OsPIP2;1*, *OsPIP2;3* and *OsPIP2;7* generally decreased during chilling in the two varieties, indicating that these PIPs might not be the key factors for the early stage of rice chilling tolerance. In addition, the chilling tolerant variety, Somewake, had a higher relative expression of *OsPIP1;1* (in shoots and roots), *OsPIP2;1* (in shoots and roots) and *OsPIP2;7* (in shoots) than Wasetoitsu during the recovery, suggesting these PIPs were probably involved in re-establishment of the disturbed water balance. Wasetoitsu, in contrast, exhibited significant increases in the relative expression of *OsPIP2;3* and *OsPIP2;7* in roots. Wasetoitsu was severely injured when subjected to chilling (7 °C) for 24 h (Figure 1), so it seems reasonable to suggest that high expression of *OsPIP2;3* and *OsPIP2;7* in roots might overcompensate for the severe chilling-induced water deficit. Taken together, aquaporins probably play complex and diversified roles in plant chilling tolerance response depending on the aquaporin isoforms, the plant tissues and the stage of chilling duration.

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