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Characterization of Four Plasma Membrane Aquaporins in Tulip Petals: A Putative Homolog is Regulated by Phosphorylation

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We suggested previously that temperature-dependent tulip (*Tulipa gesneriana*) petal movement that is concomitant with water transport is regulated by reversible phosphorylation of an unidentified plasma membrane intrinsic protein (PIP). In this study, four full-length cDNAs of PIPs from tulip petals were identified and cloned. Two PIPs, namely TgPIP1;1 and TgPIP1;2, are members of the PIP1 subfamily, and the remaining two PIPs, namely TgPIP2;1 and TgPIP2;2, belong to the PIP2 subfamily of aquaporins and were named according to the nomenclature of PIP genes in plants. Of these four homologs, only TgPIP2;2 displayed significant water channel activity in the heterologous expression assay using *Xenopus laevis* oocytes. The water channel activity of this functional isoform was abolished by mercury and was affected by inhibitors of protein kinase and protein phosphatase. Using a site-directed mutagenesis approach to substitute several serine residues with alanine, and assessing water channel activity using the methylotrophic yeast *Pichia pastoris* expression assay, we showed that Ser35, Ser116 and Ser274 are the putative phosphorylation sites of TgPIP2;2. Real-time reverse transcription–PCR analysis revealed that the transcript levels of *TgPIP1;1* and *TgPIP1;2* in tulip petals, stems, leaves, bulbs and roots are very low when compared with those of *TgPIP2;1* and *TgPIP2;2*. The transcript level of *TgPIP2;1* is negligible in roots, and *TgPIP2;2* is ubiquitously expressed in all organs with significant transcript levels. From the data reported herein, we suggest that TgPIP2;2 might be modulated by phosphorylation and dephosphorylation for regulating water channel activity, and may play a role in transcellular water transport in all tulip organs.

Keywords: Aquaporin — Phosphorylation — PIP — Tulip — Water channel activity.

Abbreviations: cRNA, complementary RNA; DMSO, dimethylsulfoxide; MBS, modified Barth's solution; OA, okadaic acid; ORF, open reading frame; P_f, osmotic water permeability coefficient; PIP, plasma membrane intrinsic protein; RACE, rapid amplification of cDNA ends; RMS, root-mean-square; RT–PCR, reverse transcription–PCR; TgPIP, *Tulipa gesneriana* PIP; TM, tramsmembrane; UTR, untranslated region

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession numbers AB305614 (TgPIP1;1), AB305615 (TgPIP1;2), AB305616 (TgPIP2;1) and AB305617 (TgPIP2;2).

Introduction

Water transport in plants occurs by apoplastic, symplastic and transmembrane pathways (reviewed by Hachez et al. 2006). Transmembrane water movement is important for changing the stomatal aperture, the rapid elongation of cells and the diurnal regulation of leaf movements (Fleurat-Lessard et al. 1997, Moshelion et al. 2002). These three functions are facilitated by aquaporins, the water channel proteins. The function and regulatory mechanism of plant aquaporins have been well investigated (for reviews, see Johansson et al. 2000, Chaumont et al. 2005, Hachez et al. 2006, Maurel 2007). Based on sequence homology, plant aquaporins are classified into four subclasses: (i) the plasma membrane intrinsic proteins (PIPs) that are localized in the plasma membrane; (ii) the tonoplast intrinsic proteins that are localized in the vacuolar membranes; (iii) the nodulin-26-like intrinsic proteins; and (iv) the small basic intrinsic proteins (reviewed by Maurel 2007). In *Arabidopsis* and maize, there are 13 PIPs that can be divided further into two subfamilies, PIP1s and PIP2s (Chaumont et al. 2001, Johanson et al. 2001). In the *Xenopus laevis* oocyte heterologous expression system, it was demonstrated that PIP1s generally exhibit no or very low water channel activity when compared with PIP2s (Chaumont et al. 2000). The difference in water permeability in members of PIP1s and PIP2s can be attributed to the changes in the conserved amino acid residues of six transmembrane (TM) α-helices and the regions of highly conserved Asn–Pro–Ala (NPA) motifs between the PIP1 and PIP2 isoforms (Chaumont et al. 2001,

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Suga and Maeshima 2004), or the presence of putative phosphorylation site(s) in PIP2s (Johansson et al. 2000). There are several reports on the water channel activity of some aquaporins and their regulation by protein phosphorylation (Maurel et al. 1995, Johansson et al. 2000, Guenther et al. 2003, Daniels and Yeager 2005, Temmei et al. 2005, Tornroth-Horsefield et al. 2006). It has been shown that the water channel activity of the SoPIP2;1 in spinach is regulated by phosphorylation at two serine residues (Johansson et al. 1998, Tornroth-Horsefield et al. 2006).

Tulip petals are closed during the night, begin to open in the late morning, remain open during the daytime and then close in the evening. The opening and closing of petals is temperature dependent and can be reproduced in the dark by changing the temperature from 5 to 20°C for opening and from 20 to 5°C for closing (Azad et al. 2004a). The temperature-dependent tulip petal opening and closing occur concomitantly with water transport and are regulated by reversible phosphorylation of an undefined PIP whose molecular mass is ~31 kDa on SDS–PAGE (Azad et al. 2004a, Azad et al. 2004b). At 20°C, this putative PIP is phosphorylated and this event probably facilitates transcellular water movement in the petals, thereby allowing the flower to open. At 5°C, this PIP is dephosphorylated, water influx is then decreased and, as a result, the flower closes. Together, these data demonstrate the existence of a direct link between phosphorylation of a PIP and a physiological process. However, it has not yet been elucidated which PIP is involved in this process. The present study was undertaken to identify the PIP whose activity may be modulated by phosphorylation. In this report, we isolated and characterized two PIP1s, TgPIP1;1 and TgPIP1;2, and two PIP2s, TgPIP2;1 and TgPIP2;2, from tulip petals, and then analyzed their water channel activity, using the *X. laevis* oocyte heterologous expression system. The effects of mercury and inhibitors of protein kinases and protein phosphatases on the water channel activity were also investigated. Site-directed mutagenesis has been employed in the *Pichia pastoris* expression system (Daniels et al. 2006) to identify the serine residues that are putative phosphorylation sites for the regulation of the water channel activity of TgPIP2;2. In addition, the organ-specific transcript levels of these four homologs were analyzed. In the light of these results, we propose that the TgPIP2;2 homolog may be associated with the temperature-dependent tulip petal opening and closing.

Results

Cloning and characterization of PIP genes from tulip petals

To isolate the full-length cDNA of PIPs, the cDNA was synthesized from total RNA of petals by reverse transcription–PCR (RT–PCR) and then amplified using degenerate primers that were designed from the highly conserved regions of PIPs that are found in other plants. After cloning, sequencing and performing 3′- and 5′-rapid amplification of cDNA ends (RACE), four genes were identified (Fig. 1). They were homologous to other plant PIPs. Based on the deduced amino acid sequences, two PIP1s, which were designated TgPIP1;1 and TgPIP1;2 in accordance with the suggested nomenclature of PIPs in plants (Chaumont et al. 2000, Chaumont et al. 2001), were identified. The remaining two PIPs were PIP2s and were designated TgPIP2;1 and TgPIP2;2. The 1,022 bp cDNA, which encoded TgPIP1;1 (accession No. AB305614), comprised a 55 bp 5′-untranslated region (5′-UTR), an 858 bp open reading frame (ORF) and a 109 bp 3′-UTR, whereas the 1,039 bp cDNA, which encoded TgPIP1;2 (accession No. AB305615), contained a 65 bp 5′-UTR, an 864 bp ORF and a 110 bp 3′-UTR. The 1,088 bp cDNA, which encoded TgPIP2;1 (accession No. AB305616), had a 47 bp 5′-UTR, an 864 bp ORF and a 177 bp 3′-UTR, whereas the 984 bp cDNA, which encoded TgPIP2;2 (accession No. AB305617), contained a 63 bp 5′-UTR, an 843 bp ORF and a 78 bp 3′-UTR. The deduced numbers of amino acid sequences of TgPIP1;1, TgPIP1;2, TgPIP2;1 and TgPIP2;2 were 286, 288, 288 and 281, respectively, with the predicted similar molecular masses of 30,515, 30,613, 30,370 and 29,852 Da, respectively.

The amino acid sequences of the TgPIPs were typical of aquaporins (Fig. 1): six hydrophobic TM α-helices [predicted by SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and the tools of ExPASy (http://kr.expasy.org/tools/)], five loops, two NPA signature motifs in loops B and E, a conserved histidine residue in loop D that is involved in pH sensing (Tournaire-Roux et al. 2003, Luu and Maurel 2005) and putative target serine(s) for phosphorylation (Johansson et al. 1998). Similar to all plant PIPs (Barone et al. 1997), TgPIPs have two conserved regions. One region is located in loop C: G-G-G-A-N-X-X-X-X-G-Y (X stands for any amino acid) and the other is located in loop E: T-G-I/T-NPA-R-S-L/F-G-A-A-I/V-I/V-F/Y-N. Moreover, TgPIPs include a signature sequence, W-I/V-F-W-V-G-P, in the sixth TM α-helix, and this sequence and location are similar to those described in the PIPs of *Arabidopsis*, maize and other plants. As previously described for PIPs (Chaumont et al. 2000), TgPIP1;1 and TgPIP1;2 had longer N-termini and shorter C-termini than TgPIP2;1 and TgPIP2;2 when the deduced amino acid sequences of the four TgPIPs were analyzed in order to predict the topology of their TM α-helices. In loop E, both TgPIP2;1 and TgPIP2;2 had the highly conserved valine residue, which has been proposed as being essential for the water channel activity of PIP2s (Suga and Maeshima 2004, Sakurai et al. 2005). Both

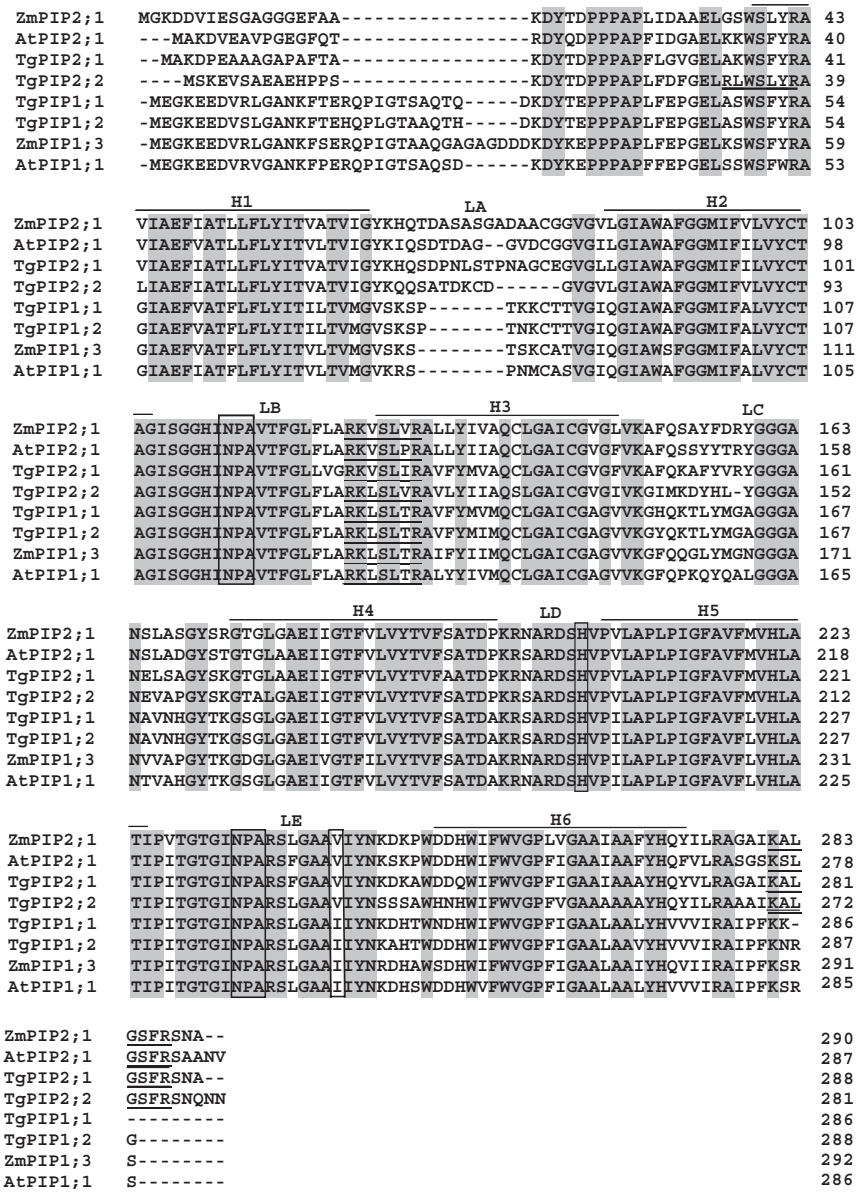

Fig. 1 Alignment of the deduced amino acid sequences of the tulip PIP homologs TgPIP1;1, TgPIP1;2, TgPIP2;1 and TgPIP2;2, and comparison with AtPIP1;1 (accession No. P61837), AtPIP2;1 (P43286), ZmPIP1;3 (Q9AQU5) and ZmPIP2;1 (Q9aTM9). Identical amino acids in all these sequences are shown on a gray background. The bars indicate the six TM α-helices, H1–H6. The connecting loops are labeled LA–LE. The two highly conserved NPA motifs that are located in loops B and E are boxed. The conserved histidine residue that is located in loop D is indicated in the box. Underlining indicates the possible consensus motifs of phosphorylation. The conserved isoleucine and valine residues that are located in loop E in PIP1s and PIP2s, respectively, are in the box.

TgPIP1;1 and TgPIP1;2 have an isoleucine residue that is found in other PIP1s at the corresponding site (Sakurai et al. 2005). A phylogenetic analysis confirmed that TgPIP1;1 and TgPIP1;2 cluster with the PIP1 subfamily, and that TgPIP2;1 and TgPIP2;2 belong to the PIP2 subfamily (Fig. 2). TgPIP1;1 and TgPIP1;2 closely resemble the ZmPIP1s which are found in maize. TgPIP2;1 closely resembles ZmPIP2s, whereas TgPIP2;2 is very homologous to AtPIP2;7 and AtPIP2;8 of *Arabidopsis* and to SoPIP2;1 of spinach.

Functional analysis of TgPIPs in Xenopus oocytes and the effect of phosphorylation

The water channel activity of the four TgPIPs was analyzed using the oocyte swelling assay following a microinjection of complementary RNA (cRNA) into the oocytes. The results were compared with those obtained following a microinjection of water (negative control) and barley PIP, HvPIP2;1 (positive control) (Katsuhara et al. 2002). After hypotonic treatment, the negative control oocytes swelled very slowly and never burst during the 2 min observation period. In contrast, the positive control (data not shown) and TgPIP2;2-expressing oocytes swelled rapidly (Fig. 3A). At least a quarter of the oocytes injected with cRNA of TgPIP2;2 burst within 2 min, and showed a >4-fold increase in the osmotic water permeability coefficient (P_f) when compared with that of the negative control oocytes (Fig. 3B). This results demonstrates that TgPIP2;2 exerts significant water channel activity. Fig. 3B shows that the increased P_f of TgPIP2;2 was completely abolished when

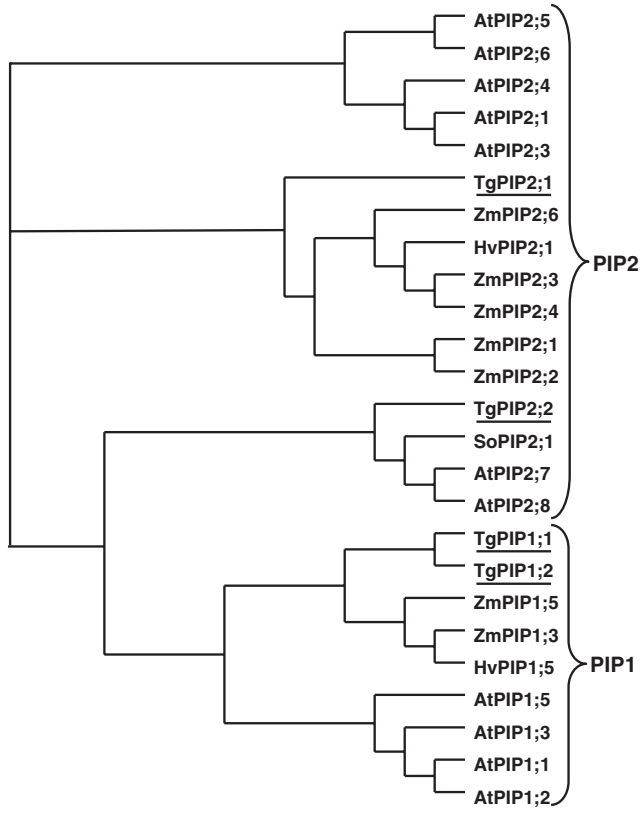

Fig. 2 Phylogenetic tree among some PIPs that includes TgPIPs. The deduced amino acid sequences of PIP-encoding genes were aligned using the ClustalW computer program (http://clustalw.ddbj.nig.ac.jp/top-e.html) and the figure was constructed using the TreeView software (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). TgPIPs (underlined) indicate the tulip PIPs that we identified. The accession numbers of PIPs of *Arabidopsis* (AtPIPs), barley (HvPIPs), maize (ZmPIPs) and spinach (SoPIP2;1, formerly PM28A) have been reported by Johanson et al. (2001), Chaumont et al. (2001), Katsuhara et al. (2002) and Johansson et al. (1996), respectively.

the oocytes were treated with $HgCl_2$, a well known inhibitor of aquaporins. On the other hand, the oocytes that were injected with cRNA of either TgPIP1;1, TgPIP1;2 or TgPIP2;1 showed only 1.2-, 1.2- and 1.4-fold increases in P_f, respectively, when compared with that of the negative control (Fig. 3B).

TgPIP2;2 conserved the putative phosphorylation serine residues in the deduced amino acid sequence (see Fig. 1). Therefore, the effects of inhibitors of protein kinases or protein phosphatases on P_f were investigated (Fig. 3C). K252a, an inhibitor of several protein kinases (Johansson et al. 1998), reduced the increased P_f by about 32%. In contrast, okadaic acid (OA), an inhibitor of protein phosphatases (Azad et al. 2004b), increased the P_f value by about 30%. However, the water channel activity of TgPIP2;1 was not changed by any of the treatments.

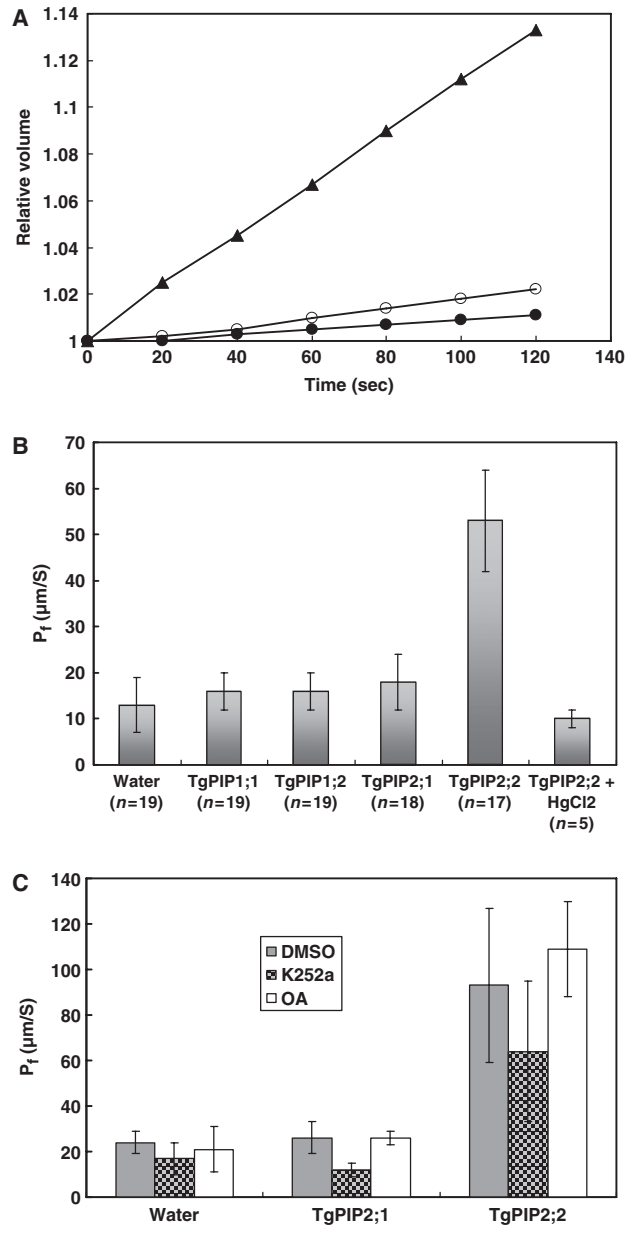

Fig. 3 Water channel activity of TgPIPs. (A) A typical change in the cell volume of *Xenopus* oocytes that express TgPIP2;2. The oocytes were injected with cRNA that encodes TgPIP2;2 and then pre-treated with $HgCl_2$ (filled circles) or without $HgCl_2$ (triangles). The oocytes that were injected with distilled water (open circles) were used as a negative control. The oocytes were monitored every 20 s for as long as 2 min or until they burst, as seen in the TgPIP2;2-expressing oocytes that were not exposed to $HgCl_2$. (B) Change in P_f of oocytes that were injected with water or with one of the four TgPIP cRNAs. For mercury inhibition, the oocytes were pre-treated with $HgCl_2$. (C) Effects of inhibitors of protein kinases and protein phosphatases on P_f of TgPIP2;2-expressing oocytes. Oocytes were pre-treated with or without inhibitors before their transfer to hypotonic MBS. The P_f values were expressed as means ± SEM and the number of replicates (n) is indicated. Experiments in A and C were independent, using different batches of oocytes, and resulted in some discrepancy of P_f in control conditions.

Conserved serine residues in TgPIP2;2 are putative phosphorylation sites for the modulation of water channel activity

The putative PIP previously identified in the petal and stem of tulip is phosphorylated at serine residue(s) (Azad et al. 2004a). Therefore, in the present study, we selected five serine residues located on the cytoplasmic face of TgPIP2;2. Some of these serine residues are known to be phosphorylated in homologs from other plants, or are located in the consensus motifs for a number of protein kinases (Johansson et al. 1998, Tornroth-Horsefield et al. 2006). Site-directed mutagenesis was used to substitute these serine residues with alanine, and the resulting mutants were designated S35A, S116A, S188A, S274A and S277A. The mutated TgPIP2;2-G_3-H_6 constructs were expressed in *P. pastoris* (Daniels et al. 2006), and water channel activity assays were performed (Materials and Methods). As shown in Fig. 4A, the water channel activity of S188A and S277A was indistinguishable from that of spheroplasts that contained wild-type TgPIP2;2-G_3-H_6. However, the water channel activity of S35A and S116A was significantly reduced and that of S274A was slightly reduced compared with that of the wild type. The expression levels of all the mutant and wild-type proteins were almost identical when analyzed by Western immunoblotting using an anti-histidine (C-terminal) monoclonal antibody (Invitrogen, Carlsbad, CA, USA) as the primary antibody and avidin–peroxidase conjugate (Sigma, St Louis, MO, USA) as the secondary antibody (data not shown). S35A, S116A, S274A and the wild-type protein demonstrated almost the same water channel activity when the spheroplasts were treated with K252a (Fig. 4B). On the other hand, OA activated the water channel activity of the three mutants, although the degree of activation was mutant dependent. These results indicate that the Ser35, Ser116 and Ser274 residues are possible phosphorylation sites in TgPIP2;2. To further our understanding, we constructed and overexpressed three double and one triple mutant, designated S35A/S116A, S35A/S274A, S116A/S274A and S35A/S116A/S274A, and analyzed their water channel activity. Fig. 4C shows that the spheroplasts prepared from the double mutants and treated with K252a exhibited an almost identical water channel activity. Although treatment with K252a did not affect the water channel activity of the S35A/S116A mutant spheroplasts, OA enhanced their water channel activity compared with that of the untreated spheroplasts (Fig. 4C). This result suggests that the phosphorylation of Ser274 is enhanced in the presence of OA, and, in turn, the water channel activity is increased. The S35A/S274A and S116A/S274A mutants showed a higher water channel activity than the S35A/S116A mutant, and OA increased the water channel activity, which supports the notion that phosphorylation also occurs at both Ser35 and Ser116.

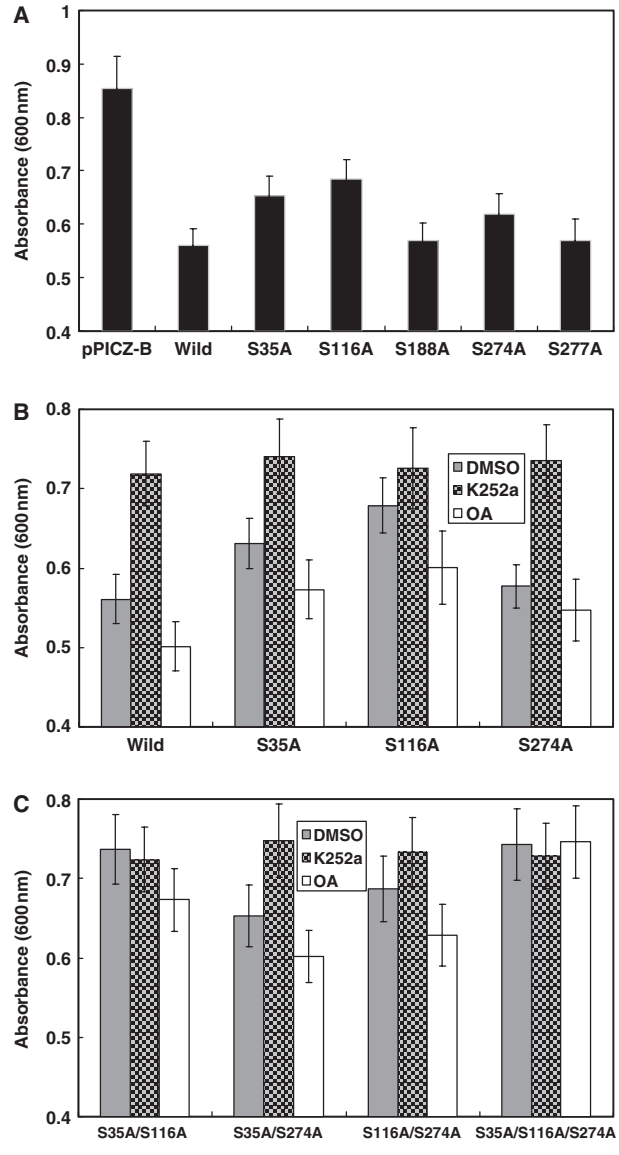

Fig. 4 Water channel activity of the wild-type and mutant forms of TgPIP2;2. (A) Water channel activity of the wild-type (wild) and single mutants of TgPIP2;2 with no inhibitor treatment. Effects of inhibitors of protein kinases and protein phosphatases on the water channel activity of the wild type and single mutants (B), and double and triple mutants (C) of TgPIP2;2. Spheroplasts were prepared from *P. pastoris* transformed with the empty vector (pPICZ-B) and *P. pastoris* expressing either wild-type or mutant TgPIP2;2. Spheroplasts used in B and C were pre-treated with inhibitors or with DMSO alone before hypo-osmotic shock. The starting value for the OD_{600} of each transformant was set to 1.0. The OD_{600} at $t = 15$ s was recorded, and the data shown are the mean ± SEM of three independent experiments.

Neither K252a nor OA had any effect on the triple mutant S35A/S116A/S274A. Together, these data suggest that Ser35, Ser116 and Ser274 are putative phosphorylation sites of TgPIP2;2.

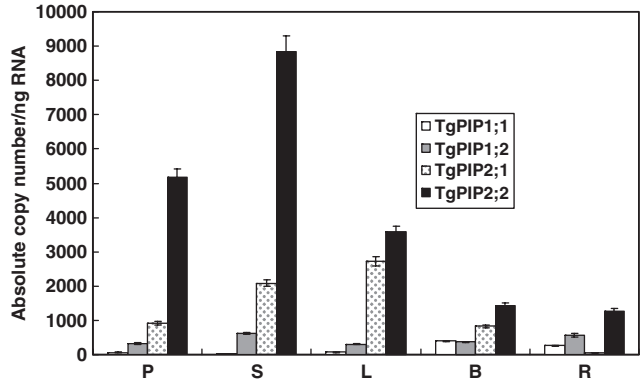

Fig. 5 Expression profile of the four *TgPIP* genes in the different organs of the tulip. Total RNA was isolated from petals (P), stems (S), leaves (L), bulbs (B) and roots (R) of the tulip when the flower was 2 d old. Total RNA (5 μg) was isolated from each organ and then used to synthesize first-strand cDNA. Real-time RT–PCR was carried out. The data are expressed as the mean ± SEM of three independent experiments for each organ.

Organ-specific expression profiles of TgPIPs

The transcript levels of the four *TgPIP* genes were analyzed using real-time RT–PCR. This technique is a highly sensitive and specific method to quantify precisely the levels of expression of transcripts of members of a homogenous gene family whose percentage identity is high and whose expression is relatively low (Jang et al. 2004, Maurel 2007). Using this approach, we demonstrated that the transcript levels of the four *TgPIP* genes, which were expressed as copy number per nanogram of total RNA in petals, stems, leaves, bulbs and roots of the tulip, differed from gene to gene in all the organs (Fig. 5). The expression levels of both *TgPIP1* genes in all organs were very low when compared with those of the two *TgPIP2* genes. The level of expression of the *TgPIP1;1* gene was negligible in petals, stems and leaves. However, its expression was slightly elevated in bulbs and roots, in which the transcript levels were 395 ± 16 (mean ± SEM) and 263 ± 15 copies ng^{-1} RNA, respectively. The transcript levels of *TgPIP1;2* in all organs, except the bulbs, were more abundant than those of *TgPIP1;1*. Its transcript levels in the petals, leaves and bulbs were almost identical, and the numbers of copies in these organs were from 300 to 380 copies ng^{-1} RNA. In the stems and roots, the expression levels were approximately 2-fold higher than that found in petals. The expression level of *TgPIP2;1* in roots was almost below the limit of detection. It was expressed abundantly in stems and leaves, and the highest level was found in the leaves. Its expression profiles in petals and bulbs were similar, and the transcript levels were 800–900 copies ng^{-1} RNA. *TgPIP2;2* was expressed ubiquitously in all organs, and there were significantly lower transcript levels found only in the bulbs and roots (1,200–1,500 copies ng^{-1} RNA). Compared with its level of expression in roots, it was more abundantly expressed in petals, stems and leaves, and the increases in the transcript levels were 4-, 7- and 3-fold, respectively.

Discussion

Homology model and functional relationship in PIPs

In the present study, we cloned four *TgPIP* genes, which are highly homologous to known plant *PIP* genes. Homology modeling analysis may be informative for the functional characterization of plant aquaporins (Wallace and Roberts 2004). Recently three-dimensional (3D) crystals of SoPIP2;1 have been visualized by X-ray crystallography (Tornroth-Horsefield et al. 2006). The homology structural models of the four TgPIPs, whose construction was based on the 3D structure of SoPIP2;1, show two pore constrictions with the water channel (Fig. 6). One such constriction is formed by the reorientation of the NPA motifs in loops B and E, to form the aqueous pore. The second constriction, which is made up of aromatic and arginine residues (Ar/R), is located on the extracellular mouth of the pore. It is formed by four residues that include aromatic residues and an arginine residue, and has been described in all PIPs of *Arabidopsis* (Wallace and Roberts 2004). This constriction functions as a selectivity filter, due to steric effects, and the arginine residue acts as a site for electrostatic repulsion of protons (Fujiyoshi et al. 2002). However, the computer-generated models predicted that the four TgPIPs will have a slightly different pore configuration (Fig. 6C–F). While the results of homology modeling will give the first clue about the expected transport properties of plant PIPs, the results of the functional analysis, which was done in *Xenopus* oocytes, showed that of the four TgPIPs, only TgPIP2;2 had water channel activity (Fig. 3A, B). Thus, the combined use of homology modeling and assessment of water channel activity indicates the necessity to establish selectivity profiles of PIPs.

Aquaporin phosphorylation modulates water channel activity

Analysis of the water channel activity of some plant aquaporins in oocytes has demonstrated that phosphorylation can enhance the P_f (Maurel et al. 1995, Johansson et al. 1998, Guenther et al. 2003). In the present study, treating TgPIP2;2-expressing oocytes with K252a and OA changed the P_f of the oocytes, and this result demonstrates the effect of phosphorylation on its water channel activity. The mechanism of action of K252a is the inhibition of phosphorylation of this homolog. In contrast, OA may increase or render its phosphorylation status stagnant by preventing protein phosphatases from using phosphorylated TgPIP2;2 as a substrate. Thus, K252a and OA may have decreased and increased the P_f of TgPIP2;2-expressing oocytes, respectively. Similar effects of K252a and OA on the P_f of

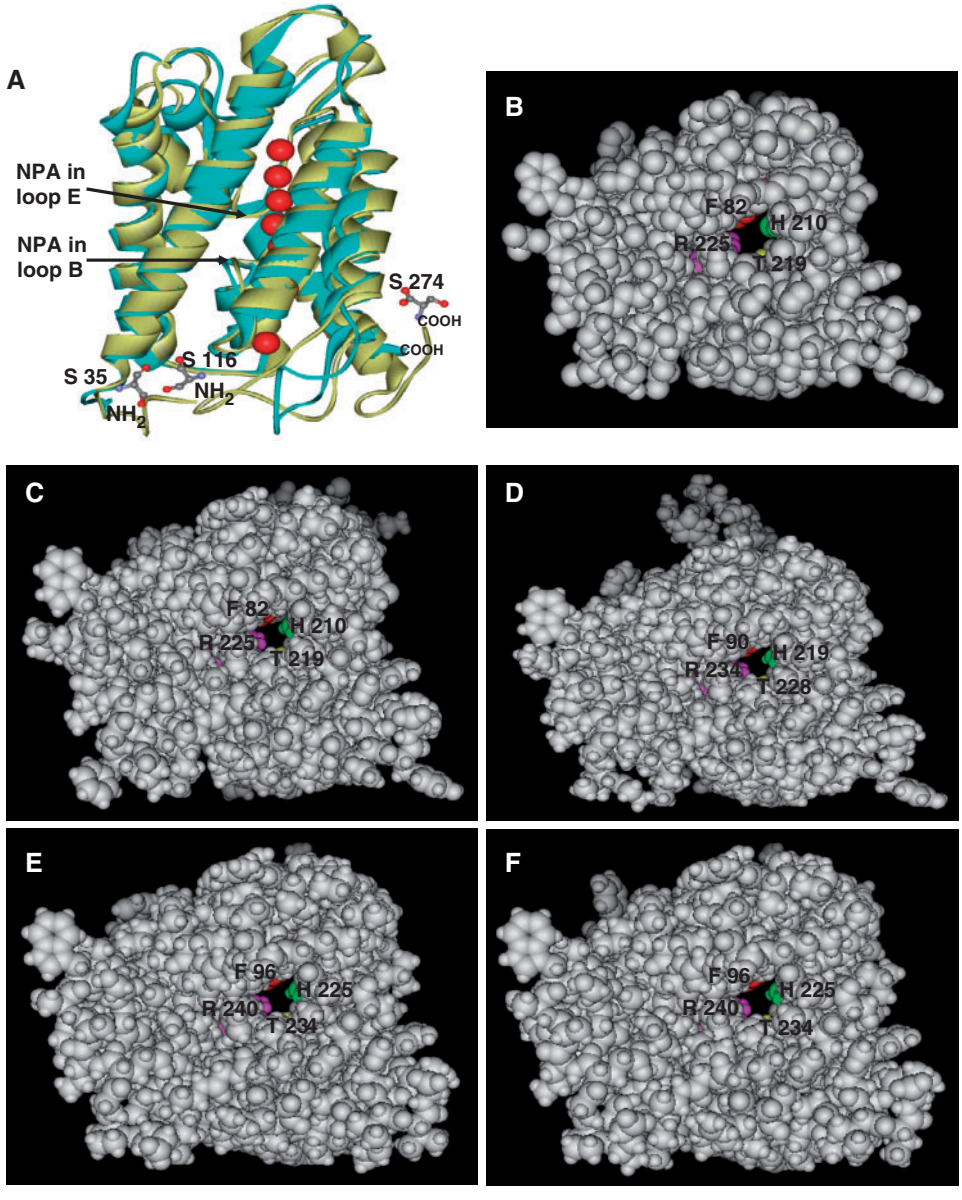

Fig. 6 Homology modeling of TgPIPs. (A) The open conformation (blue) and the closed conformation (yellow) of TgPIP2;2 (side view), and water molecules (red circles) are superimposed. The regions of the NPA motifs in loops B and E are indicated by arrows. The three serine residues, S35, S116 and S274, as the putative phosphorylation sites are shown by the balls and sticks. (B) The open conformation of SoPIP2;1 showing the pore (black hole) of the water channel and the Ar/R constriction region, as seen from the extracellular aspect along the axis of the pore. (C–F) Structural models of TgPIP2;2, TgPIP2;1, TgPIP1;1 and TgPIP1;2, respectively. Their construction was based on the open conformation of SoPIP2;1, as shown in (B), and depicts the configuration of the water channel (black hole). All atoms are shown as van der Waals space-filling spheres (B–F). Amino acid residues of the Ar/R constriction region are depicted in color, and each residue is labeled by a single letter: F (red) in H2, as described in Fig. 1, H (green) in H5, T (yellow) in LE, and R (pink) in LE.

spinach plasma membrane aquaporin were reported (Johansson et al. 1996, Johansson et al. 1998). If this is indeed so, we suggest that the water channel activity of TgPIP2;2 can be modulated through phosphorylation and dephosphorylation.

One putative phosphorylation site is a perfectly conserved serine residue that is found in loop B of all PIPs. In SoPIP2;1, this highly conserved serine residue (Ser115) and a C-terminal serine residue (Ser274) are phosphorylated, which results in gating of its water channel activity (Johansson et al. 1998, Tornroth-Horsefield et al. 2006). However, phosphorylation of N-terminal serine residues has so far been reported in TIPs (Maurel et al. 1995, Daniels and Yeager 2005), but not in PIPs.

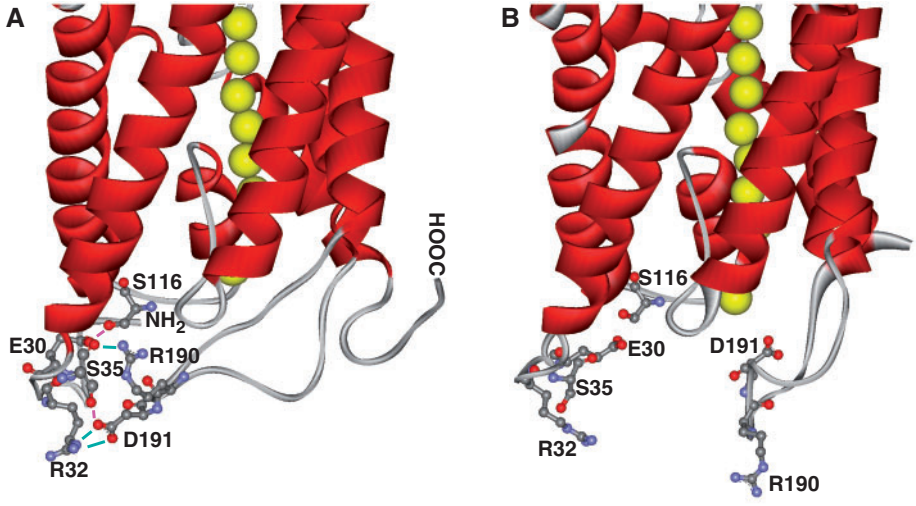

Fig. 7 3D homology models of TgPIP2;2 showing the interactions of S35 and S116 with other amino acids. The models were constructed based on the closed (A) and open (B) conformations of the crystal structure of the spinach PIP, SoPIP2;1. Interactions of S35 and S116 through hydrogen bonds (pink lines) and electrostatic interactions (blue lines) are shown in A. The model of the open conformation (B) shows no interaction of S35 and S116 with the amino acids as shown in A, and loop D is released to open the channel. Water molecules (yellow circles) superimposed in both models face the obstacle presented by loop D in the closed conformation (A) but not in the open conformation (B).

The present study reveals that the phosphorylation of Ser35, Ser116 and Ser274 influences the water channel activity of TgPIP2;2 in the spheroplast bursting assay (Fig. 4), and thus reports, perhaps for the first time, that some PIPs may also be phosphorylated in the N-terminal region. Ser35 in TgPIP2;2 is highly conserved in 12 of the 13 *Arabidopsis* PIPs (with the exception of AtPIP2;4), and in other plant PIPs. Interestingly, Ser35 in TgPIP2;2 exists in a conserved motif (R-x-x-S-x-x-R), as does Ser116 in loop B, and this motif acts as a consensus sequence for a number of protein kinases (Johansson et al. 1998, Daniels and Yeager 2005). Recently it has been shown that Ser115 and Ser274 in SoPIP2;1 are phosphorylated by two different kinases (Sjövall-Larsen et al. 2006).

Elucidation of the crystal structure of SoPIP2;1 has allowed the proposal of mechanisms by which the phosphorylation might modulate the water channel (Tornroth-Horsefield et al. 2006). Phosphorylation of Ser116 and Ser274 in TgPIP2;2 may regulate the TgPIP2;2 channel in a similar manner to that which has been proposed in SoPIP2;1 (Tornroth-Horsefield et al. 2006). Phosphorylation of Ser116 in loop B may release loop D and cause the water channel to open, by disrupting a possible anchoring network between loop D and the N-terminal tail. The anchoring network is formed by ionic and hydrogen bond interactions between the acidic residue Glu30 and the phosphorylatable Ser116 (Fig. 7A, B) (Tornroth-Horsefield et al. 2006). Phosphorylation of Ser274 may promote conformational changes in helix 5 and loop D by disrupting the hydrogen bonding between this residue and those within loop D of an adjacent monomer (Tornroth-Horsefield et al. 2006). The 3D homology model of TgPIP2;2 (Fig. 7A) in the closed conformation of SoPIP2;1 reveals that Ser35 is near to Arg190 and Asp191 in loop D. An anchoring network may exist between loop D and the N-terminal tail, because Ser35 interacts with Asp191 through hydrogen bonding and with Arg190 by an electrostatic interaction, and Arg32 forms electrostatic interactions with Asp191 (Fig. 7A). Phosphorylation of Ser35 may interrupt this anchoring network to release loop D to some extent and open the water channel (Fig. 7B). Alternatively, this serine residue may have a role in the interaction with the neighboring monomer. Its phosphorylation may disrupt this interaction, thus imparting a conformational change to the monomer that, in turn, modulates the water channel to affect the water channel activity. However, further research is necessary to elucidate the exact molecular mechanism and to understand the physiological regulation of TgPIP2;2 gating.

Expression of PIPs and physiological functions

Organ-specific expression of many PIPs is related very closely to the physiological function of each organ, and the expression of some PIPs depends on the stage of development of the plant or its organs, hormone treatment and the presence of abiotic stresses (Jang et al. 2004, Alexandersson et al. 2005, Sakurai et al. 2005). The transcript levels of *TgPIP1;1* and *TgPIP1;2* are very low, as is evident in some PIPs of other plants (Jang et al. 2004, Guo et al. 2006).

However, the transcript levels of the two *TgPIP1* genes differ between organs and may change in response to abiotic stresses or hormone treatments (Jang et al. 2004, Guo et al. 2006). Therefore, the function of TgPIP1s may be similar to that of other PIP1s, in that they may be involved either in water transport (Siefritz et al. 2002) or in the transport of other molecules, such as glycerol, urea and NH_4^+/NH_3 (Biela et al. 1999, Kaldenhoff and Fischer 2006). Despite TgPIP2;1 showing no water channel activity in oocytes, we found that its transcript levels were remarkable, especially in stems and leaves (Fig. 5). This result leads us to postulate that this homolog is not expressed or needs a physical interaction or co-expression with other TgPIPs in the oocyte expression system (Fetter et al. 2004). This homolog may also have a role in water transport in plant tissues, or have other functions that are similar to those described for HvPIP2;1, such as CO_2 conductance or CO_2 assimilation (Hanba et al. 2004, Katsuhara and Hanba 2008). However, further research is necessary using either the *Xenopus* oocytes or another expression system to elucidate its function. *TgPIP2;2* had the highest number of transcripts of the four *TgPIP* genes in all organs (Fig. 5). This agrees with the results of our previously published study, in which the putative PIP could be detected immunologically in all these organs when using 'PPAPLFEPGELSSWS' polypeptide to raise the polyclonal antibody (Azad et al. 2004a). The ubiquitous expression of *TgPIP2;2* may contribute to the water homeostasis of the whole plant (Kjellbom et al. 1999, Johansson et al. 2000) by regulating transcellular water movement (Ohshima et al. 2001) in all of the organs.

Previously it was shown that the content of the putative PIP in petals and stems was unchanged during petal opening and closing, whereas it became dephosphorylated in both organs during the closing state of petals (Azad et al. 2004a). The expression levels of both *TgPIP1* genes were negligible, especially in the petals (Fig. 5). In addition, the transcript levels of *TgPIP2;1* were about 6- and almost 5-fold lower than those of *TgPIP2;2* in the petals and stems, respectively. Furthermore, the negligible expression level of *TgPIP2;1* in roots supports the notion that the PIP, which we have detected previously (Azad et al. 2004a), is probably TgPIP2;2. Moreover, the transcript levels of *TgPIP2;2* in petals ($5{,}170 \pm 247$ copies ng^{-1} RNA) and stems ($8{,}855 \pm 440$ copies ng^{-1} RNA) did not change significantly during petal opening during the day and closing at night. Collectively, these results indicate that *TgPIP2;2* is expressed constitutively and undergoes post-translational modification, possibly by phosphorylation. The results of our previous studies (Azad et al. 2004a, Azad et al. 2004b) and a summary of the principal findings of TgPIP2;2 that are reported herein, namely (i) its water channel activity in *Xenopus* oocytes and *P. pastoris*; (i) the effects of its phosphorylation on the water channel activity; and (iii) its ubiquitous and high expression patterns, lead us to make the following conclusion. TgPIP2;2 may be the homolog or might be the most likely homolog of the TgPIPs to be modulated by reversible phosphorylation for regulating transcellular water transport that is required for mediating the temperature-dependent opening and closing movements in tulip petals. However, further research is necessary to find direct evidence in situ that TgPIP2;2 is the major PIP in tulip petals, and that it is regulated by phosphorylation and/or dephosphorylation.

Materials and Methods

Plant materials and experimental design

Tulips (*Tulipa gesneriana* L. cv. Ile de France) were grown in plastic boxes that contained horticultural soil and were watered periodically. When the flower was 2 d old, the petals, leaves, stem, bulb and roots of a whole plant were collected for total RNA isolation that was used to investigate the organ-specific transcript levels of PIP genes. Samples of at least five different plants, whose age was the same as that of the flowers, were preserved for replicate experiments. All samples were snap-frozen in liquid nitrogen immediately after collection, stored at –80°C and used within 2 weeks for RNA isolation. Total RNA extracted from petals of 2-day-old flowers was used to synthesize first-strand cDNA for full-length cDNA isolation.

RNA extraction and cDNA synthesis

Total RNA was isolated using ISOGEN reagent (Nippongene, Tokyo, Japan) and then purified using RNeasy kits and RNeasy minicolumns (Qiagen, Valencia, CA, USA), in accordance with the manufacturer's instructions. Total RNA (5 μg) was converted to cDNA using SuperScript III RNase H^- reverse transcriptase (Invitrogen), using a mixture of oligo$(dT)_{20}$ as primers, in accordance with the manufacturer's instructions.

Isolation of gene sequences encoding PIPs

Degenerate primers (sense primer, 5′-TTACTGTACCGCC GGTATCTC-3′, antisense primer, 5′-CCGGGTTGATACCGG TTCCGG-3′) were designed from the conserved sequences of PIP genes of *Arabidopsis* and other plant species. PCR was performed with the cDNA using *Taq* polymerase (TAKARA BIO INC., Shiga, Japan). After the first denaturation at 94°C for 2 min, the following steps were performed for 30 cycles: denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 5 min. The amplified products were subjected to electrophoresis, purified with a MagExtractor DNA fragment purification kit (Toyobo, Osaka, Japan), cloned into a pT7 blue T-vector (Novagen, Darmstadt, Germany) and sequenced with an ABI Prism™ 3100-Avant Genetic Analyzer (Applied Biosystems, Foster Ciry, CA, USA). The sequenced data were analyzed using the following computer programs: Chromas version 2.23, Genetyx_SV_RC_version 7, BLAST and FASTA. From the sequence information, forward gene-specific primers were designed to be used in 3′-RACE PCR. The cDNA for 3′- and 5′-RACE PCR was synthesized from RNA of petals using the GeneRacer kit (Invitrogen), in accordance with the manufacturer's instructions. 3′-RACE PCR was performed using the forward gene-specific primers (Table 1) and the GeneRacer 3′ primer (Invitrogen), in

Table 1 List of primers used for RACEs and ORFs

Primers	Sequence	Use
3R-TgPIP1;1	5′-GTTCATCTGGCGACCATCCCCATCA-3′	PIP1;1 3′ RACE
3R-TgPIP1;2	5′-GTCCATCTGGCGACCATCCCCATCA-3′	PIP1;2 3′ RACE
3R-TgPIP2;1	5′-GCATTTCAGAAGGCCTTCTACGTCAGG-3′	PIP2;1 3′ RACE
3R-TgPIP2;2	5′-GTGAAGGGGATCATGAAGGACTACCAT-3′	PIP2;2 3′ RACE
5R-TgPIP1;1	5′-CAGGAAATCCCGTAGCATGTGGACA-3′	PIP1;1 5′ RACE
5R-TgPIP1;2	5′-GATCCTCAACACTACAAGGCAGAG-3′	PIP1;2 5′ RACE
5R-TgPIP2;1	5′-GAACACTGCCCACTAGCACCCATGAGAA-3′	PIP2;1 5′ RACE
5R-TgPIP2;2	5′-CACATGACCAACCATCCCCATCACACA-3′	PIP2;2 5′ RACE
GeneRacer 3′	5′-GCTGTCAACGATACGCTACGTAACG-3′	3′ RACE
GeneRacer 5′	5′-CGACTGGAGCACGAGGACACTGA-3′	5′ RACE
OTgPIP1;1+	5′-CTGACATGGAAGATCTCTGAAGGAGTA-3′	TgPIP1;1 ORF
OTgPIP1;1–	5′-CCGTAGCATGAGATCTTGGACACTCA-3′	TgPIP1;1 ORF
OTgPIP1;2+	5′-CACGAGGACAAGATCTCTGACATGGA-3′	TgPIP1;2 ORF
OTgPIP1;2–	5′-CACTACAAGGAGATCTCAGAGAAATGC-3′	TgPIP1;2 ORF
OTgPIP2;1+	5′-CACTGACATGGAGATCTACTGAAGGAG-3′	TgPIP2;1 ORF
OTgPIP2;1–	5′-CCCACTAGCAAGATCTCCCATGAGAA-3′	TgPIP2;1 ORF
OTgPIP2;2+	5′-GACTGGAGCACAGATCTGAGGACACTG-3′	TgPIP2;2 ORF
OTgPIP2;2–	5′-GACCAACCATCCAGATCTCCATCACACA-3′	TgPIP2;2 ORF

+, forward; –, reverse.
*Bgl*II sites are underlined.

accordance with the manufacturer's protocol. From the sequence analysis of 3′-RACE clones, reverse gene-specific primers (Table 1) were designed from the 3′-UTRs. To obtain the full-length cDNA, the 5′-RACE PCR was done using reverse gene-specific primers and the GeneRacer 5′-primer (Invitrogen), in accordance with the manufacturer's instruction. The PCR products were cloned and sequenced.

Water channel activity assay in X. laevis oocytes

The entire ORF of each TgPIP, which was amplified using forward and reverse primers (Table 1), was digested with *Bgl*II and then cloned into the *Bgl*II site of a pXBG-ev1 vector, which is a pSP64T-derived pBluescript-type vector into which *Xenopus* β-globin 5′- and 3′-UTRs have been inserted (Preston et al. 1992). The constructed plasmids were linearized and the cRNAs with a cap analog [$m^7G(5')ppp(5')G$] were synthesized using the mMESSAGE mMACHINE in vitro transcription kit (Ambion, Austin, TX, USA), in accordance with the manufacturer's instructions. The water channel activity assay in which cRNA was microinjected into the oocytes was done according to a previously described method (Katsuhara et al. 2002). To investigate the effect of mercury inhibition, oocytes were pre-treated for 10 min with modified Barth's solution (MBS) [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Tris–HCl (pH 7.6), 0.3 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.41 mM $CaCl_2{\cdot}4H_2O$, 0.82 mM $MgSO_4{\cdot}7H_2O$, 10 μg ml^{-1} sodium penicillin and 10 μg ml^{-1} streptomycin sulfate] that contained 1 mM $HgCl_2$, before transferring them into the hypotonic MBS (Katsuhara et al. 2002) containing the identical concentration of $HgCl_2$, in order to perform the osmotic swelling assay. For investigation of phosphorylation and/or desphosphorylation, oocytes were pre-treated with MBS that was supplemented with 1 μM K252a (Calbiochem, San Diego, CA, USA) or 5 μM OA (Calbiochem) dissolved in 0.1% (final concentration) dimethylsulfoxide (DMSO) for 10 min before carrying out the osmotic swelling assay.

Preparation of construct and overexpression of TgPIP2;2-G_3-H_6 in P. pastoris

The construct encoding TgPIP2;2-G_3-H_6, in which a $(Gly)_3$-$(His)_6$ tag was introduced before the stop codon of TgPIP2;2, was synthesized, transformed into *P. pastoris* strain KM71H (Invitrogen) and overexpressed as described previously (Daniels and Yeager 2005), with the following modifications. Prior to yeast transformation by homologous recombination, the TgPIP2;2-G_3-H_6/pPICZ-B plasmids (pPICZ-B is the expression vector; Invitrogen) were linearized with *Pme*I (New England BioLabs, Ipswich, MA, USA). Empty pPICZ-B was also transformed into the same *P. pastoris* strain. Transformants were selected by plating on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar) containing zeocin at a concentration of 100 μg ml^{-1}. Integration of the gene of interest into the *P. pastoris* genome was determined by PCR using genomic DNA isolated from the transformants, according to the manufacturer's instructions (Invitrogen). Yeast culture, induction of protein expression and harvesting of cells were performed according to Daniels and Yeager (2005).

Spheroplasts preparation and the water channel activity assay

The *P. pastoris* cultures induced for recombinant protein expression were centrifuged at 1,500 *g* for 5 min to harvest the cells. The cell pellets were resuspended in 5 ml of BMMY medium [1% yeast extract, 2% peptone, 1.34% yeast nitrogen base (YNB), 100 mM potassium phosphate (pH 6.0), 4×10^{-5}% biotin and 1% methanol] supplemented with 1.0 M sorbitol per gram of pelleted cells. Following incubation at 30°C for 1 h with shaking at 250 r.p.m., cells were pelleted by centrifugation at 1,500*g* for

Table 2 Gene-specific primer pairs used in the real-time RT–PCR experiments

Gene	Primer	Sequence
TgPIP1;1	Forward	5′-TAGGAATTGAAGTATACATGCAAGGC-3′
TgPIP1;1	Reverse	5′-CAGGAAATCCCGTAGCATGTGGAC-3′
TgPIP1;2	Forward	5′-GGCTGAGCTGCATGATTGGTTGTA-3′
TgPIP1;2	Reverse	5′-GATCCTCAACACTACAAGGCAGAG-3′
TgPIP2;1	Forward	5′-CGTAATCTGCTCGCTCGCCTGTCA-3′
TgPIP2;1	Reverse	5′-CTAGCACCCATGAGAACTGCCAAAC-3′
TgPIP2;2	Forward	5′-GCAGCAGTGCATGGCATAACCATTG-3′
TgPIP2;2	Reverse	5′-CAACCATCCCCATCACACAACTTGA-3′

5 min and then resuspended in 5 ml of 100 mM phosphate buffer, pH 7.5 containing 1.0 M sorbitol g^{-1} of pelleted cells. Spheroplasts were generated by adding Zymolase powder (1,000 U g^{-1} pelleted cells) to the cell suspension, followed by incubation at 30°C for 2 h, with gentle shaking. An aliquot (100 µl) of spheroplasts was then transferred immediately to a spectrophotometer cuvette (1.0 cm path length). Spheroplasts were osmotically shocked by 10-fold dilution with 0.25 M sorbitol in 100 mM phosphate buffer, pH 7.5. The optical absorbance at 600 nm (OD_{600}) was monitored from $t = 0$ to the indicated subsequent time points using a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Kyoto, Japan). The decrease in OD_{600} following the hypo-osmotic shock is defined as the water channel activity. To investigate phosphorylation and/or desphosphorylation, the spheroplast suspension was pre-treated with 2 µM K252a or 5 µM OA dissolved in 0.1% DMSO (final concentration) for 10 min before hypo-osmotic shock. During all the treatments, the molarity of sorbitol (1.0 M) and the pH (7.5) were carefully maintained.

Site-directed mutagenesis of TgPIP2;2

Site-directed mutagenesis of TgPIP2;2-G_3-H_6/pPICZ-B was performed using the GeneTailor Site-Directed Mutagenesis System (Invitrogen) following the manufacturer's instructions. The forward and reverse primers used for this purpose were designed according to the instructions for the system. Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen) was used for the PCR (one cycle of 94°C for 2 min; 22 cycles of 94°C for 30 s, 55°C for 30 s, 68°C for 5 min; and one cycle of 68°C for 10 min). The mutated constructs were introduced into One-Shot MAX Efficiency DH5α^{TM}-T1^{R} *Escherichia coli* competent cells (Invitrogen). The mutagenesis was confirmed by DNA sequencing. The mutated plasmid was transformed into *P. pastoris* strain KM71H as described above.

Real-time RT–PCR and quantification of transcript copy numbers of TgPIP genes

Real-time RT–PCR was performed in a Thermal Cycler Dice^{TM} Real Time System (TAKARA SHUZO CO. LTD., Kyoto, Japan) using SYBR Green 1 SYBR Premix Ex Taq (TAKARA BIO INC.). PCR was done in a 25 µl reaction mixture (final volume) that contained 2 µl of cDNA (1 : 10 dilution and corresponding to ~50 ng of RNA), 200 nM of each gene-specific primer (Table 2) and 12.5 µl of 2× SYBR Green 1 SYBR Premix Ex Taq. For the accurate amplification of each specific target gene, the gene-specific primer set was carefully designed from the 3′-UTR of each gene, except for the forward primer for *TgPIP2;2*. The forward primer for *TgPIP2;2* was designed from the connecting region (SSSAWHNH, see Fig. 1) of the putative loop E (LE) and TM6 α-helix (H6), in which the least identity was found among the four TgPIP homologs. Every PCR was carried out in four parallel independent experiments, in accordance with the manufacturer's instructions. The negative control and the presence of contaminating genomic DNA were carefully monitored according to the method described by Jang et al. (2004). To determine the absolute transcript copy number of a specific *TgPIP* gene that was present in the total RNA sample, a plasmid that contained the target *TgPIP* as a reference matrix was used. Triplicate replicas of four dilutions of the plasmid DNA were tested under the same conditions used for the cDNA samples. The number of genes that were present in each of the four diluted samples was determined by the size and mass of the DNA, which could be used as a reference to calculate the copy number of each *TgPIP* gene that was present in the total RNA sample.

Homology modeling of TgPIPs

Each homology model was constructed using the Molecular Operating Environment software (MOE 2006.08; Chemical Computing Group, Canada). TgPIP sequences were aligned with the open conformation [Protein Data Bank (PDB) code, 2B5F] or the closed conformation (PDB code, 1z98) of the SoPIP2;1, respectively, using MOE's multiple sequence and structural alignment algorithm with the structural alignment tool and the BLOSUM62 substitution matrix. The alignment of the PIP sequences was based on the sequence, as well as the structural homology to the SoPIP2;1 experimental structure. Building 3D structures was performed using the MOE homology program, and was based on a segment matching procedure and a best intermediate algorithm, with the option to refine each individual structure enabled. A database of 10 structures, each of which was individually refined to a root-mean-square (RMS) gradient of 1 Å, was generated and the best one was chosen as the crude template for further refinement. The stereochemical quality of the models was assessed by structural analysis using the Protein Report Function of the MOE Protein Structure Evaluation, which searches for disallowed bond angles, bond lengths and side chain rotamers. Those models were then subjected to energy minimization until the RMS gradient became 0.01. The model that best matched these criteria was selected for further use in all structural analyses.

Statistics for data analysis

The data were analyzed using a Student's *t*-test. $P < 0.05$ was considered to be statistically significant. Data are presented as the mean ± SEM.

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