Heterologous Expression of Tulip Petal Plasma Membrane Aquaporins in *Pichia pastoris* for Water Channel Analysis[∇]

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Water channels formed by aquaporins (AQPs) play an important role in the control of water homeostasis in individual cells and in multicellular organisms. Plasma membrane intrinsic proteins (PIPs) constitute a subclass of plant AQPs. TgPIP2;1 and TgPIP2;2 from tulip petals are members of the PIP family. In this study, we overexpressed TgPIP2;1 and TgPIP2;2 in *Pichia pastoris* and monitored their water channel activity (WCA) either by an in vivo spheroplast-bursting assay performed after hypo-osmotic shock or by growth assay. Osmolarity, pH, and inhibitors of AQPs, protein kinases (PKs), and protein phosphatases (PPs) affect the WCA of heterologous AQPs in this expression system. The WCA of TgPIP2;2-expressing spheroplasts was affected by inhibitors of PKs and PPs, which indicates that the water channel of this homologue is regulated by phosphorylation in *P. pastoris*. From the results reported herein, we suggest that *P. pastoris* can be employed as a heterologous expression system to assay the WCA of PIPs and to monitor the AQP-mediated channel gating mechanism, and it can be developed to screen inhibitors/effectors of PIPs.

The movement of water across cell membranes has long been thought to occur by free diffusion through the lipid bilayer. However, the discovery of the membrane protein CHIP28 in red blood cells has suggested the involvement of protein channels (29), and it is now well established that transmembrane water permeability is facilitated by aquaporins (AQPs), water channel proteins that are found in bacteria, fungi, plants, and animals (1, 7, 13, 24). AQPs contain six transmembrane α -helices and five connecting loops, and both the N and C termini are located in the cytosol. The monomers assemble into tetrameric complexes, with each monomer forming an individual water channel (11, 14, 24, 33). Apart from the exceptions of AQP11 and AQP12 from mice, as described by K. Ishibashi (15), AQPs have two signature Asn-Pro-Ala motifs, which are located in the second intracellular and the fifth extracellular loops, B and E.

While 13 different AQPs have been identified in mammals (16), more than 33 AQP homologues have been discovered in plants (6, 17, 30). Plant AQPs fall into four subclasses: (i) the plasma membrane (PM) intrinsic proteins (PIPs), which are localized in the PM; (ii) the tonoplast intrinsic proteins (TIPs), which are localized in the vacuolar membranes; (iii) the nodulin-26-like intrinsic proteins; and (iv) the small basic intrinsic proteins (24). In *Arabidopsis* and maize, there are 13 PIPs, which can be divided further into two subfamilies, PIP1 and PIP2 (6, 17).

The functions and mechanisms of regulation of plant AQPs have been extensively investigated (7, 13, 18, 24). There have been several reports on the water channel activity (WCA) of specific AQPs and their regulation by protein phosphorylation

(3, 4, 8, 12, 18, 25, 32, 33). It has been shown that the WCA of the PIP2 member SoPIP2;1 from spinach is regulated by phosphorylation at two Ser residues (19, 33).

The physiologically interesting temperature-dependent opening and closing of tulip (*Tulipa gesneriana*) petals occur concomitantly with water transport and are regulated by reversible phosphorylation of an undefined PIP (4, 5). Recently, four PIP homologues were isolated from tulip petals, and their WCAs have been analyzed by heterologous expression in *Xenopus laevis* oocytes (3). It has been shown that the tulip PIP TgPIP2;2 (DDBJ/EMBL/GenBank accession no. AB305617) is ubiquitously expressed in all organs of the tulip and that TgPIP2;2 is the most likely of the TgPIP homologues to be modulated by the reversible phosphorylation that regulates transcellular water transport and mediates petal opening and closing (3, 4). However, while the members of the PIP2 subfamily are characterized as water channels (6), TgPIP2;1 (DDBJ/EMBL/GenBank accession no. AB305616) shows no significant WCA in the oocyte expression system (3). There is growing interest in research on AQPs due to their crucial roles in the physiology of plants and animals (1, 16, 21–24, 26–28, 36). The assay of AQP channel activity is usually performed using either a *X. laevis* oocyte expression system (29) or a stopped-flow light-scattering spectrophotometer (35), both of which are not widely available. Furthermore, the complexity of these methods and requirement of expertise limit their high-throughput applications. In contrast, a *Pichia pastoris* expression system is simple to use, inexpensive, and feasible and can be used in high-throughput applications. Although a *P. pastoris* expression system has been shown to assay the WCA of a TIP (9), extensive research is necessary with other AQPs such as PIPs or AQPs present in intragranular membranes to establish whether this assay system can be used to characterize a water channel and study its regulation mechanisms. With this in view, in the study reported herein, TgPIP2;1 and TgPIP2;2 have been heterologously expressed in *P. pastoris*, and their WCAs

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have been assayed. The effects of several factors, such as osmolarity, pH, and inhibitors of protein kinases (PKs) and protein phosphatases (PPs), on the WCA of the recombinant *P. pastoris* have been investigated. Based on the results, we demonstrate that the *P. pastoris* heterologous expression system can be used to rapidly characterize PIP channels, to monitor the effects of mutations, and to score the effects of inhibitors and abiotic factors.

MATERIALS AND METHODS

Preparation of constructs and overexpression of TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ in *P. pastoris*. Constructs encoding TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆, in which a Gly₃-His₆ tag was introduced before the stop codons of TgPIP2;1 and TgPIP2;2, respectively, were synthesized, transformed into *P. pastoris* strain KM71H (Invitrogen), and overexpressed as described previously (8), with minor modifications as follows. Prior to yeast transformation by homologous recombination, the TgPIP2;1-G₃-H₆/pPICZ-B and TgPIP2;2-G₃-H₆/pPICZ-B plasmids (pPICZ-B is the expression vector) (Invitrogen) were linearized with PmeI (New England BioLabs). The plasmid pPICZ-B, without insert, was also transformed into the same *P. pastoris* strain. Transformants were selected by plating on YPD agar (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) containing zeocin at a concentration of 100 µg/ml. Integration of the gene of interest into the *P. pastoris* genome was verified 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 (8). The cell pellets were frozen and stored at -80°C until required.

Preparation of the PM fraction from *P. pastoris* and Western immunoblotting. To prepare spheroplasts, cells were thawed at 4°C and resuspended in 3 ml of spheroplasting buffer (1.5 M sorbitol, 50 mM Tris-HCl [pH 7.5], 10 mM sodium azide, and 10 mM benzamidine hydrochloride) per gram of pelleted cells. β-Mercaptoethanol was added to a final concentration of 30 mM, followed by the addition of 1,000 units of the yeast lytic enzyme Zymolase (Seikagaku, Japan) per gram of pelleted cells, and the final concentration of sorbitol was maintained at 1.0 M. Following incubation at 30°C for 2 h with vigorous shaking, spheroplasts were harvested by centrifugation for 5 min at 2,000 × g. Subsequent steps were carried out at 4°C or on ice. The pellet was washed by resuspension in ice-cold spheroplasting buffer and recovered by centrifugation for 5 min at 5,000 × g. Pelleted spheroplasts were frozen at -80°C, thawed, and resuspended in 3 ml of lysis buffer (50 mM Tris-HCl [pH 7.5], 10 mM benzamidine hydrochloride, 5 mM EDTA, 5 mM EGTA, 1 mM 1,10-phenanthroline, 1 mM dithiothreitol, 10 µM leupeptin, and 5 µM pepstatin A) per gram of pelleted cells. Spheroplasts were disrupted using zirconia beads (ZB-05; Tomy Tech) and a Micro Smash MS 100R (Tomy Tech), according to the manufacturer's instructions. Phenylmethylsulfonyl fluoride in dimethyl sulfoxide (DMSO) was added to a final concentration of 1 mM immediately before spheroplasts were smashed. Cell debris was removed by centrifugation for 10 min at 10,000 × g and 4°C. The supernatant was then centrifuged at 50,000 rpm and 4°C for 1 h. The pellet, which corresponded to the membrane-enriched fraction, was resuspended in a solubilization buffer (10 mM Tris-HCl, pH 7.0, 1 mM EGTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM 1,10-phenanthroline, 10 µM leupeptin, and 5 µM pepstatin A in 20% glycerol). The PM fraction was prepared as described by Tamas et al. (31) and resuspended in the same solubilization buffer used for the membrane-enriched fraction.

Total PM protein (10 µg) was separated on a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and immunoblotting was performed according to the method described by Azad et al. (4), using an anti-His (C-terminal) monoclonal antibody (Invitrogen) as the primary antibody and avidin-peroxidase conjugate (Sigma) as the secondary antibody.

WCA 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 rpm, cells were pelleted by centrifugation at 1,500 × g for 5 min and then resuspended in 5 ml of 100 mM phosphate buffer, pH 7.5 containing 1.0 M sorbitol per gram of pelleted cells. Spheroplasts were generated by adding Zymolase powder (1,000 U/g of 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

FIG. 1. Western blot analysis of PM localization of heterologously expressed TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ in *P. pastoris*. (A) Immunodetection of TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ in the PM. The PM fractions were prepared from 30-h cultures of *P. pastoris* KM71H (lane 2) or *P. pastoris* expressing TgPIP2;1-G₃-H₆ (lane 3) or TgPIP2;2-G₃-H₆ (lane 4). Molecular masses (kDa) of the markers are shown (lane 1). (B) Time course analysis for expression of TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆. The sampling times during the time course are indicated. Results are typical of at least three different preparations.

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 density at 600 nm (OD₆₀₀) was monitored from time zero to the indicated subsequent time points (in figures and legends) using a UV-1700 PharmaSpec spectrophotometer (Shimadzu, Japan). The decrease in the OD₆₀₀ following the hypo-osmotic shock is defined as the WCA. To investigate inhibition by mercury, the spheroplast suspension was preincubated for 10 min in the presence of 2 mM HgCl₂ prior to hypo-osmotic shock. The HgCl₂ solution was prepared in 100 mM phosphate buffer, pH 7.5, supplemented with 1.0 M sorbitol. To investigate phosphorylation and/or dephosphorylation, the spheroplast suspension was pretreated with 2 µM K252a, a PK inhibitor, or 5 µM okadaic acid (OA; a PP inhibitor) 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. It was observed by viable cell count that the pretreatment of spheroplasts with 1.0 M sorbitol could not cause cell lysis.

Growth analysis. For growth assays, cells were pregrown on YPD agar containing 1.34% YNB for 3 days, resuspended in YNB medium to an OD₆₀₀ of 0.5, and then diluted in a 10-fold dilution series up to 1/10⁷. Five-microliter aliquots from the last four consecutive dilutions (1/10⁴ to 1/10⁷) were spotted onto YPD agar plates supplemented with osmotica, or at different pHs, as indicated in the figures. Growth was monitored for 2 to 3 days at 30°C.

Viable cell count. The OD₆₀₀ of spheroplasts generated from *P. pastoris* transformed with the plasmid pPICZ-B (without expression cassette) or from TgPIP-expressing *P. pastoris* was adjusted to 1.0. An aliquot of each type of spheroplast was hypo-osmotically shocked (1.0 to 0.25 M sorbitol in 100 mM phosphate buffer, pH 7.5) for 2 min and then immediately diluted 10-fold serially with sorbitol solution in the same buffer so that the final concentration of sorbitol was 1.0 M. An unshocked aliquot of each type of spheroplast was diluted in the same way with 1.0 M sorbitol in 100 mM phosphate buffer, pH 7.5. Aliquots (100 µl) from the 1/10⁴ to 1/10⁶ dilutions were spread on YPD agar plates (pH 7.0) and incubated for 60 h. The viable cell count was performed manually using three separate preparations of *P. pastoris*.

Statistical analysis. For statistical analysis, a Student's *t* test was used. A *P* value of <0.05 was considered to be statistically significant. Data are presented as the means ± standard errors of the means (SEM) or as noted in the figure legends.

RESULTS

Recombinant TgPIP2s expressed in *P. pastoris*. The expression and membrane localization of the recombinant TgPIP2s in *P. pastoris* were investigated by Western blot analysis using an anti-His (C-terminal) antibody that reacted specifically with proteins containing a C-terminal polyhistidine (His₆). Figure 1A shows that TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ in the yeast PM appear as a band of ~31 kDa, as expected for their

FIG. 2. In vivo WCA assay in *P. pastoris*. Spheroplasts were prepared from 30-h cultures of *P. pastoris* KM71H transformed with pPICZ-B or from *P. pastoris* expressing TgPIP2;1-G₃-H₆ or TgPIP2;2-G₃-H₆. The cell walls of the *P. pastoris* cells were digested, and the resulting spheroplasts were subjected to hypo-osmotic shock. Bursting of the spheroplasts due to water inflow was monitored by measuring the decrease in OD₆₀₀ over 2 min. The starting value for the OD₆₀₀ of each transformant used was set to 1.0. Data are the means $\pm$ SEM of three independent experiments.

monomeric forms. The predicted molecular masses of the homologues are 30,370 and 29,852 Da, respectively (3). The antibody did not react with proteins in the membrane fraction prepared from the host *P. pastoris* strain or with the proteins in the intragranular membranes prepared from the yeasts expressing TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ (data not shown). The time course of expression of the recombinant TgPIP2s was verified by an immunological approach (Fig. 1B). Both homologues were present at detectable levels after culturing for 18 h, and the expression levels reached a plateau after 30 h.

WCA of heterologously expressed TgPIP2s in *P. pastoris*. To monitor the WCA of the overexpressed TgPIP2s, spheroplasts were prepared from 30-h cultures of recombinant *P. pastoris* strains, the host strain, and the host strain transformed with pPICZ-B. The changes in the OD₆₀₀ values following hypo-osmotic shock were then measured. The WCA was higher in TgPIP2;1-G₃-H₆- and TgPIP2;2-G₃-H₆-expressing spheroplasts than in spheroplasts transformed with the pPICZ-B and in host-type yeast spheroplasts (Fig. 2; host type is not shown).

Overexpression of the recombinant TgPIP2s caused a rapid drop in the OD₆₀₀ after osmotic shock, which indicated increasing water influx and bursting of spheroplasts (9). Figure 2 further shows that the treatment of spheroplasts with HgCl₂, an inhibitor of AQPs, decreased the hypo-osmotic sensitivity of TgPIP2;1-G₃-H₆- and TgPIP2;2-G₃-H₆-expressing spheroplasts while it did not affect the WCA of the pPICZ-B-containing spheroplasts. However, following mercury treatment, both recombinant TgPIP homologues produced higher water flux than spheroplasts containing pPICZ-B. This might be due to incomplete inhibition resulting from too short a preincubation time with mercury. Viable cell counting was performed in order to correlate the number of intact cells with the WCAs of TgPIP2;1-G₃-H₆- and TgPIP2;2-G₃-H₆-expressing spheroplasts after hypo-osmotic shock. Following this shock, the cell count for pPICZ-B-containing spheroplasts did not change significantly (Table 1). However, the cell counts for TgPIP2;1-G₃-H₆- and TgPIP2;2-G₃-H₆-expressing spheroplasts decreased by 31 and 33%, respectively.

The WCA of PIPs analyzed in a *Xenopus* oocyte expression system or by stopped-flow spectrophotometry using membrane vesicles has been suggested to be sensitive to acidic pH because of a conserved His residue in loop D (2, 34). We investigated the effect of pH on WCA by incubating the spheroplasts in sorbitol solutions prepared in different buffers at different pHs, as indicated in Fig. 3. The spheroplasts expressing both TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ showed higher OD₆₀₀ values following treatment at acidic pH, which indicated that the WCAs of recombinant TgPIP2;1 and TgPIP2;2 were markedly inhibited due to sensitivity to acidic pH. On the other hand, spheroplasts expressing the TgPIP2 homologues that were treated with a neutral or slightly alkaline pH maintained higher WCAs.

Expression of TgPIP2s affects the growth of *P. pastoris*. *P. pastoris* transformed with the pPICZ-B or the TgPIP2;1-G₃-H₆/pPICZ-B or TgPIP2;2-G₃-H₆/pPICZ-B constructs could not be distinguished by growth on YPD plates under normal conditions (Fig. 4A). When exposed to hyperosmotic stress by the addition of sorbitol or salts to the YPD plates, cells expressing the TgPIP2s showed reduced growth compared to their growth under normal conditions. In contrast, growth of the cells that contained the pPICZ-B was not changed significantly upon exposure to the same stress. This result indicates

TABLE 1. Viable cell count of TgPIP2-expressing *P. pastoris* spheroplasts following hypo-osmotic shock

Trial	<i>P. pastoris</i> KM71H spheroplasts expressing ^a :							
	pPICZ-B			TgPIP2;1-G ₃ -H ₆			TgPIP2;2-G ₃ -H ₆	
	Viable cell count (CFU)		% Change in CFU	Viable cell count (CFU)		% Change in CFU	Viable cell count (CFU)	
	Before shock	After shock		Before shock	After shock		Before shock	After Shock
1	282	277	-2	247	170	-31	231	163
2	301	319	+6	279	184	-34	287	177
3	268	253	-6	307	238	-23	301	206
4	293	307	+5	311	203	-35	309	201
5	313	301	-4	286	197	-31	298	203
Mean			-0.2			-31		-33

^a The number of CFU following hypo-osmotic shock or without shock in each trial is the mean of five replicates.

FIG. 3. Effect of pH on the WCA of yeast spheroplasts expressing TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆. All spheroplasts were pretreated for 30 min with 1.0 M sorbitol solution prepared in buffer (100 mM) at different pHs and then subjected to hypo-osmotic shock by dilution with 0.25 M sorbitol solution at an identical pH. Sodium acetate (pH 5.5), succinic acid/NaOH (pH 6.0), Na₂HPO₄/NaH₂PO₄ (pHs 6.5, 7.0, 7.5, and 8.0), and diethanolamine/HCl (pH 8.5) buffers were used to prepare the 1.0 and 0.25 M sorbitol solutions. The starting value for the OD₆₀₀ of each transformant used was set to 1.0, and the OD₆₀₀ at the 15-s time point is shown. The data shown are the means $\pm$ SEM of three independent experiments.

that the expression of recombinant TgPIP2;1 and TgPIP2;2 confers osmosensitivity upon the cells.

As the WCA of the PIPs is inhibited at acidic pH, we investigated the effect of medium pH on TgPIP2;1-G₃-H₆- and TgPIP2;2-G₃-H₆-mediated growth inhibition. Cells transformed with the pPICZ-B plasmid grew less well at pHs 5.5 and 6.0 than at pHs 6.5 and 7.0 (Fig. 4A and B). However, at pH 5.5, the growth of TgPIP2;1-G₃-H₆- and TgPIP2;2-G₃-H₆-expressing cells was reduced more than that of cells carrying the pPICZ-B plasmid (Fig. 4B). At pH 6.0, although the growth of the TgPIP2;2-G₃-H₆-expressing cells seemed to be the same as that of the pPICZ-B-containing cells, the TgPIP2;1-G₃-H₆-expressing cells clearly showed reduced growth. However, growth of the pPICZ-B-containing cells and recombinant TgPIP2-expressing cells was almost indistinguishable at pH 6.5 to 7.5.

FIG. 4. Phenotypic growth assay of *P. pastoris* expressing TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆. Expression of TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ in *P. pastoris* suppressed growth under hyper-osmotic conditions (A) and at acidic pH (B). The pH of the medium used in the experiment shown in panel A was 7.0. The medium used in the experiment shown in panel B was buffered with either Na/succinate (for pHs 5.5 and 6.0) or Na/phosphate (for pHs 6.5 and 7.5), and the pH was adjusted with NaOH. Five-microliter aliquots from 1/10⁴ to 1/10⁷ dilutions of resuspended cells spotted onto YPD agar plates are shown. Indicated dilutions of samples are for both panels. Each experiment was repeated at least three times, and a representative result is shown.

FIG. 5. Effects of PK and PP inhibitors on the WCA in yeast spheroplasts expressing TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆. Spheroplasts were either left untreated or were pretreated with inhibitors or with DMSO alone prior to hypo-osmotic shock. The starting value for the OD₆₀₀ of each transformant used was set to 1.0. The OD₆₀₀ at the 15-s time point was recorded, and the data shown are the means $\pm$ SEM of five independent experiments.

Effects of phosphorylation on the WCA of spheroplasts expressing recombinant TgPIP2s. Ser residues corresponding to putative phosphorylation sites are conserved in TgPIP2;1 and TgPIP2;2, and the WCA of TgPIP2;2 in *Xenopus* oocytes is affected by inhibitors of PKs and PPs (3). We investigated the effects of the same inhibitors on the WCA of both TgPIP2;1- and TgPIP2;2-expressing spheroplasts (Fig. 5). K252a, a PK inhibitor (19), reduced the WCA of TgPIP2;2-expressing spheroplasts, as shown by an increase in the OD₆₀₀. In contrast, OA, a PP inhibitor (5), enhanced the WCA of TgPIP2;2-expressing spheroplasts. However, the WCA of TgPIP2;1-expressing spheroplasts was not significantly changed by any of the treatments.

DISCUSSION

In this study, we expressed two PIPs, TgPIP2;1 and TgPIP2;2, in the *P. pastoris* expression system, and their WCAs were verified by measuring spheroplast bursting after hypo-osmotic shock and by performing growth assays. Both recombinant TgPIP2s were localized in the PM fraction (Fig. 1). The spheroplast-bursting assay and the reduced viable cell counts following hypo-osmotic shock clearly demonstrate that both TgPIP2s form the water channel (Fig. 2 and Table 1). However, TgPIP2;1 does not show significant WCA in *Xenopus* oocytes (3). This might be due to abnormal localization within the PM of oocytes or other undetermined reasons. In addition to the analysis of WCA (9), this study further shows that the factors affecting or regulating the channel activity can be monitored using the *P. pastoris* expression system. For example, regulatory mechanisms, in particular the pH gating observed only in PIPs (34), could be observed using the spheroplast-bursting assay as well as the growth assay (Fig. 3 and 4). In general, acidic pH-sensitive PIPs show optimum WCA at a neutral or slightly alkaline pH in both oocyte and stopped-flow light-scattering spectrophotometric assays (2, 34). TgPIP2;1-G₃-H₆ and TgPIP2;2-G₃-H₆ showed almost the same features in the present assay system. However, the sensitivity to acidic

pH was observed only after pretreatment of the spheroplasts (at least 20 min). This indicated that the pretreatment of spheroplasts with acidic pH may have induced a drop in cytosolic pH that, in turn, imparted sensitivity to the TgPIP2-overexpressing spheroplasts. However, further research is necessary to elucidate the tendency of slightly decreased water flux observed at pHs 8.0 and 8.5 (Fig. 3). The expression of recombinant TgPIP2s in *P. pastoris* resulted in a distinct phenotype when the protein was grown in medium at acidic pH (Fig. 4B). At acidic pH, the protonation of a His residue in loop D (highly conserved in PIPs) closes the water channel (33, 34). The distinct phenotype observed for TgPIP2;1- and TgPIP2;2-expressing cells at pH 5.5 (Fig. 4B) might be due to coordinate inhibition of recombinant TgPIP2s that probably exerted a general block of water movement into the cells. The expression of TgPIP2;1 and TgPIP2;2 caused hyperosmosensitivity (Fig. 4A), probably due to a more rapid loss of water.

A role for phosphorylation in gating the WCA of four distinct AQPs in plants (GmNod26 from soybean, PvTIP3;1 from bean seed, SoPIP2;1, and TgPIP2;2) has been proposed, based on functional analysis in *Xenopus* oocytes (3, 12, 25, 19, 20). Treatment of AQP-expressing oocytes with inhibitors of PKs and/or PPs enabled the WCAs of these AQPs to be modulated while mutation of putative phosphorylation sites prevented these effects in GmNod26, PvTIP3;1, and SoPIP2;1. In the *P. pastoris* expression osmotic shock assay, K252a and OA are able to exhibit their effects on WCA by affecting PIP phosphorylation and dephosphorylation (Fig. 5). One putative phosphorylation site is a perfectly conserved Ser residue that is found in loop B of all PIPs. In SoPIP2;1, this highly conserved Ser residue (Ser-115) and a C-terminal Ser residue (Ser-274) are phosphorylated, which results in gating of its WCA (19, 33). By using site-directed mutagenesis in the *P. pastoris* expression system, we determined the putative phosphorylation sites of the TgPIP2;2 in our previous study (3). It was shown that the phosphorylation of Ser-35, Ser-116, and Ser-274 influences the WCA of TgPIP2;2 in the spheroplast-bursting assay, and thus its gating mechanism by phosphorylation and dephosphorylation was proposed (3). Although the Ser residues that form the putative phosphorylation sites are conserved in TgPIP2;1 (3), the WCA of TgPIP2;1-expressing spheroplasts was not influenced by pretreatment with PK and PP inhibitors. The reasons for this observation may include the following: (i) this isoform is not phosphorylated, or (ii) phosphorylation does not influence its WCA but may show effects upon physical interaction, or coexpression, with other TgPIP1s or TgPIP2s (10, 32). However, to understand this observation, additional research is required.

In conclusion, although the Ser residues that act as putative phosphorylation sites in TgPIP2;2 (3) have been determined, gating by phosphorylation may be homologue dependent, whereas water channel gating by pH may be common to all functional PIPs. Using the current in vivo assay system, this study ascertained the following: (i) that the WCA of PIPs/AQPs is easily assayable, (ii) that the effects of different factors influencing the channel activity can be monitored, and (iii) that AQP-mediated channel gating mechanisms can be elucidated. It has also been demonstrated here that PIP expression in *P. pastoris* causes distinct phenotypes. These phenotypes can be

used to study the functions of AQPs, to score the effects of inhibitors, and to monitor the effects of mutations.

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