Rapid Paper

Water Channel Activity of Radish Plasma Membrane Aquaporins Heterologously Expressed in Yeast and Their Modification by Site-Directed Mutagenesis

Shinobu Suga¹ and Masayoshi Maeshima²

Laboratory of Cell Dynamics, Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya, 464-8601 Japan

Plants contain a number of aquaporin isoforms. We developed a method for determining the water channel activity of individual isoforms of aquaporin. Six plasma membrane aquaporins (RsPIPs) and two vacuolar membrane aquaporins (RsTIPs) of radish (Raphanus sativus) were expressed heterologously in Saccharomyces cerevisiae BJ5458, which is deficient in endogenous functional aquaporin. Aquaporins were detected by immunoblot analysis with corresponding antibodies. Water permeability of membranes from yeast transformants was assayed by stopped-flow spectrophotometry. The water channel activity of members of the RsPIP2 and RsTIP subfamilies was about 10 times and 5 times greater, respectively, than that of the control; however, RsPIP1s had little (RsPIP1-2 and RsPIP1-3) or no activity (RsPIP1-1). Site-directed mutation of several residues conserved in RsPIP1s or RsPIP2s markedly altered the water transport activity. Exchange of Ile²⁴⁴ of RsPIP1-3 with valine increased the activity to 250% of the wild type RsPIP1-3. On the other hand, exchange of Val²⁵⁵ of RsPIP2-2, which corresponds to RsPIP1-3 Ile²⁴⁴, with isoleucine caused a marked inactivation to 45% of the original RsPIP2-2. Mutation at possible phosphorylation sites at the N- and C-terminal tails also altered the activity. These results suggest that these residues in the half-helix loop E and the tails are involved in the water transport and the functional regulation of RsPIP1 and RsPIP2.

Keywords: Aquaporin — Raphanus sativus — Saccharomyces cerevisiae — Site directed mutagenesis — Stopped flow spectrophotometry — Water channel activity.

Abbreviations: DTT, dithiothreitol; EGTA, ethyleneglycol bis(2-amino-ethyl)tetraacetic acid; NIP, NOD26-like intrinsic protein; PIP, plasma membrane intrinsic protein; RsPIP, radish plasma membrane intrinsic protein; RsTIP, radish tonoplast intrinsic protein; TIP, tonoplast intrinsic protein; SIP, small and basic intrinsic protein.

Introduction

Aquaporins facilitate water transport across membranes in an osmotic pressure-dependent manner and have been intensively studied in their structures, substrates, and water channel activity and regulation of gene expression and activity (Carbrey et al. 2003, Chaumont et al. 2000, Heymann and Engel 2000, Johansson et al. 2000, Murata et al. 2000, Quigley et al. 2001, Tournaire-Roux et al. 2003, Uehlein et al. 2003). Plant aquaporins comprise a big protein family. Arabidopsis thaliana and maize have 35 and 31 genes for aquaporins, respectively (Chaumont et al. 2001, Johanson and Gustavsson 2002, Santoni et al. 2000). Plant aquaporins are subdivided into four families, plasma membrane intrinsic protein (PIP), tonoplast intrinsic protein (TIP), NOD26-like intrinsic protein (NIP), and small and basic intrinsic protein (SIP). In most cases, the water channel activity of individual isoforms has been determined separately by using immature eggs (oocytes) of Xenopus laevis. In this system, the corresponding mRNA is injected into oocytes and translated, and then the rate of swelling or shrinking of oocytes is monitored. Although this is a fine method for determining the water channel activity of individual proteins expressed heterologously in Xenopus oocytes, there are some disadvantages. The expression levels vary with the oocyte. It is hard to quantify the accumulated protein in each oocyte because of its small quantity. Furthermore, the method is theoretically applicable for the plasma membrane aquaporins but not for those located in endomembranes.

Radish (Raphanus sativus), which belongs to the same plant family as A. thaliana, also has a large number of aquaporins. Three subfamilies of radish aquaporin, RsPIP1, RsPIP2 and RsTIP, differ in their expression in radish tissues and responses to phytohormones and abiotic stresses (Suga et al. 2001, Suga et al. 2002, Suga et al. 2003). Thus, to elucidate their physiological significance, we need to evaluate their individual water channel activity by the same method.

In the present study, we developed a combination method of heterologous expression of plant aquaporins in yeast and stopped-flow spectrophotometrical assay of water channel activity.
Structure-function relationship of radish PIPs

Activity. First, we confirmed that endogenous aquaporin of Saccharomyces cerevisiae is not expressed or has no function. Then we expressed six isoforms of radish PIPs (RsPIP1-1, 1-2, 1-3, 2-1, 2-2, and 2-3) and two isoforms of radish TIPs (RsTIP1-1 and 2-1) and quantified their expressed levels in yeast with isoform-specific antibodies prepared in this study. Furthermore, we analyzed the biochemical roles of several characteristic residues by site-directed mutagenesis of RsPIP1-3 and RsPIP2-2, and found them to be critical for the water channel activity and for distinguishing the RsPIP1 from the RsPIP2 subfamily.

Results

Endogenous aquaporins of S. cerevisiae BJ5458 strain

To measure the water transport activity of radish aquaporins by the yeast expression system, we expressed radish aquaporins in a S. cerevisiae strain BJ5458, which lacks vacuolar proteases to avoid degradation of translation products. S. cerevisiae has two aquaporin genes, AQY1 and AQY2 (Bonhivers et al. 1998, Carbrey et al. 2001). Thus, we determined the sequences of AQY1 and AQY2 of a laboratory strain BJ5458 and the wild type (Σ1278b) are compared. The conserved residues among three sequences are in gray. The black boxes indicate Met121 and Thr255, the mutated residues in the laboratory strains. (C) Alignment of AQY2 sequences of BJ5458, S288C and Σ1278b. AQY2 of S288C and BJ5458 had a stop codon in the middle.

Expression of radish aquaporins in yeast and quantification of their protein levels

To express radish aquaporin in this strain, we prepared nine constructs using a yeast expression vector pKT10 (Fig. 2). To detect the translation product in yeast transformants, we newly prepared antibodies specific to the C-terminal parts of RsPIP2s, RsTIP1-1 and RsTIP2-1. Anti-RsTIP1-1 and anti-RsTIP2-1 antibodies recognized only RsTIP1-1 (γ type, 23 kDa) and RsTIP2-1 (δ type, 22 kDa), respectively (Fig. 3A). These antibodies did not react with α- and β-type TIPs, since their sequences do not match the peptide sequences of the target antigens. Fig. 3B shows that the anti-RsPIP1 and anti-RsPIP2s antibodies recognized the corresponding isoforms. A
A faint band of RsPIP2-1 was detected at 28 kDa. This band may be of a degradation product of 30-kDa protein, since its level varied with the experiment. The intensity of immunostained bands of RsPIP1-1, 1-2, and 1-3 were approximately equal. Also, the anti-RsPIP2s antibody gave the equal intensity of RsPIP2-1, 2-2, and 2-3 in an immunoblot (Fig. 3B). Thus, expression of eight radish aquaporin isoforms in yeast was confirmed.

As shown in Fig. 4A, both RsPIP1-1 and RsPIP2-1 were detected in the plasma membrane fraction and RsTIP1-1 was detected in the vacuolar membrane fraction, indicating their correct intracellular localization. A faint immunostained band at 28 kDa may be a degradation product of RsPIP2-1.

We prepared myc-RsPIP1-1 and myc-RsPIP2-1 by tagging RsPIP1-1 and RsPIP2-1 with c-myc at their N-termini to express them in yeast. Both myc-RsPIP1-1 and myc-RsPIP2-1 were detected in yeast membrane fractions with the anti-myc antibody (Fig. 4B). From the intensity of immunoblots with anti-myc, anti-RsPIP1 and anti-RsPIP2-1 antibodies, the relative amount of RsPIP2-1 was calculated to be the same as that of RsPIP1-1 (Table 1). Judging from the consideration of the results shown in Fig. 3B and this calculation, all six RsPIPs were accumulated in relatively equal amounts. We also measured the diameter of membrane vesicles containing RsPIP1-1, 2-1 or the control vacant vector by negative staining electron microscopy (Fig. 4C). There was no marked difference in the diameter between RsPIP1-1 (209±69 nm), RsPIP2-1 (206±53 nm), and vacant vector pKT10 (210±72 nm).

Table 1 Relative amount of RsPIP1-1 and RsPIP2-1 expressed in *S. cerevisiae* cells

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Ratio $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc-RsPIP2-1/myc-RsPIP1-1 $^b$</td>
<td>2.02</td>
</tr>
<tr>
<td>RsPIP1-1/myc-RsPIP1-1 $^c$</td>
<td>1.06</td>
</tr>
<tr>
<td>RsPIP2-1/myc-RsPIP2-1 $^d$</td>
<td>0.519</td>
</tr>
<tr>
<td>RsPIP2-1/RsPIP1-1 $^e$</td>
<td>0.989</td>
</tr>
</tbody>
</table>

$^a$ Ratio was calculated from the intensity of immunostained bands in immunoblots with the corresponding antibodies; anti-myc antibody ($^*$), anti-RsPIP1 ($^*$), anti-RsPIP2-1 ($^*$).

$^b$ Value was calculated from the above three ratios.
Osmotic water channel activity of radish aquaporins expressed in yeast

Membrane vesicles prepared from yeast cells expressing radish aquaporins were assayed for osmotic water channel with a stopped-flow light scattering spectrophotometer. The swelling rate of vesicles in the hypotonic solution was monitored as decrease in the scattered light intensity. The vector control showed a slow influx of water into the membrane vesicles (Fig. 5). RsPIP1-1 did not enhance the permeability. RsPIP1-2 and RsPIP1-3 enhanced the permeability 70% and 220%, respectively. This was not due to the absence of the corresponding isoforms in yeast membranes, since the expression and accumulation were confirmed (Fig. 3). On the other hand, the water permeability of the vesicles containing RsPIP2-1, RsPIP2-2 or RsPIP2-3 was 9.2, 9.7, and 8.4 times, respectively, higher than that of the control (Fig. 5B). The background value of the control is thought to be due to the simple diffusion of water across the membrane.

The water transport activity was decreased by addition of HgCl$_2$ (Fig. 5C). Laizé et al. (1995) reported that pCMBS at 5 mM relatively inhibited the water permeability of secretory vesicles prepared from yeast cells expressing AQP1 but not that at 1 mM, which markedly increased the permeability. In our experiments, the water permeability of the vesicles containing a vacant vector was also increased by 1 mM HgCl$_2$. Thus, we treated the membrane vesicles with 5 mM HgCl$_2$. This treatment strongly inhibited the water transport activity of RsPIP2s and RsTIPs (Fig. 5C). All RsPIPs have four cysteine residues at the second and third transmembrane domains (Suga et al. 2001) and one of the residues may be a mercury-sensitive site. Cys$^{116}$ of RsTIP2-1 and Cys$^{119}$ of RsTIP1-1 may be the mercury-sensitive sites, since Cys$^{116}$ of δ-TIP and Cys$^{119}$ of γ-TIP were the mercury-reactive sites (Daniels et al. 1996).

These results indicate that three members of the RsPIP2 subfamily function as a water channel. RsTIP1-1 and RsTIP2-1 were previously demonstrated to have water channel activity by the Xenopus oocyte expression system (Higuchi et al. 1998). The present results indicated that RsTIPs had water channel activity in both Xenopus oocytes and yeast cells and confirmed the applicability of the yeast expression/stopped-flow assay procedure.

Functional analysis of radish PIPs by site-directed mutagenesis

We applied the heterologous expression system in yeast to mutagenic analysis of radish aquaporins in order to examine which residues give the differences in the water channel activity between the RsPIP1 and RsPIP2 types. Here, we changed several characteristic amino acid residues; namely, Ser$^{27}$, Ile$^{244}$, Lys$^{238}$, and Asp$^{249}$ of RsPIP1-3, and Val$^{235}$, Glu$^{230}$, Ser$^{240}$, and Ser$^{278}$ of RsPIP2-2.

The wild-type and mutant forms of RsPIPs were expressed in yeast and detected by immunoblotting with anti-

RsPIP1 and anti-RsPIP2-2 antibodies (Fig. 6A). The expressed protein levels of RsPIPs other than the S27A and I244V mutant forms were similar to the wild type. The water channel activity of the mutant forms were determined by the stopped-flow spectrophotometrical assay of the osmotic water permeability of the membranes (Fig. 6B). First we focused on Ser$^{27}$, because the residue is conserved among RsPIP1s and a candidate of pho-
The water channel activity of S27A of RsPIP1-3 was 60% greater than that of wild type. Then, we selected several amino acid residues; namely, three residues of RsPIP1s (Ile244, Lys248, and Asp249) and four residues of RsPIP2s (Val235, Glu239, and Ser240) (Fig. 7A). The residues are characteristic to the RsPIP1 and RsPIP2 types, respectively (Suga et al. 2001). These are located around the second NPA motif and estimated to be in the extracellular side of the channel molecule (Fig. 7B). In the sequence alignment, the Ile244, Lys248, and Asp249 of RsPIP1-3 correspond to the Val235, Glu239, and Ser240 of RsPIP2-2, respectively. Therefore we exchanged the residues mutually between RsPIP1-3 and RsPIP2-2 (I244V, K248E, and D249S). Although the water channel activities of K248E and D249S were not changed, the activity of I244V was increased to 250% of the RsPIP1-3 wild type. Whereas, the activities of V235I, E239K, and S240D mutants of RsPIP2-2 decreased to 45%, 72%, and 59%, respectively, compared with the RsPIP2-2 wild type. Thus the residues of Val235, Glu239, and Ser240 of the RsPIP2 type may be essential to express the full activity of water transport.

The serine residue at 278 within a consensus phosphorylation site serine-arginine of RsPIP2-2 corresponds to Ser274 of spinach PM28A that is phosphorylated by the plasma membrane Ca2+-dependent protein kinase (Johansson et al. 1998). For RsPIP2-2, the exchange of Ser278 with alanine or aspartic acid resulted in the decrease the water channel activity to 64% or 63%, respectively (Fig. 6B). Therefore, Ser278 is necessary for the water transport.

**Discussion**

In this study, we determined the osmotic water channel activity of radish aquaporin isoforms by the combination method of yeast expression and stopped-flow spectrophotometry. Since yeast has a vacuole, plant TIPs can be correctly local-
ized to the vacuole in yeast cells. We used the laboratory strain BJ5458 for two reasons. First, this strain does not have a functional aquaporin as demonstrated in this study. Second, BJ5458 lacks some vacuolar proteases. The latter property is essential for expression and accumulation of exogenous proteins. Indeed, some vacuolar membrane enzymes, such as Ca\(^{2+}\)/H\(^+\) antipporter (Kamiya and Maeshima 2004), H\(^+\)-pyrophosphatase (Nakanishi et al. 2001) and pumpkin TIP (Hara-Nishimura et al. 1997), were successfully localized to the vacuolar membrane of the strain deficient in vacuolar proteases. We demonstrated that RsPIP1-1 and RsPIP2-1 were accumulated in plasma membranes, and RsTIP1-1 in vacuolar membranes, whose amounts are sufficient to assay the water channel activity (Fig. 3).

RsPIP2s showed a high water channel activity (Fig. 5). Therefore, we conclude that the RsPIP2 subfamily members are constitutively active water channels. In plants, the protein level of at least RsPIP2-1 varies with the tissue, stage of growth and environmental conditions, while the levels of RsPIP1s and RsTIPs are relatively constant (Suga et al. 2001, Suga et al. 2002). RsPIP2-1 is highly accumulated in young roots and leaves. In seedling roots, the RsPIP2-1 level is increased under salt stress and decreased under osmotic stress. Therefore, the quantitative but not functional regulation is a key step for water channel activity of the RsPIP2 subfamily. The changes in the level of RsPIP2s might be directly reflected in the water channel activity of plasma membranes in plants.

The water channel activity of the RsPIP1 subfamily members was low or negligible. *Zea mays* ZmPIP1 members had no water channel activity in the *Xenopus* oocyte system (Chau mont et al. 2000). Furthermore, *Arabidopsis* AtPIP1;1, 1;3 and 1;4 did not show water channel activity and only AtPIP1;2 shows low activity (Tournaire-Roux et al. 2003). RsPIP1-1 is estimated to correspond to AtPIP1;1, and both RsPIP1-2 and RsPIP1-3 correspond to AtPIP1;2 judging from their sequence identities. Therefore the present observations are consistent with the results of *Arabidopsis* AtPIP1 subfamily.

The site-directed mutagenesis revealed that the exchange of Ile\(^{244}\) with valine produced an active form of RsPIP1-3 with the activity of 250% of the wild type (Fig. 6). In contrast to the mutant form I244V of RsPIP1-3, exchange of the corresponding residue of RsPIP2-2 with isoleucine (V235I) caused a marked decrease of the water channel activity by 45%. It should be noted that Ile\(^{244}\) and Val\(^{235}\) are conserved in the RsPIP1 and RsPIP2 subfamily, respectively. The residues are located in the pore-forming half helix HE that includes the second NPA motif (Fig. 7). Therefore, Ile\(^{244}\) of RsPIP1s and Val\(^{235}\) of RsPIP2s may be specific residues that distinguish the type 1 and 2 of PIPs. There is a possibility that the isoleucine residue causes a steric hindrance for the water channel funnel, since isoleucine is more bulky than valine. Recently, Fetter et al. (2004) reported that the half helix HE may be involved in the subunit–subunit interaction of maize PIPs. Furthermore, they demonstrated that the interaction causes activation of the PIP function. This is not consistent with our estimation that the half helix HE is essential for water channel funnel. However, there is a possibility that modification of this part by amino-acid exchange may cause the configuration of TM5 and/or TM6. The TM5 and TM6 have been demonstrated to be essential for the subunit–subunit interaction of aquaporins and for the water channel function (Duchesne et al. 2002). Thus, we cannot deny an indirect effect of the amino-acid exchange, such as I244V and V235I, on the water channel activity.

In conclusion, the present study demonstrated that the half helix HE is essential for water channel funnel. However, there is a possibility that modification of this part by amino-acid exchange may change the configuration of TM5 and/or TM6. The TM5 and TM6 have been demonstrated to be essential for the subunit–subunit interaction of aquaporins and for the water channel function (Duchesne et al. 2002). Thus, we cannot deny an indirect effect of the amino-acid exchange, such as I244V and V235I, on the water channel activity.
aquaporins. Dispersion of the obtained values is small compared with assay of individual oocytes. We propose this method as a new standard system for assay of aquaporins.

Materials and Methods

Analysis of yeast aquaporin genes

Yeast aquaporin genes (AQY1 and AQY2) were amplified by PCR using gene-specific primers. The PCR products from the genome DNA were sequenced with an ABI PRISM 3100-Avant capillary sequencer.

Expression of radish aquaporins in yeast

EcoRI–SalI or EcoRI–PvuII fragments of radish aquaporin cDNA (RsPIP and RsTIP2-1; EcoRI–SalI, RsTIP1-1; EcoRI–PvuI) were inserted into yeast expression vector pKT10 (Tanaka et al. 1990, Nakanishi et al. 2001). EcoRI–SalI fragments of RsPIP1-1 and RsPIP2-1 were inserted into pKT10-mycN vector, which have a c-myc epitope. The obtained plasmid was introduced into S. cerevisiae strain BJ5458, which is deficient in major vacuolar proteinases. Positive Ura+ colonies were selected, and the expression of radish aquaporin was confirmed by immunoblotting.

Crude membrane and vacuolar membrane fractions were prepared from yeast (Nakanishi et al. 2001). Crude membranes were suspended in 0.45 M mannitol, 90 mM KCl, 1 mM EDTA, and 20 mM Tris-HCl, pH 7.2. The initial rate constant was calculated from single-exponential fits.

Determination of the osmotic water permeability of membranes

The size of membrane vesicles was determined by electron microscopic analysis. Membrane vesicles were prepared from yeast cells expressing RsPIP1-1, RsPIP2-1 and pKT10. A droplet of membrane vesicles was placed on a carbon-film grid for 2 min, and then excess liquid was removed. After the grid was partially dried, sample vesicles were stained using a 2% uranyl acetate solution for 2 min. Membrane vesicles were observed under a JEOL electron microscope (model, JEM-2000EX, TEM).

Site-directed mutagenesis of radish aquaporins

Site-directed mutagenesis of RsPIP1-3 and RsPIP2-2 was performed using a QuickChange site-directed mutagenesis kit (Stratagene) as described previously (Kamiya and Maeshima 2004). The identity of the mutated nucleotides was confirmed by DNA sequencing. The obtained plasmid was introduced into S. cerevisiae strain BJ5458 as described above.

Acknowledgments

We are grateful to Drs Yoichi Nakanishi and Takehiro Kamiya for their valuable advice on heterologous expression in yeast. This work was supported by Grants-in-Aid for Scientific Research 13142203, 13CE2005, and 14COE4A2 (to M.M.) from the Ministry of Education, Sports, Culture, Science and Technology of Japan.

References


(Received: May 6, 2004; Accepted May 13, 2004)