Tonoplast vesicles of *Beta vulgaris* storage root show functional aquaporins regulated by protons

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**Background information.** Water is crucial for plant development and growth, and its transport pathways inside a plant are an ongoing topic for study. Plants express a large number of membrane intrinsic proteins whose role is now being re-evaluated by considering not only the control of the overall plant water balance but also in adaptation to environmental challenges that may affect their physiology. In particular, we focused our work on water movements across the root cell TP (tonoplast), the delimiting membrane of the vacuole. This major organelle plays a central role in osmoregulation.

**Results.** An enriched fraction of TP vesicles from *Beta vulgaris* (red beet) storage roots obtained by a conventional method was used to characterize its water permeability properties by means of the stopped-flow technique. The preparation showed high water permeability (485 µm·s⁻¹), consistent with values reported in the literature. The water permeability was strongly blocked by HgCl₂ (reduced to 16%) and its energy activation was low. These observations allow us to postulate the presence of functional water channels in this preparation. Moreover, Western-blot analysis demonstrated the presence of a tonoplast intrinsic protein. With the purpose of studying the regulation of water channels, TP vesicles were exposed to different acidic pH media. When the pH of a medium was low (pH 5.6), the water permeability exhibited a 42% inhibition.

**Conclusions.** Our findings prove that although almost all water channels present in the TP vesicles of *B. vulgaris* root are sensitive to HgCl₂, not all are inhibited by pH. This interesting selectivity to acidification of the medium could play a role in adapting the water balance in the cell-to-cell pathway.

**Introduction**

In mammals, as many as 11 different aquaporins have been reported to be involved in physiological processes as widely apart as water reabsorption in the kidney to water balance in corneal epithelia (Agre et al., 1995). It is therefore remarkable to observe that in the plant field, in a single plant species (*Arabidopsis thaliana*) more than 35 MIPs (membrane intrinsic proteins), which include aquaporins, have been identified (Johanson et al., 2001; Javot and Maurel, 2002; Maurel et al., 2002). This is the situation prevailing since the first plant aquaporin was functionally expressed in *Xenopus laevis* oocytes by Maurel et al. (1993) and it is therefore clear why plant water transport is now being revisited.

Water movement in roots (as in other organs) can be separately described as occurring along three main pathways: first, the apoplastic path that mainly includes water flow across cell walls and intercellular spaces; secondly, the symplastic path that involves water movement within the cytoplasmic continuum, mediated by plasmodesmata and where no membranes have to be crossed; and finally, the third path or transcellular path that includes water flow across the membranes. Usually the last two pathways are referred as cell-to-cell pathways. However, increasing evidences are pointing out that the relative contribution of each pathway to the overall water...
flow may differ, depending on the plant tissue, on its developmental stage or environmental conditions (biotic or abiotic stress).

How aquaporins are involved in physiological responses still remains elusive (Chaumont et al., 2001; Hill et al., 2004). Moreover, compared with ion channels, little is known about signals and mechanisms that affect aquaporin activity.

It is also interesting to note that the MIP family in plants can be divided into four main groups: PIPs (PM (plasma membrane) intrinsic proteins), TIPs (TP (tonoplast) intrinsic proteins), the more recently defined groups NIPs (NOD26-like MIPs, first described in the peribacteroid membrane of soya bean root nodules) and SIPs (small and basic intrinsic proteins) (Chaumont et al., 2001; Baiges et al., 2002). Another remarkable aspect is not only that there is a high number of plant MIP genes, but that also these proteins are extremely abundant. Particularly, TIPs can account for more than 10% of the TP total protein (Higuchi et al., 1998; Jauh et al., 1999; Karlsson et al., 2000).

The central vacuole occupies 90% of the total cell volume and plays diverse functions including solute storage, cell-volume regulation and pH homeostasis between the cytoplasm and the normally acidic vacuole content (Taiz, 1992; Marty, 1999). Both in isolated vacuoles and in a purified fraction of TP vesicles, the reported \( P_f \) (water permeabilities) values are usually high indicating active water channels (Maurel et al., 1997; Niemietz and Tyerman, 1997; Morillon and Lasalles, 1999). Why are there so many different TIPs expressed in this membrane? Why are they so abundantly expressed? How are they regulated? It is postulated that movement of water inside cells is likely to dominate under stress conditions and therefore the presence of a pore for water flow allows control and regulation of this pathway (Tyerman et al., 1999, 2002). To achieve this goal, three main mechanisms can be triggered: (i) a significant change in the pattern expression of the protein, (ii) recycling with membrane trafficking resembling the vasopressin-regulated internalization of AQP2 in kidney (Verkman and Mitra, 2000; Vera-Estrella et al., 2004) and (iii) directly affecting the gating of the channel. In particular, significant advances have been achieved in the latter mechanism. It has been proposed that the activity of the channel can be affected by phosphorylation and dephosphorylation (Maurel et al., 1995; Johansson et al., 1996, 1998), calcium concentration (Gerbeau et al., 2002), medium acidification (Amodeo et al., 2002; Gerbeau et al., 2002, Tournaire-Roux et al., 2003) and mechanical stimuli (Wan et al., 2004).

The aim of our present work is to understand the role of aquaporins in water transport in _Beta vulgaris_ root, a salt-tolerant species. In root sections water-flow studies were carried out and a transcellular water pathway sensitive to mercurial compounds was described (Amodeo et al., 1999). Furthermore, in isolated vacuoles it was demonstrated how acidification affects water and solute transfers (Amodeo et al., 2002). In the present study, we pursue the characterization of aquaporin-mediated water transport in the TP and investigate its regulation properties. To achieve this goal, we purified TP vesicles from _B. vulgaris_ storage roots following a conventional method, and used this preparation to characterize its water permeability properties to study its regulation by pH.

### Results

An enriched fraction of TP vesicles from the root parenchyma of _B. vulgaris_ was obtained by separation of a microsomal fraction of root tissue in a sucrose gradient. Marker enzyme activities were assayed to rule out putative contamination with PM or other endomembranes. As shown in Table 1, the purified fraction shows an enrichment factor of 1.56 from the initial microsomal fraction in nitrate-inhibited ATPase activity (a TP marker). Moreover, PM contamination is low as indicated by the reduced activity of the PM marker, vanadate-inhibited ATPase. Also, endomembranes are depleted or low [ER (endoplasmic reticulum) and MITOC (mitochondria), see Table 1].

The purified TP fraction was also analysed by electron microscopy (Figure 1A), allowing to visualize spherical vesicles with a mean diameter of \( 142 \pm 4 \text{ nm (±S.E.M., } n = 340) \). As shown in Figure 1(B), vesicles have a homogenous size distribution, which is essential for measuring water permeability by means of the stopped flow-technique. The obtained average diameter was used for all permeability calculations.

Stopped-flow experiments were carried out to find out if water channels are present in TP vesicles. The \( P_f \) (osmotic water permeability coefficient) of TP vesicles
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Table 1 | Biochemical characterization of a purified tonoplast fraction from B. vulgaris roots

<table>
<thead>
<tr>
<th></th>
<th>Nitrate-sensitive H(^+) ATPase</th>
<th>Vanadate-sensitive H(^+) ATPase</th>
<th>Cyt c reduced</th>
<th>Cyt c oxidase</th>
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<tr>
<td></td>
<td>[µmol of Pi·h(^{-1})·(mg of protein(^{-1})]</td>
<td>[µmol·h(^{-1})·(mg of protein(^{-1})]</td>
<td>[µmol·min(^{-1})·(mg of protein(^{-1})]</td>
<td>[µmol·min(^{-1})·(mg of protein(^{-1})]</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>11.58 ± 1.92 (n = 4)</td>
<td>5.38 ± 0.37 (n = 4)</td>
<td>3.16 ± 0.85 (n = 5)</td>
<td>ND</td>
</tr>
<tr>
<td>TP fraction</td>
<td>18.09 ± 1.66 (n = 4)</td>
<td>1.98 ± 0.82 (n = 4)</td>
<td>1.23 ± 0.22 (n = 5)</td>
<td>ND</td>
</tr>
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The TP vesicles were isolated as described in the Material and methods section. Marker enzyme activities of the microsomal and tonoplast fractions are indicated. Specific activities are expressed as µmol substrate·mg of protein\(^{-1}\). Values are means ± S.E.M. The number of independent membrane preparations is shown in parentheses. ND indicates non-detectable values. ER, endoplasmic reticulum; MITOC, mitochondria.

was calculated from their osmotic shrinking kinetics. A typical experiment of the time course of the scattered light intensity when B. vulgaris TP membrane vesicles shrink as a consequence of being exposed to an inward mannitol gradient (200 mosmol·kg\(^{-1}\)) is shown in Figure 2(A). In the same figure, it can be seen that no changes in scattered light occurred when the vesicles are exposed to an iso-osmotic medium. Volume/surface ratio estimation and stopped-flow kinetic measurements allowed to calculate a mean water permeability coefficient of 485 ± 69 µm·s\(^{-1}\) (±S.E.M., n = 5) at 26°C. The amplitude of change in light scattering increased with the size of the imposed inwardly osmotic gradient and was proportional to the ratio of extra/intravesicular osmolarities (Figure 2B). Consequently, the observed changes in light scattering reflect volume changes in accordance to van Heeswijk and van Os (1986).

To further characterize water transport in root cell TP, the temperature dependence of \(P_f\) was measured between 10 and 30°C. Data for a representative experiment are shown in an Arrhenius plot where a linear fit to experimental data is indicated (Figure 3A). Activation energy was 5.4 kcal·mol\(^{-1}\) (1 cal = 4.184 J) indicating that water is predominantly using proteinaceous structures to cross the root cell TP.

To demonstrate that these pores are aquaporins, two different approaches were followed: first, blocking water permeation using a known water channel blocker and secondly, assessing the presence of aquaporins, more specifically TIPs.

\(\text{HgCl}_2\) is a widely used inhibitor for water transport studies in animals and plants that works by binding to the thiol group of cysteine residues located close to the aqueous pore (Agre et al., 1999). Therefore two different \(\text{HgCl}_2\) concentrations (0.1 mM and 1 mM) were assayed for water transport in TP vesicles. As shown in Figure 3(B), \(P_f\) was dramatically reduced, presenting respectively 76.4 ± 0.3% (±S.E.M., n = 4) and 84.4 ± 2.2% (±S.E.M., n = 4) inhibition for the tested concentrations.

Western-blot analysis was carried out to confirm the presence of TIPs in the TP fraction. Figure 4 shows immunoblotting of different membrane fractions of B. vulgaris root cells. The anti-αTIP (TIP3;1) antibody recognized two bands in the TP-enriched fraction corresponding to approx. 25 and 27 kDa. This double band was previously described using the same antibody by Johnson et al. (1989). The diffuse immunostaining pattern in the high molecular mass range, including the discernible band at 41 kDa, was also described by these authors, and represents various levels of aggregation of the 25 kDa TIP.

Although there is no previous information about the presence of TIP3;1 in red beet vacuoles, the antibody we used here is yet to be employed by others (Jauh et al., 1999) to test the presence of TIP3;1 in vacuoles. Also an antibody suggested to have specificity to TIP (TIP-MA27) that was raised by immunization with whole red beet TP membrane was tested (Marty-Mazars et al., 1995). However, other authors have reported that this antibody predominantly recognized proteins of >45 kDa rather than the expected 27 kDa for a TIP (Jauh et al., 1999). In our preparation (results not shown), in addition to the proteins of >45 kDa, a 22 kDa protein is detected instead of the 27 kDa.

Although it is assumed that PIP and TIP sub-families are mainly localized in the PM and TP respectively, PIP could also be found in subcellular fractions, reflecting contamination, or both routing along the secretory pathway or intracellular
localization, e.g. the perinuclear compartment (Chaumont et al., 2000; Santoni et al., 2000). As shown in Figure 4(B), antibodies against PIP labelled exclusively the PM fraction but did not bind to the TP, which might allow us to discard the presence of PIP.

In order to interpret the results, it must be noted that not only the specificity of the antibodies but also putative cross-reactions cannot be discarded. For this reason, the data presented here pursue mainly the goal of demonstrating that the TP fraction has water channels related to TIPs and not to PIPs rather to determine which type of the TIP subfamily could be present in the fraction. These results, along with the
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Figure 3 | (A) Energy activation and (B) water permeability for water transport in TP vesicles

(A) Energy activation is low indicating water moving through pores. Temperature dependence of $P_f$ in TP vesicles determined between 10 and 30 °C. A linear fit to the data indicated an apparent activation energy ($E_a$) of 5.4 kcal·mol$^{-1}$, suggesting that a path for facilitated diffusion of water (aquaporins) predominates in this membrane. (B) Water permeability is highly inhibited with mercurial compounds. Effect of HgCl$_2$ on water transport. Values are shown as percentage of the control (without HgCl$_2$). $P_f$ was reduced to 24 or 16% ($n$ = 4) of the control values after adding 0.1 or 1 mM HgCl$_2$ respectively to the TP equilibration solution. The HgCl$_2$ concentration was maintained during the experiments as both solutions (injected versus the hyperosmotic solution) contained the same inhibitor concentration. Data are mean water permeability values (±S.E.M., $n$ = 4) expressed as a percentage of the control (without HgCl$_2$).

Figure 4 | Western-blot analysis of PM extracts purified from B. vulgaris roots with different antibodies

(A) M (total microsomal fraction), TP and PM membranes were tested using antibodies for TP aquaporin (anti-αTIP from P. vulgaris). A 25 kDa band is observed in the microsomes and only in the TP fraction. Dilution for the antibody was 1:1000. (B) M, TP and PM were tested using antibodies for PM aquaporin (anti-PIP1 from wheat and anti-PIP2 from A. thaliana) to discard putative contamination in the TP preparation (1:1000 for both antibodies). M is used as a control. PM fraction is enriched in PIP while TP is depleted. See the Materials and methods section for details. Sizes in kDa are indicated on the left or right of the panels.

In conclusion, all these results point out to the existence of active TIPs with high $P_f$ and high sensitivity to mercurial compounds on the TP of B. vulgaris root cells.

Since in a previous work (Amodeo et al., 2002), we observed that acidification affects volume changes in isolated vacuoles, we performed an experiment in which the pH of the medium was reduced to 5.6, although the buffer strength or medium osmolarity in the iso- and hyperosmotic solutions was maintained. Under these conditions, water permeability was reduced to 58 ± 3% (±S.E.M., $n$ = 6), showing that medium acidification partially blocks water transport in TP vesicles (Figure 5A). Furthermore, when different pH values varying from 5.6 to 9.5 were assayed, the highest water permeability was observed at pH 7.3 (Figure 5B), a pH usually found in the cytoplasm and not in the intravacuolar space, where pH is usually more acidic.

Discussion

Previous studies in B. vulgaris storage root sections allowed us to identify a transcellular water pathway...
Figure 5 | Acidification partially inhibits water permeability

(A) Effect of pH on water transport. Values are shown as percentage of control (pH 8.3). Rate constant was reduced to 58% (n = 6) of control values when pH of the equilibration solution was 5.6. (B) Water transport measurements at different pH values. Membrane vesicles were first equilibrated at the indicated pH. Stopped-flow experiments were performed by applying an inwardly directed osmotic gradient, also at the same pH value. Values are the average of (n) independent determinations, as indicated in brackets with error bars showing S.E.M. The pH was adjusted using Tris/Mes buffers at a final concentration of 10 mM.

sensitive to mercurial compounds (Amodeo et al., 1999). These results were then extended at a cellular level, seeking for a putative regulation of water movement. Using videomicroscopy, volume changes of individual vacuoles were tracked in response to osmotic challenges. The observations confirmed what was observed at the tissue level: osmotic water transport can be reversibly blocked by mercurial compounds (Amodeo et al., 2002). Moreover, a novel contribution with respect to regulatory mechanisms was found, i.e. medium acidification reversibly reduces water permeability of isolated vacuoles. In the experiments, minimum calculated water permeability value was $19 \pm 2 \mu m \cdot s^{-1}$ (±S.E.M.). It was probably, as stated by Amodeo et al. (2002), a result of the presence of an adherent water layer transferred together with the vacuole into the hypo-osmotic drop. To confirm further our results and continue the characterization of the water permeability properties of the TP of B. vulgaris root, we isolated a purified fraction of TP vesicles and performed experiments using the stopped-flow technique. This approach allowed control and manipulation of intra- and extravesicular content, essential for studying regulatory mechanisms involved in channel gating.

The present study shows that TP vesicles exhibit a high $P_f$, low Arrhenius activation energy and high inhibition by mercurial compounds. These findings, together with the detection of a band of approx. 25 kDa by the anti-αTIP antibody, confirm unequivocally the existence of active water channels in the TP of B. vulgaris root parenchyma.

The reported $P_f$ is also in agreement with other TP preparations (Maurel et al., 1997; Niemietz and Tyerman, 1997), and together with our previous observations in isolated vacuoles (Amodeo et al., 2002) and root sections (Amodeo et al., 1999) confirm that aquaporins are involved in the root parenchyma water transport.

For many years, particularly in animal cells, it was proposed that the pH of the medium could regulate water permeability in an on–off process (Parisi et al., 1983; Parisi and Bourguet, 1984; Parisi and Wietzerbin, 1984). In addition to this, a pH effect was also observed in cloned aquaporin genes expressed in X. laevis oocytes (Yasui et al., 1999; Zeuthen and Klaerke, 1999; Németh-Cahalan and Hall, 2000). Significant advances were also made in seeking for a putative pH regulation of water transport in plants (Amodeo et al., 2002; Gerbeau et al., 2002; Tournaire-Roux et al., 2003). Tournaire-Roux et al. (2003) described a novel molecular mechanism for PIP regulation by cytoplasmic pH, which appears to be highly conserved in all plant PM aquaporins. More specifically, it was demonstrated that these proteins are blocked by cytosolic acidosis through protonation of a conserved histidine residue. The findings provide new insights for the organ and cell bases of inhibition of root-water uptake under hypoxic stress. The
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In conclusion, *B. vulgaris* root cell TP shows active aquaporins that are partially blocked by acidification, probably because only certain TIP isoforms can be blocked by protons. These observations open new insights in water permeability regulation at the TP level. As it is known, vacuoles can occupy as much as 90% of the total cell volume (Marty, 1999). It is therefore comprehensible why the cytoplasm, reduced to a minor fraction of the cell volume, can be subject to fast and significant volume changes detrimental to cell metabolism. Several *in vitro* experiments have suggested that water permeability of the TP is higher than that of the PM (Maurel et al., 1997; Niemietz and Tyerman, 1997). It has been proposed that high water permeability in the TP would help to minimize short-term fluctuations in cytoplasmic volume, avoiding metabolic or structural damage (Javot and Maurel, 2002). Therefore regulation of aquaporin gating can be one of the mechanisms for optimal water balance. In particular, the co-ordinate inhibition of PM aquaporins by cytosol acidosis provides a mechanism that results in the inhibition of root water transport as was observed by measurements of root hydraulic conductivity (Birner and Steudle, 1993; Tournaire-Roux et al., 2003). If all PIPs shut down in case of cytoplasmic acidification, should TIPs remain open (pH insensitive)? If TIPs are completely open, the buffering capacity in between the cytoplasm and the vacuole will be efficient. However, this efficiency will be even greater for the symplastic water redistribution if a fraction of TIPs are sensitive to pH. In this hypothesis, this fine tuning will allow water balance and distribution inside the cell and between cells via plasmodesmata (symplastic pathway).

Materials and methods

Plant material

*B. vulgaris* (red beet) plants were commercially obtained, planted in individual pots and maintained under semi-controlled conditions until use.

Isolation of TP vesicles

The isolation procedure was adapted from Poole et al. (1984). Approximately 400 g (fresh weight) of roots were cut into small pieces and washed in water. Then, they were sliced into small pieces and homogenized in a buffer containing 10 mM HEPES-KOH, pH 7.5, 250 mM sucrose, and 1 mM EDTA. The homogenate was centrifuged at 2000 g for 10 minutes, and the supernatant was further centrifuged at 30,000 g for 15 minutes. The resulting pellet was washed twice with the same buffer and then resuspended in 500 µL of the same buffer. A 27 kDa polypeptide (Marty-Mazars et al., 1995), which does not seem to correspond to the subclass αTIP. This information together with the presented results, allows us to speculate that more than one isoform could be present in the preparation.
pieces and homogenized using an adapted commercial blender in 70 mM Tris, 250 mM sucrose, 5 mM EDTA, 2.4% PVP-40, 25 mM 2-mercaptoethanol and 0.1% (w/v) BSA (pH 8). The obtained homogenate was filtered through several layers of cheesecloth and centrifuged for 10 min at 13,000 g. The supernatant was then filtered with Miracloth (Calbiochem, San Diego, CA, U.S.A.) and centrifuged for 36 min at 80,000 g. The final pellet (crude microsomal fraction) was resuspended in a media containing 124 mM sucrose, 5 mM DTT (dithiothreitol), 15 mM Mes (pH 6.8) with Bis–Tris–propane to a final volume of 8 ml. Each 4 ml were layered on to each of two-step gradients consisting of 4 ml of 10% (w/v) sucrose over 4 ml of 23% sucrose (both containing 1 mM DTT and 15 mM Mes, pH 6.8) and centrifuged for 2 h at 80,000 g. The TP fraction was recovered from the interface of the 10–23% layer, diluted in 250 mM sucrose, 1 mM EGTA, 1 mM MgCl₂, 2 mM DTT and 20 mM Tris/Mes (pH 7.5) and centrifuged at 80,000 g for 36 min. The pellets obtained were resuspended in the previous buffer and then frozen in liquid nitrogen and stored at −70°C for later use. Vesicles used for all the experiments were thawed only once to minimize the damage. All procedures were carried out at 4°C or in an iced bath.

Isolation of PM vesicles
An enriched PM fraction was isolated from the root parenchyma following a method based on partitioning in an aqueous two-phase system (Larsson et al., 1994; Gerbeau et al., 2002). Briefly, 150 g (fresh weight) of roots were cut into small pieces and homogenized using an adapted commercial blender in 200 ml of 50 mM Tris, 500 mM sucrose, 10 mM EDTA, 10 mM EGTA, 10% (v/v) glycerol, 0.6% (w/v) PVP, 0.5 µg · ml⁻¹ leupeptin, 5 mM DTT and 10 mM ascorbic acid, pH 8.0 (homogenization media). The obtained homogenate was filtered through several layers of cheesecloth and centrifuged for 10 min at 10,000 g. The supernatant was then filtered with Miracloth (Calbiochem) and centrifuged for 36 min at 50,000 g. The final pellet (crude microsomal fraction) was resuspended in a medium containing 350 mM sucrose, 2 mM DTT and 5 mM potassium phosphate buffer (pH 7.8). PM vesicles were obtained from this fraction by partitioning in an aqueous two-phase system [6.6% (w/w) dextran T500, 6.6% (w/w) poly(ethylene glycol) 3350, 5 mM KCl, 330 mM sucrose and 5 mM potassium phosphate buffer (pH 7.8)] as detailed in Larsson et al. (1994) and Gerbeau et al. (2002). The final PM fraction was diluted in 10 mM boric acid, 9 mM KCl, 300 mM sucrose and 10 mM Tris (pH 8.3), and centrifuged at 50,000 g for 36 min. The pellets obtained were resuspended in the previous buffer and then frozen in liquid nitrogen and stored at −70°C for later use.

Vesicles used for all the experiments were thawed only once to minimize the damage. All procedures were carried out at 4°C or in an iced bath.

Enzymatic assays
Membrane markers to identify the TP and assess contamination by other membrane components were employed by a method described by Briskin et al. (1987). Briefly, Cyt c (cytochrome c) oxidase and reductase were measured kinetically using K₂HPO₄/KH₂PO₄ buffer (pH 7.5) with or without 1.5 mM KCN. The substrate used for Cyt c oxidase assay was reduced 4 mM Cyt c in the presence of Na₂O₄S₂, whereas the one used for Cyt c reductase assay was non-reduced Cyt c and 120 mM NADH. To analyse the enzyme activity, changes in absorbance at 550 nm were followed. Vanadate-inhibited ATPase activity was determined using 50 mM Mes, 160 mM sucrose, 0.1 mM (NH₄)₆Mo₇O₂⁴, 5 mM MgSO₄, 1 mM NaN₃, 25 mM K₂SO₄ and 1 mM DTT (pH 6.5) with or without 0.6 mM sodium-orthovanadate. Nitrate-inhibited ATPase was determined in the same media but with 50 mM KCl instead of K₂SO₄ (pH 8) and in the presence or absence of 25 mM KNO₃. The substrate used for ATPase assays was 3 mM ATP. The Pᵢ production was evaluated by Fiske and Subbarow (1925) reaction.

Electron microscopy
The interface of the 10–23% gradient was diluted in 100 mM phosphate buffer (pH 7.4), centrifuged at 80,000 g and resuspended with 0.25% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 7.4) for 120 min at 4°C. Samples were washed with this buffer and post-fixed for 1 h with 1% (w/v) OsO₄ in 100 mM phosphate buffer (pH 7.4) at room temperature (23°C). Then the preparation was washed twice with distilled water for 10 min, stained with 5% (w/v) uranyl acetate for 2 h at room temperature, dehydrated in ethanol and embedded in Durcupam. Samples were examined in a microscope at ×20,000.

Stopped-flow light scattering
Volume changes in TP vesicles were followed by 90° light scattering at 500 nm in a stopped-flow spectrophotometer (Applied Photophysics, Leatherhead, Surrey, U.K.) essentially as described previously (Verkman et al., 1985; van Heeswijk and van Os, 1986). TP vesicles were diluted into an equilibration buffer (50 mM NaCl, 50 mM mannitol and 10 mM Tris/Mes, pH 8.3). The hypo-osmotic shock induced a transient opening of the vesicles, so that the interior of the vesicles is equal in composition to the extravesicular solution (Biber et al., 1983; Gerbeau et al., 2002). Water transport was assessed by mixing the equilibrated TP vesicles to an equal volume of the same solution present in the interior of the vesicles but with 500 mM mannitol. This results in an inwardly osmotic gradient responsible for volume changes. Data were fitted to single- and multi-exponential functions and the osmotic permeability coefficient was calculated according to the following equation:

\[ P_f = k V_o / S V_{sc} C_{out} \]

where \( k \) is the faster exponential rate constant, \( V_o \) the initial mean vesicle volume, \( S \) the partial molal volume of water, \( S \) the mean vesicle surface area and \( C_{out} \) the external osmolarity. In each experiment, data from 10 to 12 time course traces were averaged. Replicates were used from a single preparation and then repeated for at least 3–4 different preparations or as indicated. All measurements were performed at room temperature except for those determining the activation energy of water transport \( E_a \). Size of vesicles was determined by electron microscopy.

Activation energy of water transport
\( E_a \) was determined as described by Agre et al. (1999), where assay temperatures were varied between 10 and 30°C. \( E_a \) was deduced from the linear fit of an Arrhenius representation of temperature dependence of \( P_f \) between these values. For this experiment, two replicates were used from a single preparation to cover the different temperatures. The experiment was repeated for three different preparations.
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Inhibition of water transport
A stock solution of 100 mM HgCl₂ was freshly prepared and TP vesicles were preincubated for 2–4 min in the presence of HgCl₂; at a final concentration of either 0.1 mM or 1 mM HgCl₂. This concentration was maintained during the experiments as was present both in the equilibration medium as well as in the hyperosmotic solution.

pH effect
Experiments were carried out by changing the pH value of the medium (pH 5.6–pH 9.5) for both sides of the membrane of the vesicle and manipulating the pH buffer (Tris/Mes). In each run, vesicles were preincubated with the desired osmotic buffer were mixed with an equal amount of the hyperosmotic mannitol solution at the same pH value. Final buffer concentration was 10 mM.

Western blot
TP vesicles were used as samples. Protein (30 µg) diluted in loading buffer containing 2.5% (w/v) 2-mercaptoethanol was loaded for each lane and separated in a SDS/PAGE (10% polyacrylamide gel). Then the proteins were transferred onto to a nitrocellulose membrane for 2 h at 100 V. The membrane was blocked with PBST [PBS containing 3% (w/v) BSA and 0.1% (v/v) Tween 20] at room temperature and then incubated overnight at 4°C for anti-αTIP or 2 h at room temperature for the anti-PIP. After washing for 45 min with three changes of PBST, the blots were incubated with secondary antibody, 1:7500 dilution of goat anti-rabbit IgG-horseradish peroxidase (Sigma). Detection of antibody conjugates was performed using a chemiluminescent substrate (Western Lightning Chemiluminescence Reagent Plus; PerkinElmer LifeSciences, Boston, MA, U.S.A.).

An enriched PM fraction was isolated from the root parenchyma following a method based on partitioning in an aqueous two-phase system (Larsson et al., 1994; Gerbeau et al., 2002) and used as a control.

Rabbit antisera raised against αTIP (antisera for Phaseolus vulgaris αTIP, Johnson et al., 1989), PIP2 (antisera for A. thaliana PIP, Daniels et al., 1994) and PIP1 (likely to recognize all the wheat PIP1 aquaporins; Aroca et al., 2005) were provided by M. Daniels et al. (Scripps Research Institute, La Jolla, CA, U.S.A.) and M. Daniels (Scripps Research Institute, La Jolla, CA, U.S.A.).

Antibody for TIP-MA 27 kindly provided by Dr F. Marty (Université de Bourgogne, Dijon Cedex, France) was also tested (Marty-Mazars et al., 1995). Dilution for the antibodies was 1:1000 for αTIP, PIP1 and PIP2 and 1:5000 for TIP-MA 27.

General analytical methods
Protein concentration was determined using a method of Bradford (1976) with BSA used as a protein standard. Osmolarities of all solutions were determined using a vapour pressure osmometer by Wescor Osmometer (5520C, Wescor, Logan, UT, U.S.A.). Chemicals were purchased from Sigma unless otherwise indicated.

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