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RESEARCH PAPER

Plasma membrane of *Beta vulgaris* storage root shows high water channel activity regulated by cytoplasmic pH and a dual range of calcium concentrations

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Abstract

Plasma membrane vesicles isolated by two-phase partitioning from the storage root of *Beta vulgaris* show atypically high water permeability that is equivalent only to those reported for active aquaporins in tonoplast or animal red cells (P_f=542 μm s^{-1}). The values were determined from the shrinking kinetics measured by stopped-flow light scattering. This high P_f was only partially inhibited by mercury ($HgCl_2$) but showed low activation energy (E_a) consistent with water permeation through water channels. To study short-term regulation of water transport that could be the result of channel gating, the effects of pH, divalent cations, and protection against dephosphorylation were tested. The high P_f observed at pH 8.3 was dramatically reduced by medium acidification. Moreover, intravesicular acidification (corresponding to the cytoplasmic face of the membrane) shut down the aquaporins. De-phosphorylation was discounted as a regulatory mechanism in this preparation. On the other hand, among divalent cations, only calcium showed a clear effect on aquaporin activity, with two distinct ranges of sensitivity to free Ca^{2+} concentration (pCa 8 and pCa 4). Since the normal cytoplasmic free Ca^{2+} sits between these ranges it allows for the possibility of changes in Ca^{2+} to finely up- or down-regulate water channel activity. The calcium effect is predominantly on the cytoplasmic face, and inhibition corresponds to an increase in the activation energy for water transport. In conclusion, these findings establish both cytoplasmic pH and Ca^{2+} as important regulatory factors involved in aquaporin gating.

Key words: Aquaporin regulation, *Beta vulgaris*, calcium, cytoplasmic acidification, plasma membrane, water channels.

Introduction

One of the key regulatory points for water movement through the whole plant is water transport across root cells. Depending on the environmental conditions and the plant–water balance, plants can modify the relative contributions of apoplastic and cell-to-cell pathways of water flow across roots to adjust the overall hydraulic conductivity (Javot and Maurel, 2002; Tyerman *et al*., 2002). The different components of the free energy gradient driving water flow, together with the relative importance of water pathways, has already been analysed by Steudle and co-workers (the composite transport model; see Steudle, 1994, 2000; Steudle and Peterson, 1998).

Aquaporins are essential in the cell-to-cell pathway as their presence allows not only higher water permeability but also control and regulation of water flow (Maurel, 1997; Tyerman *et al*., 2002). They can therefore provide a fine control of the hydraulic conductivity of the cell-to-cell pathway in response to biotic and abiotic challenges, as

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well as internal control systems (Uno *et al*., 1998; Katsuhara *et al*., 2002; Morillon and Lasalles, 2002).

Different mechanisms of regulation have been postulated for aquaporins (reviewed by Vander Willingen *et al*., 2004): (i) changes in their abundance, i.e. levels of gene expression, protein translation or degradation; (ii) protein targeting, i.e. recycling within membranes (Verkman and Mitra, 2000; Vera-Estrella *et al*., 2004); and (iii) directly affecting the gating of the channel. The last provides the basis for short-term regulation allowing fast fine-tuning of water permeability.

To mediate direct gating of the channel, one of the first processes described was phosphorylation and dephosphorylation (Maurel *et al*., 1995; Johansson *et al*., 1998; Guenther *et al*., 2003). Medium acidification has also been shown to reduce water permeability (Amodeo *et al*., 2002; Gerbeau *et al*., 2002; Tournaire-Roux *et al*., 2003; Sutka *et al*., 2005), with the molecular mechanism being linked to key histidine residues in plasma membrane intrinsic proteins (PIPs) (Tournaire-Roux *et al*., 2003). Calcium has also been ascribed to modulating water channels (Gerbeau *et al*., 2002), although it has not yet been elucidated if it is directly involved in the pore gating or acting through a signalling process or via calmodulin, as described for AQP0 (Nemeth-Cahalan *et al.*, 2004). More recently it has been proposed that mechanical stimuli also gate aquaporins (Wan *et al*., 2004).

To enter the cell-to-cell pathway, water must cross the plasma membrane, facilitated to variable degrees, by plasma membrane aquaporins (PIPs) (Maurel *et al*., 1997; Tyerman *et al*., 2002). Tonoplast aquaporins (tonoplast intrinsic proteins, TIPs) are probably also important in establishing the intracellular water pathway, due to the large cross-sectional surface area the vacuole offers to radial water flow in mature parts of roots. Several groups of researchers have therefore focused their efforts on understanding water transport properties of aquaporins in tonoplast and plasma membranes. Very high values for water permeability have been observed in the tonoplast of diverse plant species, isolated as either vesicles or vacuoles (Maurel *et al*., 1997; Niemietz and Tyerman, 1997; Morillon and Lasalles, 1999). These observations contrast with generally lower water permeabilities reported in plasma membrane vesicles or protoplasts, despite the probable presence of PIPs (Maurel *et al*., 1997; Ramahaleo *et al*., 1999; Dordas *et al*., 2000; Gerbeau *et al*., 2002; Moshelion *et al*., 2002). It was suggested that regulatory mechanisms lead to inactivation of aquaporins in the plasma membrane during the isolation of vesicles and possibly protoplasts (Gerbeau *et al*., 2002), as high water permeabilities could be measured in a subpopulation of isolated protoplasts (Ramahaleo *et al*., 1999; Suga *et al*., 2003) or in intact cells using the pressure probe (Zhang *et al*., 1999). Also, in the case of isolated protoplasts, it has been proposed that a change in the number and/or activity of aquaporins (up-regulation?) in the plasma membrane can occur quite rapidly (Moshelion *et al*., 2004). These observations indicate that gating of aquaporins is very likely, and, furthermore, that whole-cell studies which propose direct effects of various treatments on aquaporin activity may, in reality, be mediated by cytoplasmic signalling molecules such as protons and calcium.

Previous work allowed a mercury-sensitive trans-cellular pathway of water transport to be identified in root sections of *Beta vulgaris*, demonstrating that the cell-to-cell pathway may play an important role in the storage root of this halophyte (Amodeo *et al*., 1999). This work pointed to a major role for osmotic gradients in driving water transport according to the composite water transport model of roots (Steudle and Peterson, 1998). Although PIP aquaporins have been reported from *Beta vulgaris* storage roots (Qi *et al*., 1995; Barone *et al*., 1997, 1998), their functional characterization in native membranes and regulatory mechanisms has not been investigated. The present study was therefore conducted to investigate plasma membrane aquaporin activity, and its regulation in *Beta vulgaris* storage roots as an example of a halophyte storage root system. The results revealed unprecedented high water permeability for isolated plasma membrane, which was highly sensitive to pH and pCa on the cytoplasmic face of the membrane.

Materials and methods

Isolation of plasma membrane vesicles

Plant roots from commercial *Beta vulgaris* L. were separated and washed briefly. Approximately 150 g (fresh weight) of roots were cut into small pieces and homogenized using an adapted commercial blender in 200 ml 50 mM TRIS, 500 mM sucrose, 10 mM EDTA, 10 mM EGTA, 10% (v/v) glycerol, 0.6% (w/v) PVP, 0.5 μM leupeptin, 5 mM DTT, 1 mM sodium vanadate, and 10 mM ascorbic acid, pH 8.0 (homogenization medium). The homogenate obtained was filtered through several layers of cheesecloth and centrifuged for 10 min at 10 000 *g*. The supernatant was then filtered through miracloth and centrifuged for 36 min at 50 000 *g*. The final pellet (crude microsomal fraction) was resuspended in a medium containing 330 mM sucrose, 2 mM DTT, and 5 mM phosphate buffer, pH 7.8. Plasma membrane vesicles were obtained from this fraction by partitioning in an aqueous two-phase system (6.6% Dextran T500, 6.6% polyethylene glycol 3350, 5 mM KCl, 330 mM sucrose, and 5 mM phosphate buffer, pH 7.8) as detailed in Larsson *et al.* (1994) and Gerbeau *et al.* (2002). The final plasma membrane 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. Where the dose–response relationship for Ca^{2+} was tested, phase-partitioned vesicles were washed and resuspended in 300 mM sucrose, 50 mM TRIS, and 10 mM EGTA, pH 8.3. The pellets obtained were resuspended in the previous buffer and then frozen in liquid nitrogen and stored at –70 °C for later use. When indicated, an alternative protocol (protected membranes) with cation chelators and phosphatase inhibitors was employed. In this case, the homogenization media included 20 mM EDTA, 20 mM EGTA, 50 mM NaF, 5 mM β-glycerophosphate, and 1 mM phenanthroline, and the resuspension buffer was complemented with 5 mM EDTA and 5 mM EGTA.

Vesicles used for all the experiments were thawed only once to minimize damage. The plasma membrane-enriched fraction

contained 9.27±0.78 mg protein ml^{-1} (n=23). All procedures were carried out at 4 °C or on ice.

General analytical methods

Protein concentration was determined according to Bradford (1976) with bovine serum albumin used as a protein standard. Marker enzyme activities were vanadate-sensitive H^+-ATPase for the plasma membrane, nitrate-sensitive H^+-ATPase for the tonoplast, IDPase for the Golgi bodies, cyt *c* oxidase for the mitochondria, and NADH cyt *c* reductase for endoplasmic reticulum, as described elsewhere (Briskin *et al.*, 1987). The enrichment in the plasma membrane enzyme marker, vanadate-inhibited H^+-ATPase activity, was calculated from the increase in activity between the crude microsomal fraction and membranes recovered in the upper phase after PEG/Dextran phase partitioning. The percentage of right-side-out vesicles, i.e. cytoplasmic side-in, was determined following the latency of vanadate-inhibited H^+-ATPase in the presence of 0.05% (w/v) Brij 58 detergent (Larsson *et al.* 1994). Osmolarities of all solutions were determined using a vapour pressure osmometer (5520C Wescor, Logan, UT, USA).

Vesicle size

The size of the vesicles was determined with dynamic light scattering using a NICOMP 380 particle sizer (PSS-NICOMP Particle Sizing Systems, Santa Barbara, CA, USA). The instrument was calibrated against latex beads of known diameter distributions, following the instructions of the manufacturer to give the absolute dimensions of the vesicles. Measurements of vesicle size were carried out in membranes that had been submitted to the same dilution protocol as those used in stopped-flow measurements. Electron micrographs were also used for some preparations to determine vesicle diameters. In this case, vesicles were 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) OsO_4 in 100 mM phosphate buffer, pH 7.4, at room temperature. 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.

Measurement of permeability coefficient of water: stopped-flow light scattering

Kinetics of vesicle volume was followed by 90° light scattering at 500 nm in an Applied Photophysics stopped-flow fluorimeter, essentially as described previously (Verkman *et al.*, 1985; van Heeswijk and van Os, 1986). Briefly, vesicles were diluted 100-fold into an equilibration buffer (50 mM NaF (protected vesicles) or 50 mM NaCl (non-protected vesicles), 50 mM mannitol, and 10 mM TRIS-MES, pH 8.3) in order to induce a transient opening of vesicles and equilibration of their interior with the extra-vesicular solution (Biber *et al.*, 1983; Gerbeau *et al.*, 2002). Water transport was assayed by mixing the equilibrated vesicles with the same volume of a hyperosmotic mannitol medium complemented with 500 mM mannitol. This resulted in an outward water flow responsible for volume changes. Data were fitted to a single or multi-exponential function using commercial software provided by Applied Photophysics and/or MicroCal ORIGIN version 5, and the osmotic water permeability (P_f) was calculated according to the following equation:

$$P_f = k.V_o/S.V_w.C_{out}$$

where k is the faster exponential rate constant (accounting for >90% of the change in volume), V_o is the initial mean vesicle volume, V_w is the molar volume of water, S is the mean vesicle surface area, and C_{out} is the external osmolarity.

In each experiment, data from 10–12 time-course traces were averaged and fitted to single or multi-exponential functions. Replicates were used from a single experiment and then repeated for at least three or four different preparations. All measurements were performed at room temperature (23 °C) except for those determining the activation energy of water transport (E_a).

Activation energy of water transport (E_a)

The E_a was determined according to Agre *et al.* (1999), in which assay temperatures were varied between 10 °C and 30 °C. E_a was deduced from the linear fit of an Arrhenius representation of temperature-dependent P_f between the above-mentioned temperature values. For this experiment three replicates were used from a single preparation to cover the different temperatures. The experiment was repeated for three different preparations or as indicated.

Inhibition of water transport

To test mercury inhibition, a stock solution of 100 mM $HgCl_2$ was freshly prepared and plasma membrane vesicles were pre-incubated for 2 min in the presence of $HgCl_2$ at a final concentration of 0.1 mM $HgCl_2$. This concentration was maintained during the experiments.

Regulation of water permeability

To test regulatory mechanisms that shut down aquaporins present in the vesicles, different assays were first carried out by changing the pH on both sides of the vesicle membrane. In each run, vesicles prepared with the desired osmotic buffer were mixed in the stopped-flow chamber with an equal amount of the hyperosmotic mannitol solution at the same pH. The final buffer concentration was 10 mM. In some experiments, the vesicles loaded with 10 mM TRIS-MES at a certain pH were exposed to a hyperosmotic mannitol solution containing 100 mM TRIS-MES at the same or different pH in order to maintain external pH according to the hyperosmotic solution values after mixing both equal volumes in the run. To test divalent cations, vesicles were exposed to 2 mM $CaCl_2$ or $MgCl_2$ or $BaCl_2$, adding 0.1 mM EDTA and 0.1 mM EGTA to the equilibration buffer.

To test which face of the plasma membrane may be responding to calcium, different calcium gradients were established. Vesicles loaded with 2.5 mM $CaCl_2$ were mixed with a hyperosmotic solution containing the same calcium concentration (2.5 mM $CaCl_2$) or containing 50 mM EDTA to substantially lower the external free calcium concentration. Vesicles with low internal calcium concentration were buffered with 5 mM EGTA and 5 mM EDTA, and mixed with a hyperosmotic solution either containing 2.5 mM free calcium (to expose only the outside to calcium) or buffered with 5 mM EGTA and 5 mM EDTA to obtain the minimum calcium concentration; this latter situation was considered to be the 'no calcium' condition.

The dose–response relationship for the inhibition by Ca^{2+} was performed by incubating the vesicles in a medium of varying free $CaCl_2$ concentration. Briefly, 20 µl of thawed vesicles in 300 mM sucrose, 50 mM TRIS, 10 mM EGTA were mixed with 80 µl H_2O containing different Ca^{2+} concentrations, which resulted in a final buffered solution of 60 mM sucrose, 10 mM TRIS-HCl, and 2 mM EGTA, pH 8.3 and the desired Ca^{2+} concentration. After 1 min, the volume of the vesicle suspension was brought up to 800 µl by the addition of 700 µl of 60 mM sucrose, 10 mM TRIS-HCl, and 2 mM EGTA, pH 8.3 and the desired Ca^{2+} concentration, and after 2 min the vesicles diluted in this way were injected with an identical solution plus extra 400 mM mannitol (resulting in a 200 mM mannitol osmotic gradient). The pH of all calcium-buffered solutions was carefully monitored and buffered to keep pH in the range where it did not affect water permeability. Free Ca^{2+} concentration in the reaction medium was calculated using Fabiato and Fabiato (1979) and GEOCHEM software programs.

Results

To ensure a highly purified outside-out preparation of *Beta vulgaris* root plasma membrane vesicles, enriched plasma membrane preparations were isolated by partitioning the microsomal fraction in an aqueous two-phase system (Larsson *et al.*, 1994; Gerbeau *et al.*, 2002). Although this purification method has been used already in the literature for the same plant material (Baizabal-Aguirre *et al.*, 1997; Lino *et al.*, 1998) some modifications that were essential to control the quality of the preparation, the homogeneity of the size of the vesicles, and the membrane orientation were introduced. Marker enzyme activities were assayed to rule out contamination from tonoplast membrane or other endomembranes. The plasma membrane vesicle fraction showed an enrichment factor of 4 from the initial microsomal fraction in vanadate-inhibited H^+-ATPase activity (a plasma membrane marker) (Table 1). Tonoplast membrane contamination was low as indicated by the reduced activity of the tonoplast membrane marker, nitrate-inhibited H^+-ATPase. Markers for Golgi, endoplasmic reticulum, and mitochondria were IDPase, cytochrome *c* reductase, and cytochrome *c* oxidase, respectively, and, as shown in Table 1, contamination with these other endomembranes was also low.

Although the two-phase system used for vesicle isolation provides primarily a purification of outside-out vesicles (Larsson *et al*., 1994), therefore exposing the apoplastic side of the membrane to the external solution, it was essential for the present studies to test vesicle sidedness. This is usually performed by assaying a marker for the cytoplasmic surface in the absence/presence of a suitable detergent. Vanadate-sensitive H^+-ATPase latency was assayed in six independent preparations showing that 75±4% of the plasma membrane vesicles presented an outside-out orientation.

To determine water permeability values, another essential feature is to know as accurately as possible the size of the vesicles. Therefore, the size analysis of vesicles was done employing two methodologies: electron microscopy and quasi-elastic light scattering. In both cases, the population was mainly a homogeneous distribution. Electron microscopy gave a mean diameter of 222±11 nm [±standard error of the mean (SEM), *n*=140], with similar results for quasi-elastic light scattering. These values are also in agreement with the ones reported in the literature (Niemietz and Tyerman, 1997; Dordas *et al.*, 2000; Gerbeau *et al.*, 2002). The important aspect of this comparison is that the latter technique allowed measurements of vesicle size in membranes that have been submitted to the same dilution protocol as those used in stopped-flow measurements, and this could be done in parallel with the experimental runs. Therefore the size of vesicles was determined in every experiment and for every protocol to control putative initial volume modifications upon exposing the vesicles to different protocols (i.e. acidification, calcium concentration, etc.).

Characterization of water transport in root plasma membrane vesicles

The osmotic water permeability (P_f) of plasma membrane vesicles was measured after rapidly increasing the extravesicular osmolarity by means of the stopped-flow technique and recording the light-scattering signal at 500 nm (Niemietz and Tyerman, 1997). Figure 1A shows a typical example of the time-course of the (reversed) scattered light intensity that occurred when *Beta vulgaris* plasma membrane vesicles shrink as a consequence of being exposed to an inwardly directed mannitol gradient of 200 mOsm. The ideal osmotic behaviour of the plasma membrane vesicles was confirmed by the observations that (i) no time-dependent change in the light signal was observed after exposure of the vesicles to an iso-osmotic medium (Fig. 1A) and (ii) the amplitude of change in light scattering increased with the size of the imposed osmotic gradient and was proportional to the ratio of initial osmolarities (data not shown).

Isolated *Beta vulgaris* root plasma membrane vesicles generally presented a high osmotic water permeability coefficient (P_f) of 542±40 μm s^{-1} (±SEM, *n*=7 independent vesicle preparations) at 23 °C. This value is consistent to water moving through pores (Tsai *et al.*, 1991; Agre *et al*., 1999). The flux of water from the plasma membrane vesicles was partially inhibited by incubation with 100 μM $HgCl_2$ (Fig. 1B), P_f being reduced to 80±4% (±SEM, *n*=3) of its initial value in the absence of $HgCl_2$.

A putative contamination of the preparation by tonoplast vesicles was discarded, based on the following evidence:

Table 1. *Biochemical characterization of the purified plasma membrane fraction from* Beta vulgaris *root parenchyma*

The plasma membrane vesicles were isolated using an aqueous two-phase partitioning system as described in the Materials and methods. Marker enzymes activities of the microsomal and plasma membrane fractions are indicated. Values are specific activity expressed as mean ±SEM. The numbers of independent membrane preparations tested are shown in parentheses.

	Vanadate-sensitive H^+ ATPase (μmol h^{-1} mg^{-1} protein)	Nitrate-sensitive H^+ ATPase (μmol h^{-1} mg^{-1} protein)	IDPase latency (μmol h^{-1} mg^{-1} protein)	Cyt *c* oxidase (μmol min^{-1} mg^{-1} protein)	NADH Cyt *c* reductase (μmol min^{-1} mg^{-1} protein)
Microsomal fraction	6.13±1.00 (*n*=6)	3.17±1.29 (*n*=6)	20.37±3.06 (*n*=3)	6.64±5.34 (*n*=3)	0.63±0.23 (*n*=3)
Plasma membrane fraction	25.38±4.78 (*n*=8)	2.46±0.62 (*n*=8)	23.26±4.27 (*n*=3)	0.70±0.71 (*n*=6)	0.28±0.09 (*n*=6)

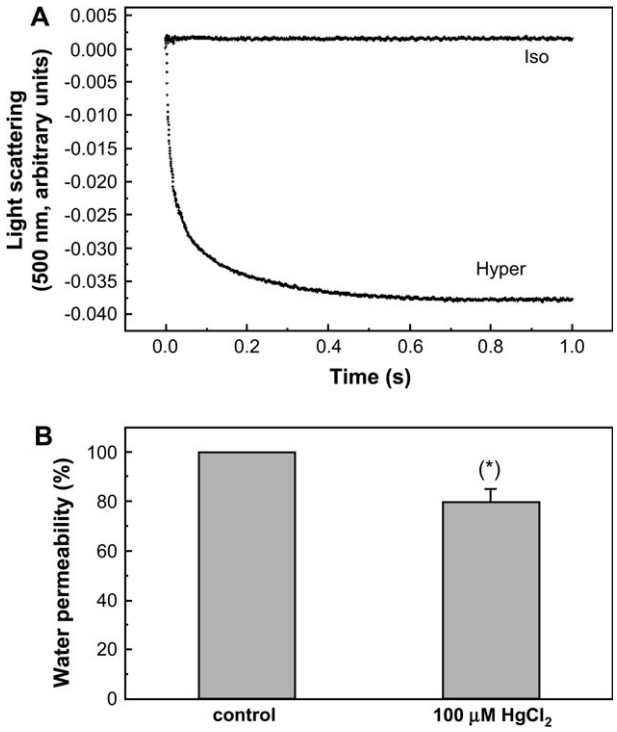

Fig. 1. Plasma membrane vesicles show high water permeability. (A) Representative time-course of water efflux in *Beta vulgaris* plasma membrane vesicles. The plasma membrane vesicles were abruptly mixed in a stopped-flow fluorimeter with an equal volume of a hyperosmotic solution, thus creating an inwardly directed osmotic gradient of 200 mosm kg^{-1}. Changes in vesicle volume were followed by light scattering (arbitrary units). As a control, vesicles were mixed with an iso-osmotic solution where no volume change was detected. The traces correspond to an average of 12–14 individual time-courses of scattered light intensity at 23 °C. (B) Inhibition of water permeability by $HgCl_2$. Plasma membrane vesicles were pre-incubated for 2 min with 100 μM $HgCl_2$. This concentration was maintained throughout the experiments. Membrane vesicles were mixed with a hyperosmotic solution containing the same inhibitor concentration. Data are mean water permeability values (±SEM, n=4) expressed as a percentage of the control (without $HgCl_2$).

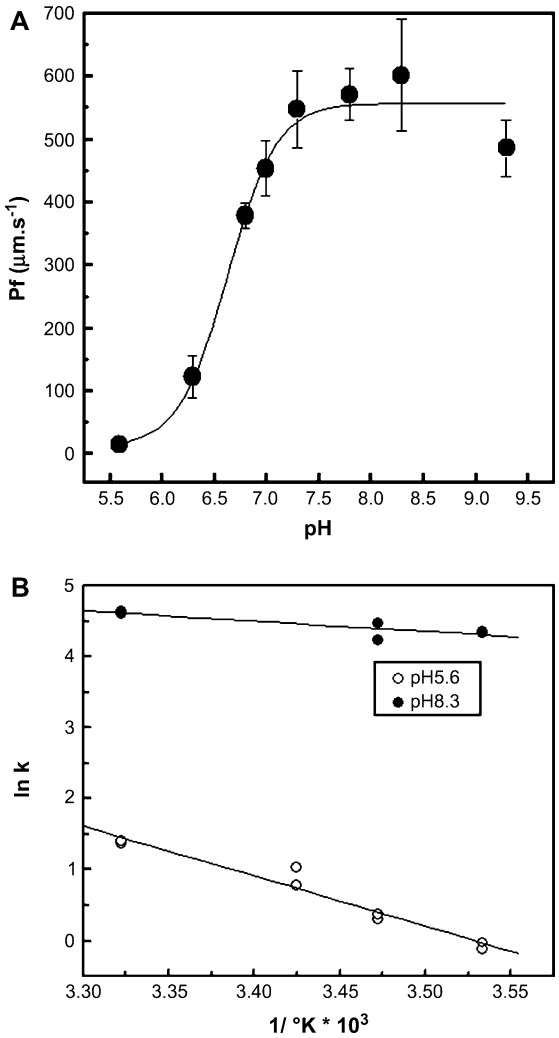

Fig. 2. Effect of pH on water transport in plasma membrane vesicles. (A) Membrane vesicle equilibration and water transport measurements were performed at the indicated pH. The pH was adjusted using TRIS-MES buffers at a final concentration of 10 mM. The vesicles were transferred with the pH adjusted as indicated and stopped-flow experiments were performed by applying an inwardly directed osmotic gradient. Values are the average of four independent determinations with error bars showing SEM. (B) Temperature dependence of water transport in plasma membrane vesicles. A typical Arrhenius plot of water efflux from plasma membrane vesicles exposed to two different pH values is shown. ln k represents the natural log of the rate constant determined from single exponential fit of the permeability data (plasma membrane vesicles at pH 8.3, closed circles, and at pH 5.6, open circles). The inverse temperature (10, 15, 23, and 27 °C) is plotted as Kelvin degrees (×1000). E_a values obtained at pH 5.6 are consistent with values for water transport through lipid bilayers.

(i) Table 1 shows an enriched plasma membrane fraction with a low level of tonoplast contamination; (ii) experiments carried out with a purified tonoplast fraction obtained from *Beta vulgaris* root, employing a sucrose gradient protocol (Poole *et al.*, 1984), showed a high P_f but essentially different responses to mercury and low pH (Sutka *et al.* 2005).

To characterize water transport further, the temperature dependence of P_f was measured between 10 °C and 30 °C. Data for a representative experiment are shown as an Arrhenius plot where a linear fit to the transformed experimental data is shown (Fig. 2B). The activation energy deduced from the slope of such fits was E_a=2.9± 0.3 kcal mol^{-1} (±SEM, n=3).

A high rate of water transport and a low E_a demonstrate that the plasma membrane of *Beta vulgaris* root cells has active aquaporins facilitating water efflux. Low mercurial sensitivity may reflect the presence of aquaporins that are not inhibited by mercurial compounds (Daniels *et al.*, 1994; Biela *et al.*, 1999).

Regulation of water permeability

To assess the impact of pH on water transport, P_f was determined at different pH values varying from 5.6 to 9.5 (Fig. 2A). The P_f was reduced when the pH was lower than 7, with a half-maximal reduction obtained at pH

6.6±0.1 (±SEM, *n*=4), and the data were fitted using a Boltzman equation. Maximal inhibition was obtained at a pH of 5.5, where P_f drops to 14.04±0.45 μm s^{-1} (98% reduction from maximal value). The temperature dependence at pH 5.6 was also assayed (Fig. 2B). The E_a obtained was 14.1±5.9 kcal.mol^{-1} (±SEM, *n*=4).

To test the sidedness of the pH effect and also if pH gradients were important in regulating P_f, an experimental protocol was designed where pH gradients were established across the plasma membrane during the osmotically induced water efflux (Fig. 3). To ensure an initial pH gradient when mixing solutions in the stopped-flow apparatus, different buffer concentrations were used in both intra- and extra-vesicular media. Vesicles must be kept in an initial buffer solution during handling that is the same as the desired intra-vesicular solution (i.e. 10 mM TRIS-MES, pH 5.6 or pH 8.3). To impose a temporary pH gradient after mixing in the stopped-flow apparatus required that a higher concentration of buffer was injected against the vesicle storage buffer, i.e. initial solution equal to 10 mM TRIS-MES which after mixing with the same volume of 100 mM TRIS-MES gave a final extra-vesicular concentration of 55 mM TRIS-MES at a different or the same pH. Two extreme pH values, 5.6 and 8.3, were compared (Fig. 3). Results showed that when the interior of the vesicles was acid, P_f was markedly reduced independent of the existence of a pH gradient across the membrane. When the internal pH was basic, P_f was maintained at a high value which was also independent of external pH (Fig. 3A, B). The possible effect of the buffer concentration gradient on water transport was discarded, because vesicles equilibrated at pH 8.3 with intra-vesicular media of 100 mM TRIS-MES and then exposed to an iso-osmotic solution with 10 mM TRIS-MES displayed no volume change (data not shown). In the literature, proton transport rates have been measured in *Beta vulgaris* root plasma membrane vesicles under different experimental conditions by analysing the rate of change of acridine orange or quinacrine fluorescence intensity (Bennett and Spanswick, 1984; Blumwald *et al.*, 1987; Lino *et al.*, 1998). These reports showed that the time scale of proton gradient dissipation was >1 s in relatively weakly buffered solutions; this lag was much longer than the shrinking kinetics reported here of <200 ms. Use of highly buffered solutions ensured that the pHs imposed were maintained during the vesicle shrinkage experiments. Overall, and since the vesicles were 75% right side out, the

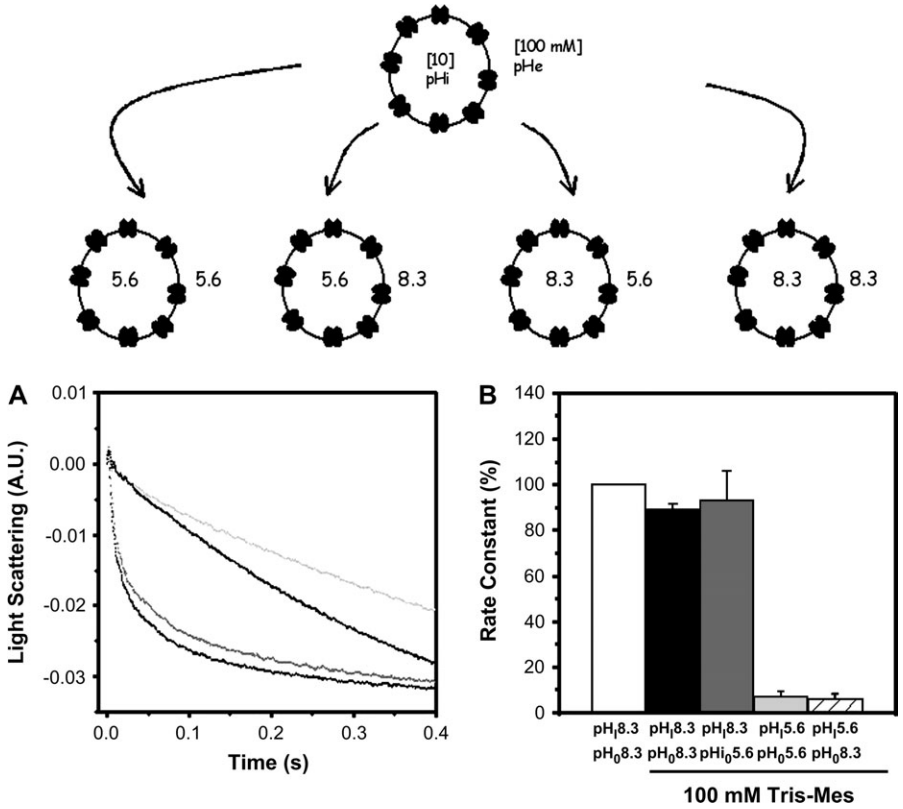

Fig. 3. The P_f of plasma membrane vesicles is dependent on intra-vesicular but not extra-vesicular pH. To test which face of the plasma membrane may be responding to pH, a pH gradient was established (10 mM/55 mM TRIS-MES, outside/inside; see text and diagram at the top). (A) Changes in light scattering intensity of plasma membrane vesicles as a result of exposure to a trans-membrane gradient (iso/hyper) were measured subsequently. A typical experiment is shown. (B) Mean water permeability values expressed as a percentage of the control condition (in/out pH 8.3; 10 mM TRIS-MES) are shown. The water permeability is inhibited, specifically in the case where pH is acid in the interior of the vesicles (right-side-out oriented, i.e. the cytoplasmic side). Data are ±SEM, *n*=4 independent membrane preparations.

results indicate that P_f was sensitive only to low pH on the cytoplasmic face of the plasma membrane (as reported in the literature, Tournaire-Roux *et al*., 2003) and was not sensitive to the pH gradient.

Divalent cations

Another proposed regulatory mechanism of aquaporins is the concentration of divalent cations (Johnson and Chrispeels, 1992; Johansson *et al.,* 1996; Gerbeau *et al*., 2002). Initially, water permeability was measured in the presence of different divalent cations (2 mM $CaCl_2$, or $MgCl_2$ or $BaCl_2$) on both faces of the membrane. No inhibition was detected in the presence of Mg^{2+}, a slight one for Ba^{2+} (30%), and a strong one for Ca^{2+} (95%) (Fig. 4A). When the temperature dependence of water transport was measured in the presence of calcium, the E_a obtained was 10.9±2.8 kcal mol^{-1} (±SEM, n=3), which was higher than that of the control (2.9 kcal mol^{-1}) (Fig. 4B), and clearly indicated that calcium was reducing the water pathway through pores.

Experiments analogous to those shown in Fig. 3 were performed to test the sidedness of the calcium effect (Fig. 5). When calcium was not present (high pCa) on both sides of the membrane, the highest water permeability was obtained. When calcium was initially loaded into the vesicles at a high concentration (low pCa) with either no (buffered to high pCa in the presence of chelators) or high calcium on the outside, there was strong inhibition of water transport. With the reverse gradient, where no calcium was present on the inside of the vesicles but high calcium was present on the outside, the result was only a slight inhibition of water transport.

Protected versus unprotected protocol for isolation of plasma membrane vesicles

Two different protocols were tested: a standard protocol (unprotected) and a second one (protected), where cation chelators and protein phosphatases inhibitors were present during the vesicle isolation and experiments (see Materials and methods). No differences were found in P_f or in calcium inhibition for vesicles isolated with the protecting protocol with respect to vesicles isolated with the standard one, although in both cases there was some variation in P_f between preparations. In both cases the mean P_f values were very high and not significantly different (t-test, P <0.05), protected vesicles presented a P_f value of 526±27 μm s^{-1} (±SEM, n=6), while the P_f corresponding to non-protected plasma membrane vesicles was 542±40 μm s^{-1} (±SEM, n=7). Also, in both cases inhibition at 2 mM calcium was over 90%.

Dose–response curve for calcium

The effect of Ca^{2+} on water transport was also analysed by dose–response experiments where concentrations of free

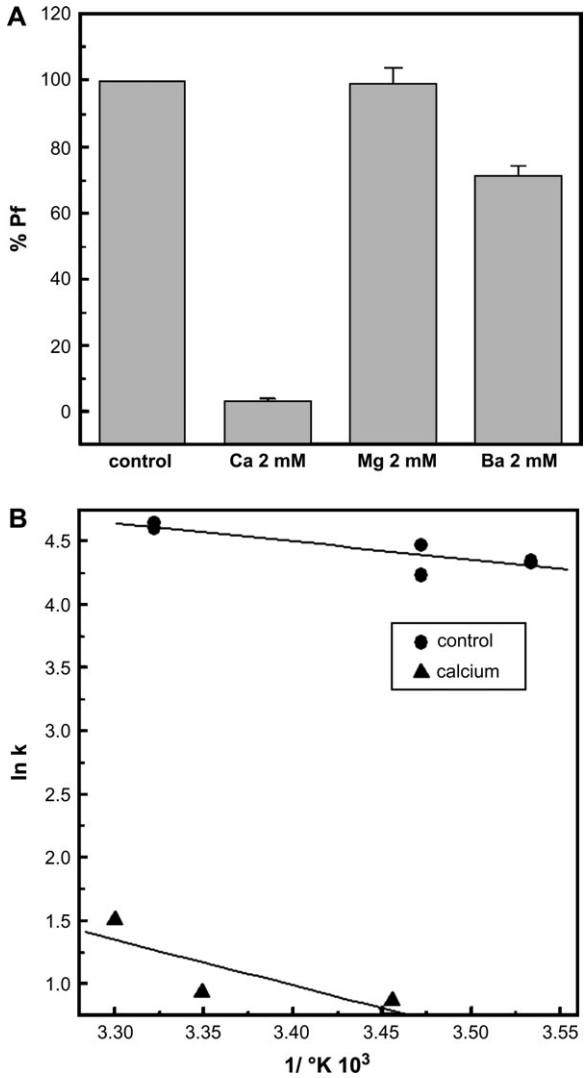

Fig. 4. Inhibition by divalent cations. (A) The osmotic permeability coefficient of plasma membrane vesicles was measured in the presence of 2 mM $CaCl_2$, $MgCl_2$, or $BaCl_2$ in both, intra- and extra-vesicular media. Data are mean P_f values (±SEM, n=4) expressed as a percentage of the control (without divalent cations). (B) Temperature dependence of water transport in plasma membrane vesicles in the presence of Ca^{2+}. A typical Arrhenius plot of water efflux from plasma membrane vesicles is shown. Water transport measurements were performed at the indicated temperature in the presence (closed triangles) or absence (closed circles) of Ca^{2+}. Linear fits to the experimental data are indicated. E_a values obtained in the presence of Ca^{2+} are consistent with values for transport through lipid bilayers.

Ca^{2+} between 0 mM and 2 mM were tested (Fig. 6). A biphasic dose–response curve was obtained with a component in the nanomolar range (Fig. 6A, B) and a second component in the micromolar range (Fig. 6C, D). This dual effect of Ca^{2+} was observed in each of five separate membrane preparations. The results showed a high apparent affinity component with an IC50 of 3.3 nM (SEM ±0.29 nM) and a lower apparent affinity component with an IC50 of 280 μM (SEM ±172 μM). The IC50s were significantly different between the low and high concentration

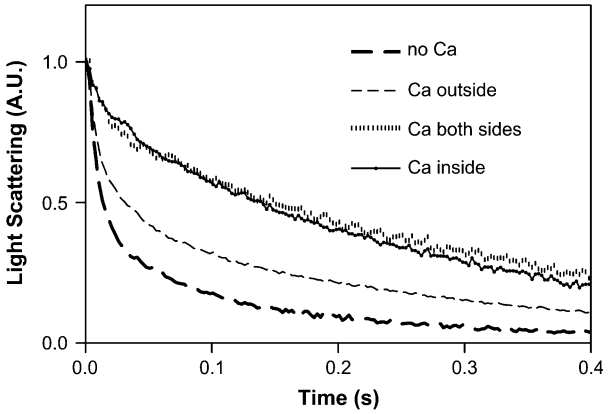

Fig. 5. The P_f of plasma membrane vesicles is dependent on intravesicular but not extra-vesicular calcium. Change in the light-scattering intensity of plasma membrane vesicles as a result of exposure to a transmembrane osmotic gradient (iso/hyper) with different calcium gradients. To test which face of the plasma membrane may be responding to calcium, different calcium gradients were established. Vesicles were loaded with 2.5 mM $CaCl_2$ and mixed with a hyperosmotic solution containing the same calcium concentration (Ca both sides) or a strong calcium buffer (50 mM EDTA) to lower the external free calcium concentration substantially (Ca inside). Vesicles buffered with 5 mM EGTA and 5 mM EDTA to a low internal calcium concentration were mixed with a hyperosmotic solution containing either 2.5 mM free calcium (Ca outside) or with a low calcium concentration (buffered with 5 mM EGTA and 5 mM EDTA) (no Ca). Average traces from several vesicle injections are shown for each combination.

ranges as is quite evident from inspection of the data in Fig. 6 (P <0.0001, t-test). The results showed a high apparent affinity component with an IC50 of 5 nM and a lower apparent affinity component with an IC50 of 200 μM. The apparent Hill coefficients for these components are 38 and 1, respectively, reflecting a steep and sensitive effect in the low concentration range and a more gradual inhibition in the high concentration range. Despite variable initial P_f between different vesicle preparations shown in Fig 6A, that in the extreme varied by 4-fold, the calcium dose response was similar, and the final inhibited level of P_f was similar, presumably reflecting variable levels of initial aquaporin activity on similar basal membrane water permeability.

Discussion

The present work provides new insights on water transport through aquaporins in root-derived plasma membrane. Although the plant plasma membrane possesses multiple aquaporins (Maurel *et al.*, 2002) most reports have found lower aquaporin activity in this membrane when compared with the tonoplast (Table 2). The isolation of plasma membrane vesicles with high water transport activity has not been very successful. A hypothesis proposed and tested by Gerbeau *et al.* (2002) was that aquaporins were inhibited

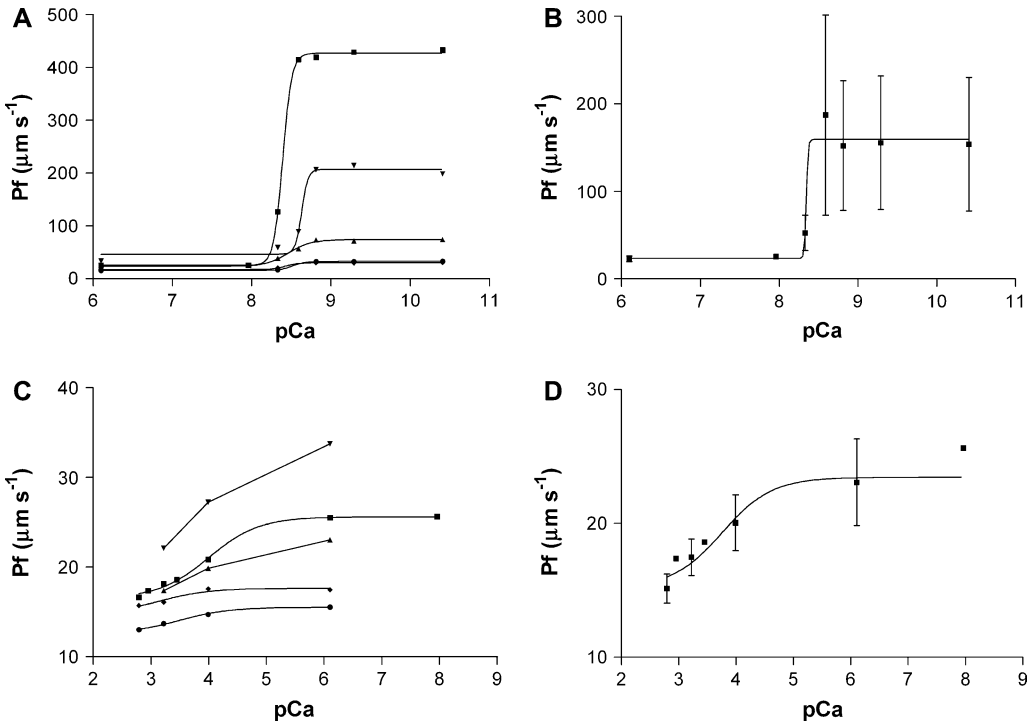

Fig. 6. Evidence for a dual concentration effect of free calcium on P_f in beet root plasma membrane vesicles. (A) Five independent vesicles isolations from different beets using protected and non-protected protocols with the range in free calcium confined to the low concentration range (pCa 6–11). (B) Mean P_f from five independent isolations, the IC50 for Ca^{2+} was pCa 8.34, Hill slope was 38. (C) As for (A), but in the high concentration range of free Ca. (D) As for (B), but the IC50 was pCa=3.7, Hill slope=1.

during the isolation of plasma membrane vesicles due to exposure to non-physiological conditions. The comparison of P_f values in isolated protoplasts and vacuoles provided another means by which to estimate the relative water permeability of the tonoplast and plasma membrane. Although there are some exceptions (protoplasts isolated from hypocotyls and rape roots; Maurel *et al*., 2002), data for protoplasts and isolated plasma membrane vesicles report lower water permeability independently of the tissue or species considered than that reported for the tonoplast—vacuoles or tonoplast vesicles (Table 2). Therefore, the prevailing concept is that the plasma membrane must be the limiting barrier for water uptake, but that the aquaporins situated in the plasma membrane are highly regulated.

In particular, the molecular identification of aquaporins in the plasma membrane of cells from *Beta vulgaris* storage tissue has already been described by Qi *et al.* (1995) and Barone *et al.* (1997, 1998), but a functional analysis of these proteins was not demonstrated. The present results show that osmotic water permeability of *Beta vulgaris* plasma membrane is 542 μm s^{-1}, a high value in comparison with P_f reported for other plant species, and in the same order as the values reported for the tonoplast, including red beet tonoplast vesicles (Table 2). This high P_f, together with the low E_a obtained, indicates, by definition, the presence of active water channels, presumed to be aquaporins, mediating water transport across these membranes.

Table 2. *Water permeability values across different plant membranes*

The table shows mean water permeability values (expressed in μm s^{-1}) across different isolated plasma membrane vesicles (PMV) or tonoplast vesicles (TPV) measured by stopped flow or from isolated protoplasts and vacuoles, measured by videomicroscopy as reported in the literature. Superscript letters indicate the references listed in the footnote. The value reported in this work is shown in bold and underlined.

Plant material	PMV	TPV	Protoplasts	Vacuoles
Wheat root	12.5[a]	86[a]	2.5[b]	–
Tobacco	6.1[c]	690[c]	27.12[d]	–
Arabidopsis	11.2[e]	–	69[h]	–
Rape leaf	–	–	–	623[g]
Rape hypocotyl	–	–	370[b]	1100[g]
Rape root	–	–	2 to 500[b,k]	656[g]
Red beet root	**542**	485[o]	–	19.87[f] to *270*[g]
Onion leaf	–	–	9[b]	184[g]
Melon root	–	–	11[i]	–
Maize root	–	–	15 to 45[n]	–
Petunia leaf	–	–	–	955[g]
Radish root	–	–	>300[m]	–
Samanea motor cells	–	–	3 to 5[j]	–
Squash root	23.9[l]	–	–	–

[a] Niemietz and Tyerman (1997); [b] Ramahaleo *et al.* (1999); [c] Maurel *et al.* (1997); [d] Siefritz *et al.* (2002); [e] Gerbeau *et al*. (2002); [f] Amodeo *et al.* (2002), with higher values if correcting unstirred layers; [g] Morillon and Lassalles (1999); [h] Morillon and Chrispeels (2001); [i] Martinez-Ballesta *et al.* (2000); [j] Moshelion *et al.* 2002; [k] Morillon and Lasalles (2002); [l] Dordas *et al.* (2000); [m] Suga *et al*. (2003); [n] Aroca *et al*. (2005); [o] Sutka *et al*. (2005).

For *Beta vulgaris* plasma membrane, a reduction of 20% was only found in water transport with mercuric chloride. By contrast Niemietz and Tyerman (2002) have shown that Ag^+ was more effective as a blocker, with almost complete inhibition similar to the effects of pH and Ca^{2+} reported here. When the authors tested $AgNO_3$ on red beet plasma membrane vesicles, they found 74% of P_f inhibition with an EC50 of 13.2 μM. Due to the great abundance of aquaporins in plants, it is reasonable to assume that the preparation used contains some aquaporins sensitive to mercury, while other ones are less sensitive to mercury but are more sensitive to Ag^+. This is supported by the observation that, although mercurials are found to block most plant aquaporins, there have been some cases where no mercurial inhibition was observed (Daniels *et al.*, 1994; Biela *et al.*, 1999).

Plant aquaporin regulation

Changes in membrane water permeability are likely to be critical for plant adaptation to different stress situations (Javot and Maurel, 2002; Tyerman *et al.*, 2002) and the regulation of aquaporins is one of the determinant points to control this membrane permeability. In this work, studies have been focused on short-term regulatory mechanisms exploring the roles of protons and calcium ions as signalling molecules in the cell that are likely to change concentration during various stress responses. The isolated vesicle system has been employed because this can be used to test gating factors on aquaporins directly and without the complication of indirect effects caused by the cell metabolic machinery and second messenger systems within the cytoplasm. There are other reported apparent direct gating responses of aquaporins to mechanical stress and reactive oxygen species that may well be mediated via cytoplasmic pCa and pH changes.

As a first approach, medium acidification was tested in the system. It is well known that pH is an important factor in modulating properties of membrane transport (Netting, 2002). It has been reported that acidic cytoplasmic pH has an inhibitory effect on the water transport, reducing water permeability of some animal membranes (Parisi *et al.*, 1983, 1984*a*, *b*) and plant membranes (Amodeo *et al*., 2002; Gerbeau *et al.*, 2002; Sutka *et al*., 2005). Furthermore, it was demonstrated that cytoplasmic acidification completely shuts down activity of PIP overexpressed in *Xenopus* oocytes (Tournaire-Roux *et al.*, 2003). The gating mechanism of the inhibitory effect was explained by the protonation of a conserved histidine residue located on an extra-membrane loop of PIPs. Because this residue is perfectly conserved in all plant PIP aquaporins, P_f inhibition was expected to be observed in the plasma membrane preparation at low pH.

The results reported here show that *Beta vulgaris* plasma membrane vesicles are extremely sensitive to acidic pH with P_f half-maximum inhibition at pH 6.6. It has been

reported that *Arabidopsis thaliana* roots reduce their hydraulic conductivity under anoxia, one of the main factors that triggers cytoplasmic acidification (Tournaire-Roux *et al.*, 2003). Therefore, it is likely that *Beta vulgaris* roots also react to anoxic stress by closure of plasma membrane aquaporins. There are other stresses that are putative candidates to cause cytoplasmic acidification. Although the effects of salt stress on cytoplasmic pH are controversial (Colmer *et al.*, 1994; Halperin and Lynch, 2003) some evidence involving chilling (Yoshida *et al.,* 1999) and pathogen elicitors (Viehweger *et al.*, 2002) have been described. Due to the high amount of PIPs and the conserved pH response, these conditions are also likely to reduce plasma membrane water permeability through closure of aquaporins.

The present findings provide a complementary approach to study the pH effect on PIP gating beyond the already described overexpression of the proteins in *Xenopus* oocytes. It was possible to discriminate the sidedness of the pH effect due to the vesicle preparations having a high proportion of right-side-out vesicles (outside-out, therefore exposing the apoplastic side of the membrane) and through the use of temporary pH gradients induced by the stopped-flow technique. The present results show that only when the pH was low inside the vesicles, was the water permeability strongly reduced, in agreement with the location of the pH sensor in PIPs (Tournaire-Roux *et al*., 2003).

Another interesting aspect that characterizes this preparation is that, despite the low sensitivity to mercury, water permeation is all but shut down with low pH and/or high calcium concentrations. It is therefore possible to postulate that a significant population of plasma membrane aquaporins is insensitive to $HgCl_2$ and that tests of PIP activity should also incorporate the use of acidic pH and/or calcium.

Sensitivity to divalent cations was also tested on *Beta vulgaris* plasma membrane vesicles. The effect found for calcium was strongest compared with magnesium and barium, reducing water permeability by up to 95%. A calcium inhibitory effect has also been reported for *Arabidopsis thaliana* (Gerbeau *et al*., 2002), suggesting a direct effect of calcium on some aquaporins but, in this case, only a low sensitivity range of calcium concentrations was observed. A direct action of calcium on water channels has also been suggested in animal systems. Fotiadis *et al*. (2002) point to the existence of a putative Ca^{2+}-binding site at the C-terminus of AQP1 due to the striking sequence homology found between the AQP1 C-terminus and EF-hands from Ca^{2+}-binding proteins belonging to the calmodulin superfamily.

Evidence in favour of a blocking action of calcium on aquaporins in the present system is not only supported by the high specificity but also by the increase in the E_a. When plasma membrane vesicles are exposed to high concentrations of calcium, E_a increases indicating that, under these experimental conditions, water movements through a proteinacous pore are minimized. Further experiments demonstrated that calcium present in the intra-vesicular solution had the main effect. Therefore the present results show, for the first time in plant aquaporins, that it is the cytoplasmic face of the protein that is sensitive to calcium. This experimental approach is validated by the above-mentioned results describing the sidedness of the pH effect.

Interestingly, the dose–response curve for calcium sensitivity of P_f in *Beta vulgaris* plasma membrane is a biphasic one, with one component in the nanomolar range and another in the micromolar range. Although the involvement of a modifying enzyme or a protein partner with calcium-dependent activity or affinity cannot be completely discounted, the dose–response curve could be interpreted as PIPs presenting two binding sites with high and low sensitivity for calcium, respectively, or a mixed population of PIPs with different calcium sensitivity. In the nanomolar range of Ca^{2+} concentrations the largest effect was observed on P_f with an IC50 of pCa 8.3, corresponding to a free calcium concentration of 5 nM. This is rather low compared with physiological Ca^{2+} concentrations in the cytoplasm of around 100 nM; however, there may be other factors *in vivo* that counteract this strong Ca^{2+} sensitivity that are missing in vesicle preparations used in the present study. Because the relationship is so steep, the large Hill coefficient must be considered the best approximation derived from the fit. In some particular cases, high Hill coefficients have also been reported (Gilabert *et al*., 2001). The classic interpretation for this coefficient is commonly used to estimate the magnitude of co-operativity in ligand binding. However, recently it was reported that this coefficient could also be theoretically interpreted as an indication of channel gating behaviour, estimating the magnitude of co-operativity in gating transitions (Yifrach, 2004). It is not possible to come to any definitive explanation for this behaviour shown with P_f in the present work, but the results point unambiguously to P_f showing high sensitivity to low calcium concentrations.

The reduction in P_f that occurred at higher calcium concentrations (100–200 μM) is similar to that already reported by Gerbeau *et al.* (2002) for *Arabidopsis* suspension cell plasma membrane vesicles, with very similar characteristics in terms of IC50 (~100 μM) and a Hill coefficient near 1. By contrast, Gerbeau *et al.* (2002) did not observe the other water channel component with high calcium sensitivity, which may not be expressed in suspension cells. It remains to be seen what the free Ca^{2+} concentration is in beet root cells under normal physiological conditions, but if it is assumed that it is in the order of 100 nM, this sits between the two sensitivity ranges that have been observed for P_f and may indicate that PIPs could be turned on and off by changes in cytosolic Ca^{2+} concentrations.

Phosphorylation/dephosphorylation processes are known to be an important point in regulating water channel activity

(Javot and Maurel, 2002). Several aquaporins that contain consensus sequences for phosphorylation have been shown to be regulated in this way, at least when expressed in *Xenopus* oocytes (Maurel *et al.*, 1995; Johansson *et al.*, 1998, Guenther *et al.*, 2003). Considering the potential for loss of water transport activity in standard plasma membrane vesicle preparations, Gerbeau *et al.* (2002) developed an alternative vesicle isolation protocol in which possible dephosphorylation of the channel was prevented. With the aim of comparing the present results with those of other researchers, *Beta vulgaris* plasma membrane vesicles were isolated following Gerbeau's modified protocol. No P_f augmentation or decrease was found when vesicles were isolated in the presence of dephosphorylation-protecting agents. These results could suggest the absence of control of *Beta vulgaris* root plasma membrane aquaporins by phosphorylation. Alternatively, there may be no significant membrane-associated phosphatase during isolation of vesicles that could dephosphorylate the aquaporins in *Beta vulgaris* root cells.

In conclusion, *Beta vulgaris* plasma membrane vesicles show high P_f, which is completely inhibited at low pH and calcium, two short-term regulatory mechanisms that can be triggered from the cytoplasmic side.

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References

Agre P, Mathai JC, Smith BL, Preston GM. 1999. Functional analyses of aquaporin water channel proteins. *Methods in Enzymology* **294,** 550–572.

Amodeo G, Dorr R, Vallejo A, Sutka M, Parisi M. 1999. Radial and axial water transport in sugar beet storage root. *Journal of Experimental Botany* **50,** 509–516.

Amodeo G, Sutka M, Dorr R, Parisi M. 2002. Protoplasmatic pH modifies water and solute transfers in *Beta vulgaris* root vacuoles. *Journal of Membrane Biology* **187,** 175–184.

Aroca R, Amodeo G, Fernandez-Illescas S, Herman EM, Chaumont F, Chrispeels MJ. 2005. The role of aquaporins and membrane damage in chilling and hydrogen peroxide induced changes in the hydraulic conductance of maize roots. *Plant Physiology* **137,** 341–353.

Baizabal-Aguirre VM, Gonzalez de la Vara LE. 1997. Purification and characterization of a calcium regulated protein kinase from beet root *Beta vulgaris* plasma membranes. *Physiologia Plantarum* **99,** 135–143.

Barone LM, Mu HH, Shih CJ, Kashlan KB, Wasserman BP. 1998. Distinct biochemical and topological properties of the 31- and 27-kilodalton plasma membrane intrinsic protein subgroups from red beet. *Plant Physiology* **118,** 315–322.

Barone LM, Shih C, Wasserman BP. 1997. Mercury-induced conformational changes and identification of conserved surface loops in plasma membrane aquaporins from higher plants: topology of PMIP31 from *Beta vulgaris* L. *Journal of Biological Chemistry* **272,** 30672–3077.

Bennet AB, Spanswick RM. 1984. H^+-ATPase activity from storage tissue of *Beta vulgaris*. *Plant Physiology* **74,** 545–548.

Biber J, Malmström K, Scalera V, Murer H. 1983. Phosphorylation of rat kidney proximal tubular brush border membranes: role of AMPc dependent protein phosphorylation in the regulation of phosphate transport. *Pflügers Archiv* **398,** 221–226.

Biela A, Grote K, Otto B, Hoth S, Hedrich R, Kaldenhoff R. 1999. The *Nicotiana tabacum* plasma membrane aquaporin NtAQP1 is mercury-insensitive and permeable for glycerol. *The Plant Journal* **18,** 565–570.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Briskin DP, Leonard RT, Hodkes TK. 1987. Isolation of the plasma membrane: membrane markers and general principles. *Methods in Enzymology* **148,** 542–558.

Blumwald E, Cragoe EJ, Poole RJ. 1987. Inhibition of Na^+/H^+ antiport activity in sugar beet tonoplast by analogs of amiloride. *Plant Physiology* **85,** 30–33.

Colmer TD, Fan TWM, Higashi RM, Lauchli A. 1994. Interactions of Ca^{2+} and NaCl stress on the ion relations and intracellular pH of *Sorghum bicolor* root-tips: an *in-vivo* P31-NMR study. *Journal of Experimental Botany* **45,** 1037–1044.

Daniels MJ, Mirkov TE, Chrispeels MJ. 1994. The plasma membrane of *Arabidopsis thaliana* contains a mercury-insensitive aquaporin that is homolog of the tonoplast water channel protein TIP. *Plant Physiology* **106,** 1325–1333.

Dordas C, Chrispeels MJ, Brown PH. 2000. Permeability and channel-mediated transport of boric acid across membrane vesicles isolated from squash roots. *Plant Physiology* **124,** 1349–1362.

Fabiato A, Fabiato F. 1979. Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal of Physiology* **75,** 463–505.

Fotiadis D, Suda K, Tittmann P, Jeno P, Philippsen A, Muller DJ, Gross H, Engel A. 2002. Identification and structure of a putative Ca^{2+}-binding domain at the C terminus of AQP1. *Journal of Molecular Biology* **318,** 1381–1394.

Gerbeau P, Amodeo G, Henzler T, Santoni V, Ripoche P, Maurel C. 2002. The water permeability of Arabidopsis plasma membrane is regulated by divalent cations and pH. *The Plant Journal* **30,** 71–81.

Gilabert JA, Bakowski D, Parekh AB. 2001. Energized mitochondria increase the dynamic range over which inositol 1,4,5-trisphosphate activates store-operated calcium influx. *EMBO Journal* **20,** 2672–2679.

Guenther JF, Chanmanivone N, Galetovic MP, Wallace IS, Cobb JA, Roberts DM. 2003. Phosphorylation of soybean nodulin 26 on serine 262 enhances water permeability and is regulated developmentally and by osmotic signals. *The Plant Cell* **15,** 981–991.

Halperin SJ, Lynch JP. 2003. Effects of salinity on cytosolic Na^+ and K^+ in root hairs of *Arabidopsis thaliana*: *in vivo* measurements using the fluorescent dyes SBFI and PBFI. *Journal of Experimental Botany* **54,** 2035–2043.

Javot H, Maurel C. 2002. The role of aquaporins in root water uptake. *Annals of Botany* **90,** 301–313.

Johansson I, Karlsson M, Shukla VK, Chrispeels MJ, Larsson C, Kjellbom P. 1998. Water transport activity of the plasma

membrane aquaporin PM28A is regulated by phosphorylation. *The Plant Cell* **10,** 451–459.

Johansson I, Larsson C, Ek B, Kjellbom P. 1996. The major integral proteins of spinach leaf plasma membranes are putative aquaporins and are phosphorylated in response to Ca^{2+} and apoplastic water potential. *The Plant Cell* **8,** 1181–1191.

Johnson KD, Chrispeels MJ. 1992. Tonoplast-bound protein kinase phosphorylates tonoplast intrinsic protein. *Plant Physiology* **100,** 1787–1795.

Katsuhara M, Akiyama Y, Koshio K, Shibasaka M, Kasamo K. 2002. Functional analysis of water channels in barley roots. *Plant Cell Physiology* **43,** 885–893.

Larsson C, Sommarin M, Widell S. 1994. Isolation of highly purified plant plasma membranes and separation of inside-out and right-side-out vesicles. *Methods in Enzymology* **228,** 451–469.

Lino B, Baizabal-Aguirre VM, Gonzalez de la Vara LE. 1998. The plasma-membrane H^+-ATPase from beet root is inhibited by a calcium-dependent phosphorylation. *Planta* **204,** 352–359.

Martinez-Ballesta MDC, Martinez V, Carvajal M. 2000. Regulation of water channel activity in whole roots and in protoplasts from roots of melon plants grown under saline conditions. *Australian Journal of Plant Physiology* **27,** 685–691.

Maurel C. 1997. Aquaporins and water permeability of plant membranes. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 399–429.

Maurel C, Javot H, Lauvergeat V, Gerbeau P, Tournaire C, Santoni V, Heyes J. 2002. Molecular physiology of aquaporins in plants. *International Review of Cytology* **215,** 105–148.

Maurel C, Kado RT, Guern, Chrispeels MJ. 1995. Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP. *EMBO Journal* **14,** 3028–3035.

Maurel C, Tacnet F, Güclü J, Guern J, Ripoche P. 1997. Purified vesicles of tobacco cell vacuolar and plasma membranes exhibit dramatically different water permeability and water channel activity. *Proceedings of the National Academy of Sciences, USA* **94,** 7103–7108.

Morillon R, Chrispeels MJ. 2001. The role of ABA and the transpiration stream in the regulation of the osmotic water permeability of leaf cells. *Proceedings of the National Academy of Sciences, USA* **98,** 14138–14143.

Morillon R, Lassalles JP. 1999. Osmotic water permeability of isolated vacuoles. *Planta* **210,** 80–84.

Morillon R, Lassalles JP. 2002. Water deficit during root development: effects on the growth of roots and osmotic water permeability of isolated root protoplasts. *Planta* **214,** 392–399.

Moshelion M, Becker D, Biela A, Uehlein N, Hedrich R, Otto B, Levi H, Moran N, Kaldenhoff R. 2002. Plasma membrane aquaporins in the motor cells of *Samanea saman*: diurnal and circadian regulation. *The Plant Cell* **14,** 727–739.

Moshelion M, Moran N, Chaumont F. 2004. Dynamic changes in the osmotic water permeability of protoplast plasma membrane. *Plant Physiology* **135,** 2301–2317.

Nemeth-Cahalan KL, Kalman K, Hall JE. 2004. Molecular basis of pH and Ca^{2+} regulation of aquaporin water permeability. *Journal of General Physiology* **123,** 573–580.

Netting AG. 2002. pH, abscisic acid and the integration of metabolism in plants under stressed and non-stressed conditions. II. Modifications in modes of metabolism induced by variation in the tension on the water column and by stress. *Journal of Experimental Botany* **53,** 151–173.

Niemietz CM, Tyerman SD. 1997. Characterization of water channels in wheat root membrane vesicles. *Plant Physiology* **115,** 561–567.

Niemietz CM, Tyerman SD. 2002. New potent inhibitors of aquaporins: silver and gold compounds inhibit aquaporins of plant and human origin. *FEBS Letters* **531,** 443–447.

Parisi M, Bourguet J. 1984*a.* Effects of cellular acidification on ADH-induced intramembrane particle aggregates. *American Journal of Physiology* **246,** C157–C159.

Parisi M, Wietzerbin J. 1984*b.* Cellular pH and the ADH-induced hydrosmotic response in different ADH target epithelia. *Pflügers Archiv* **402,** 211–215.

Parisi M, Wietzerbin J, Bourguet J. 1983. Intracellular pH, transepithelial pH gradients and ADH-induced water channels. *American Journal of Physiology* **244,** F712–F718.

Poole RJ, Brinskin DP, Krátký Z, Johnstone RM. 1984. Density gradient localization of plasma membrane and tonoplast from storage tissue of growing and dormant red beet. *Plant Physiology* **74,** 549–556.

Qi X, Tai CH, Wasserman BP. 1995. Plasma membrane intrinsic protein of *Beta vulgaris* L. *Plant Physiology* **108,** 387–392.

Ramahaleo T, Morillon R, Alexandre J, Lassalles JP. 1999. Osmotic water permeability of isolated protoplast: modifications during development. *Plant Physiology* **119,** 885–896.

Siefritz F, Tyree MT, Lovisolo C, Schubert A, Kaldenhoff R. 2002. PIP1 plasma membrane aquaporins in tobacco: from cellular effects to function in plants. *The Plant Cell* **14,** 869–876.

Steudle E. 1994. Water transport across roots. *Plant and Soil* **167,** 79–90.

Steudle E. 2000. Water uptake by roots: an integration of views. *Plant and Soil* **226,** 45–56.

Steudle E, Peterson CA. 1998. How does water get through roots? *Journal of Experimental Botany* **49,** 775–788.

Suga S, Murai M, Kuwagata T, Maeshima M. 2003. Differences in aquaporin levels among cell types of radish and measurement of osmotic water permeability of individual protoplasts. *Plant Cell Physiology* **44,** 277–286.

Sutka M, Alleva K, Parisi M, Amodeo G. 2005. Tonoplast vesicles of *Beta vulgaris* storage root show functional aquaporins regulated by protons. *Biology of the Cell* **97,** 837–846.

Tournaire-Roux C, Sutka M, Javot H, Gout E, Gerbeau P, Luu DT, Bligny R, Maurel C. 2003. Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. *Nature* **425,** 393–397.

Tsai ST, Zhang RB, Verkman AS. 1991. High channel-mediated water permeability in rabbit erythrocytes: characterization in native cells and expression in *Xenopus* oocytes. *Biochemistry* **30,** 2087–2092.

Tyerman SD, Niemietz CM, Bramley H. 2002. Plant aquaporins: multifunctional water and solute channels with expanding roles. *Plant, Cell and Environment* **25,** 173–194.

Uno Y, Urano T, Yamaguchi-Shinozaki K, Kanechi M, Inagaki N, Maekawa S, Shinozaki K. 1998. Early salt-stress effects on expression of genes for aquaporin homologues in the halophyte sea aster (*Aster tripolium* L.). *Journal of Plant Research* **111,** 411–419.

Vander Willigen C, Verdoucq L, Boursiac Y, Maurel C. 2004. Aquaporins in plants In: Blatt MR, ed. *Membrane transport in plants*. Annual Plant Reviews **8,** Sheffield: Blackwell Publishing, 221–250.

van Heeswijk MP, van Os CH. 1986. Osmotic water permeability of brush border and basolateral membrane vesicles from rat renal cortex and small intestine. *Journal of Membrane Biology* **92,** 183–193.

Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O. 2004. Novel regulation of aquaporins during osmotic stress. *Plant Physiology* **135,** 2318–2329.

Verkman AS, Dix JA, Seifter JL. 1985. Water and urea transport in renal microvillus membrane vesicles. *American Journal of Physiology* **248,** F650–F655.

Verkman AS, Mitra AK. 2000. Structure and function of aquaporin water channels. *American Journal of Physiology* **278,** F13–F28.

Viehweger K, Dordschbal B, Roos W. 2002. Elicitor-activated phospholipase A_2 generates lysophosphatidylcholines that mobilize the vacuolar H^+ pool for pH signaling via the activation of Na^+-dependent proton fluxes. *The Plant Cell* **14,** 1509–1525.

Wan XC, Steudle E, Hartung W. 2004. Gating of water channels (aquaporins) in cortical cells of young corn roots by mechanical stimuli (pressure pulses): effects of ABA and of $HgCl_2$. *Journal of Experimental Botany* **55,** 411–422.

Yifrach O. 2004. Hill coefficient for estimating the magnitude of cooperativity in gating transitions of voltage-dependent ion channels. *Biophysical Journal* **87,** 822–830.

Yoshida S, Hotsubo K, Kawamura Y, Murai M, Arakawa K, Takezawa D. 1999. Alterations of intracellular pH in response to low temperature stresses. *Journal of Plant Research* **112,** 225–236.

Zhang WH, Tyerman SD. 1999. Inhibition of water channels by $HgCl_2$ in intact wheat root cells. *Plant Physiology* **120,** 849–857.