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ORIGINAL ARTICLE

Two different effects of calcium on aquaporins in salinity-stressed pepper plants

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Abstract Two different effects of calcium were studied, respectively, in plasma membrane vesicles and in protoplasts isolated from roots of control pepper plants (*Capsicum annuum* L cv. California) or of plants treated with 50 mM NaCl, 10 mM $CaCl_2$ or 10 mM $CaCl_2$ + 50 mM NaCl. Under saline conditions, osmotic water permeability (P_f) values decreased in protoplasts and plasma membrane vesicles, and the same reduction was observed in the PIP1 aquaporin abundance, indicating inhibitory effects of NaCl on aquaporin functionality and protein abundance. The cytosolic Ca^{2+} concentration, $[Ca^{2+}]_{cyt}$, was reduced by salinity, as observed by confocal microscope analysis. Two different actions of Ca^{2+} were observed. On the one hand, increase in free cytosolic calcium concentrations associated with stress perception may lead to aquaporin closure. On the other hand, when critical requirements of Ca^{2+} were reduced (by salinity), and extra-calcium would lead to an upregulation of aquaporins, indicating that a positive role of calcium at whole plant level combined with an inhibitory mechanism at aquaporin level may work in the regulation of pepper root water transport under salt stress. However, a link between these observations and other cell signalling in relation to water channel gating remains to be established.

Keywords Aquaporin · Calcium · Osmotic water permeability · Salinity

Abbreviations

DTT	Dithiothreitol
BSA	Bovine serum albumine
SDS	Sodium dodecyl sulphate
DAB	3,3′Diaminobenzidine
DMSO	Dimethyl sulfoxide

Introduction

Roots show a remarkable capacity to adjust their properties to the physiological water demand of the whole plant and can alter their water permeability over the short term in response to several environmental stresses (see Maurel et al. 2002 for review; Javot and Maurel 2002). In most conditions, an intense radial transport of water occurs across living root tissues, along three main paths, namely the apoplastic, symplastic, and transcellular paths. This transport allows maintaining an optimal water balance, and supports transpiration and growth (Steudle and Peterson 1998; Steudle 2000). In most of the plant species investigated, pharmacological and/or genetic evidence has suggested that aquaporins represent a major path during water uptake by roots (Javot and Maurel 2002).

One of the primary responses of plants to salt stress is the inhibition of their root water uptake capacity (see Martínez-Ballesta et al. 2006 for review). Plants grown under salt stress reduce root hydraulic conductance (L_0),

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involving aquaporins, but the response of aquaporin gene expression and the upstream signalling mechanism are still unclear. Salinity has been shown to decrease the amounts of mRNA encoding PIP aquaporins in *Arabidopsis* (Maathuis et al. 2003; Martínez-Ballesta et al. 2003; Boursiac et al. 2005) and barley (Katsuhara et al. 2002, 2003a, b). However, Suga et al. (2002) showed, in radish seedlings, that the mRNA and protein levels of different PIPs and TIPs remained unchanged after NaCl addition. The up-regulation by salt stress of certain aquaporin isoforms in *Arabidopsis* and in rice plants was also observed in other reports (Liu et al. 1994; Seki et al. 2002). In *Arabidopsis*, aquaporin protein levels significantly dropped with a delay of several hours with respect to mRNAs (Boursiac et al. 2005). In addition, immunodetection of specific aquaporin isoforms was reported in certain studies, but a great difficulty was to obtain isoform-specific antibodies due to the high sequence homology between members of the same family (Barkla et al. 1999; Kirch et al. 2000).

The molecular and cellular mechanisms for regulation of aquaporins under normal or stress conditions have been studied by many laboratories (for reviews, see Chaumont et al. 2005; Luu and Maurel 2005). Different stimuli can induce changes in aquaporin phosphorylation or protonation, thus playing an important role in aquaporin gating under stress conditions (Johansson et al. 1998; Guenther et al. 2003; Tournaire-Roux et al. 2003). Experiments with purified membranes and organelles have shown that water transport in isolated sugar beet vacuoles is inhibited by acidic pH (Amodeo et al. 2002) and that the water permeability of *Arabidopsis* and *Beta vulgaris* plasma membrane is down-regulated by Ca^{2+}, in addition to low pH (Gerbeau et al. 2002; Alleva et al. 2006). Therefore, an increase in osmotic water permeability of the *Arabidopsis* plasma membrane was observed when isolation of plasma membrane vesicles was performed under protective conditions that prevented desphosphorylation or the effect of divalent ions (Gerbeau et al. 2002).

Calcium is an essential divalent cation and plant nutrient, which plays structural roles in the cell wall and membranes, and acts as a counter-cation for inorganic and organic anions in the vacuole. Calcium is also a crucial intracellular messenger (Marschner 1995; White and Broadley 2003). In a recent report, it has been demonstrated that the inhibitory effects of $HgCl_2$ (a common aquaporin blocker but also a general metabolism inhibitor) on root water transport in maize, were only seen provided that the plant nutrient solution contained Ca^{2+} (Ionenko et al. 2006). These data were interpreted to mean that Ca^{2+} plays an important role in the up-regulation of water channel activity. They are consistent with the results in spinach (*Spinacia oleracea*) by Johansson et al. (1996, 1998), who demonstrated the dependence of aquaporin phosphorylation and therefore water transport activity on sub-micromolar concentrations of Ca^{2+}. Recent results with pepper plants have also shown that calcium seems to be involved in plasma membrane aquaporin regulation via a chain of processes within the cell but not by alteration of the stability of the plasma membrane (Cabañero et al. 2006).

Although most of the previous studies have focused on individual roles of Ca^{2+} on water transport regulation, either at the membrane (Gerbeau et al. 2002; Alleva et al. 2006) or at the cell or root level (Carvajal et al. 2000; Ionenko et al. 2006), the aim of this paper was to investigate how Ca^{2+} can have multiple, and possibly opposite effects in influencing the function of aquaporins. This question was addressed in pepper (*Capsicum annuum* L.) plants grown in control and salinity conditions. Following a previous work by Cabañero et al. (2006), we investigated here the possible co-existence of a regulatory pathway operating through Ca^{2+} availability for the plant with the direct influence of Ca^{2+} on aquaporin protein gating. For this, studies were carried out with purified vesicles and protoplasts isolated from roots of plants exposed to various calcium or salt supplies. We then compared the osmotic water permeability coefficients (P_f) of the purified vesicles and protoplasts, as a function of the plant growth conditions and/or of the presence of exogenous calcium. Further, studies on the effect of Ca^{2+} on aquaporin abundance and root radial distribution were performed, together with studies of intracellular Ca^{2+} availability with confocal microscopy.

Materials and methods

Plant culture

Seeds of pepper (*Capsicum annum* L cv. California) kindly provided by Ramiro Armedo S.A (Calahorra, La Rioja, Spain) were imbibed with de-ionised water for 2 days and placed in an incubator chamber at 30°C, in darkness. The seeds were placed in trays with vermiculite as substrate. After 4–5 days, they were placed in 15-L containers (about 30 plants per container), containing Hoagland nutrient solution (Epstein 1972), and transferred to the definitive controlled environment chamber, with a 16-h light, 8-h dark cycle and air temperatures of 25°C and 20°C, respectively. The relative humidity (RH) was 60% (light period) and 80% (dark), and the photosynthetically active radiation (PAR) was 400 μmol m^{-2} s^{-1}, provided by a combination of fluorescent tubes (TLD 36 W/83, Philips, Hamburg, Germany, and F36 W/GRO, Sylvania, Danvers, MA, USA) and metal-halide lamps (HQI, T 400 W; Osram, München, Germany). After approximately 15 days of growth, plants were transferred to new containers (with the same

characteristics), where different treatments were applied: control untreated plants, plants treated for 3 days with 50 mM NaCl, 10 mM $CaCl_2$, or 10 mM $CaCl_2$ prior to exposure to 50 mM NaCl for three additional days.

Protoplast isolation

Chopped root segments from the apical 3–6 cm of the primary roots were digested in a solution containing 0.6 M mannitol, 1 mM $CaCl_2$, 0.5 mM dithiothreitol (DTT), 2% (w/v) cellulase, 0.1% (w/v) pectolyase, and 0.05% (w/v) BSA (pH 5.6). We placed approximately 5 g of roots in 20 mL of this solution and finally added 0.05 g of polyvinylpyrrolidone (PVP). After 3.5 h of gentle shaking at 27°C, protoplasts were separated from partially digested tissue by repeated filtration through 60-μm nylon mesh. The filtrate was centrifuged at 150*g* for 6 min. The pellet was carefully suspended in a suspension medium containing 0.7 M mannitol, 0.1 mM $CaCl_2$, 0.5 mM DTT, and 5 mM Tris-MES, pH 6.5, and centrifugation was repeated. The pellet was then suspended in a final solution containing 0.7 M mannitol, 0.2 mM KNO_3, 10 μM $CaCl_2$, 0.1 mM NaH_2PO_4, 0.5 mM DTT, and 5 mM Tris-MES, pH 6.5. Protoplasts selected for P_f measurements had diameters between 20 and 40 μm.

Measurement of osmotic water permeability

Protoplast suspension (15 μL) was dispersed on the central grid part of a counting chamber, and a single protoplast was held by the tip of a microcapillary. Cortical cells were chosen for the experiments as they contribute highly to water uptake (Steudle and Peterson 1998). Protoplasts obtained from cortical cells could be recognised easily by their bigger size. The same volume of water was added, to reduce the osmotic potential of the medium. Changes in the cell volume were photographed with a digital camera to obtain 16 images in 5 s. Calculations of cell volume were carried out using the Leica Q500 computer programme (Leica Microsistemas, SA, Barcelona, Spain) (Carvajal et al. 2000). The initial changes in protoplast volume with time (0–3 s) were used for the determination of a linear expansion rate, *k*. The osmotic water permeability coefficient, P_f, was calculated according to the following equation (Zhang and Verkman 1991):

$$P_f = (V_0 / S_0)[k / Vw\ (Osmol_{in} - Osmol_{out})] \quad (1)$$

where V_0 is the initial cell volume, S_0 is the initial cell surface area, V_w is the molar volume of water (18×10^{-6} m^3 mol^{-1}), *k* is the expansion rate of the initial phase of the swelling (fitted from recordings with 5–12% increase in volume) and $Osmol_{in}$ and $Osmol_{out}$ are the internal and external osmolalities, respectively (Zhang and Verkman 1991). Measurements of osmolality (Osmol kg^{-1} H_2O) were carried out using an osmometer (Digital Osmometer, Roebling, Berlin, Germany). A sample of cells was centrifuged (150*g*), and the resulting pellet frozen and centrifuged again (1,000*g* for 5 min) in an Eppendorf tube. The cell sap was obtained from the supernatants after centrifugation at 15,000*g* and osmolality was then measured. To study the functionality of aquaporins, mercuric chloride was added as an aquaporin blocker. In all cases, 50 μM $HgCl_2$ was added to the cell suspensions for 5 min. The suspensions were then centrifuged twice at 1,000*g* for 2 min, and the cell pellets resuspended in fresh culture medium. An aliquot was taken for P_f measurements and DTT (2 mM) was added to the cell suspension. After 20 min, P_f was measured again (Carvajal et al. 2000).

Plasma-membrane purification

Plasma membranes were isolated, using the two-phase aqueous polymer technique, for Western-blot assays (method I) according to Larsson et al. (1987) or for stopped-flow light scattering measurements (method II) according to Gerbeau et al. (2002). All procedures were performed at 4°C. Approximately 20 g of fresh root material were chopped finely and vacuum-infiltrated with 40 mL of 50 mM Hepes and 0.5 M sucrose, adjusted to pH 7.5 with NaOH, plus 1 mM DTT, 5 mM ascorbic acid and 0.6% insoluble PVP (w/v) (method I) or 40 mL of 500 mM sucrose, 10% glycerol, 20 mM Na_2EDTA, 20 mM EGTA, 50 mM NaF, 5 mM *β*-glycerophosphate, 1 mM 1,10-phenantroline, 1 mM Na_3VO_4, 0.6% PVP, 10 mM ascorbic acid, 5 mM DTT, 0.5 mg L^{-1}, leupeptin and 50 mM Tris-MES, pH 8.0 (method II). In both methods, the buffer-saturated material was homogenised, using a pestle and mortar, and filtered through a 240-μm nylon cloth. The filtrate was centrifuged at 10,000*g* for 15 min and the supernatant further centrifuged at 55,000*g* for 35 min, at 4°C, to yield a microsomal pellet, which was resuspended in 1 mL of 0.33 M sucrose in 5 mM phosphate buffer pH 7.8 (method I) or 0.33 M sucrose, 2 mM DTT, 10 mM NaF and 5 mM potassium phosphate, pH 7.8 (method II). The suspension (2 mL) was added to 6 g of an aqueous two-phase mixture, producing an 8 g two-phase system with a final composition of 5.8% (w/v) Dextran T500 (Pharmacia), 5.8% (w/v) polyethylene-glycol (PEG) 3.350 (Sigma), 3 mM KCl, 5 mM phosphate buffer (pH 7.8) and 0.33 M sucrose. The phase-system was centrifuged for 5 min at 4,000*g*. The resulting plasma membranes (upper phase) were purified using a batch procedure (Larsson et al. 1987). The third upper phase was diluted with phosphate buffer, pH 7.8 (method I) or washed with 9 mM KCl, 300 mM sucrose, 0.2 M EDTA, 0.2 M EGTA, 0.5 M NaF and 10 mM Tris-borate, pH 8.3 (method II) and centrifuged at

55,000*g* for 35 min; the resulting pellet was resuspended in 1 mL of 5 mM MES-Tris plus 0.25 M sucrose, pH 6.5 (method I) or 9 mM KCl, 300 mM sucrose, 0.2 M EDTA, 0.2 M EGTA, 0.5 M NaF, 2 mg L^{-1} leupeptin, 1 M DTT and 10 mM Tris-borate, pH 8.3 (method II), and stored at −80°C before use.

Plant plasma membrane isolation was performed 3 days after the ionic (NaCl, $CaCl_2$, $CaCl_2$ + NaCl) treatments. Yield (0.1 mg proteins g^{-1} FW) and purity of plasma membrane preparation was checked by marker enzymes (data not shown) and was similar for all the treatments. Protein concentration was determined by the *RC DC* protein assay kit (Bio-Rad) following the recommendations of the manufacturer.

Stopped-flow light scattering

The kinetics of PM vesicle volume adjustment were followed by 90° light scattering at $\lambda_{ex} = 515$ nm. Measurements were carried out at 20°C in an SFM3 stopped-flow spectrophotometer (Biologic, Claix, France) as previously described (Maurel et al. 1997; Gerbeau et al. 2002). Purified membrane vesicles were diluted 100-fold into a buffer containing 30 mM KCl and 20 mM Tris-MES, pH 8.3 (90 mOsmol kg^{-1} H_2O). Alternatively, membranes were diluted in the same medium but adjusted to pH 6.0 or containing 0.3 mM Ca^{2+}. The hypo-osmotic shock associated with membrane dilution induced a transient opening of vesicles and equilibration of their interior with the extravesicular solution (Biber et al. 1983). Therefore, although the sidedness of the vesicles was unknown, the transient opening of the vesicles should have allowed an access of Ca^{2+} to the cytosolic side of the membrane (Gerbeau et al. 2002). Vesicles were mixed with an equal volume of the same buffer used for membrane vesicle equilibration but with a sucrose concentration of 540 mM (630 mOsmol kg^{-1} H_2O). This resulted in a 270 mOsmol kg^{-1} H_2O inward osmotic gradient. The P_f was computed from the light scattering time-course according to the following equation:

$$P_f = k_{exp}\ V_0 / A_v\ V_w\ C_{out} \quad (2)$$

where k_{exp} is the fitted exponential rate constant, V_0 is the initial mean vesicle volume, A_v is the mean vesicle surface, V_w is the molar volume of water, and C_{out} is the external osmolality (Maurel et al. 1997). For each plant treatment, we characterised at least two independent membrane preparations with a total of $n = 5$ measurements.

Size of vesicles

The size of the membrane vesicles was estimated by microscopy in one representative preparation of each ionic treatment. For this, plasma membrane (1.0 mg membrane protein ml^{-1}) was fixed by addition of 5% (v/v) glutaraldehyde, 5 mM potassium phosphate, pH 7.8, and 330 mM sucrose, in a volume ratio of 1:1, and incubated at 4°C for 1 h. Then, the membranes were collected by centrifugation at 100,000*g* for 45 min. The pellets were transferred to Eppendorf tubes and washed (six times) in 0.15 M potassium phosphate, pH 7.2. After post-fixation in 1% (w/v) OsO_4 for 2 h, the pellets were again washed twice with 0.15 M potassium phosphate buffer. Dehydration was carried out stepwise in 50, 75, 96% and absolute ethanol (2 × 10 min for each concentration). Then, the pellets were washed twice in propylene oxide during 20 min and incubated in Spurr resin and propylene oxide in a volume ratio of 1:1 during 1 h. After a new Spurr resin infiltration overnight, polymerisation took place in Spurr resin at 70°C for 24 h. Ultra-thin sections were post-stained in 2% (w/v) uranyl acetate and Pb nitrate and studied in a Philip technique 12 electronic microscope.

Gel electrophoresis and immunoblotting

The same amount of plasma membrane proteins (12 μg per lane) were loaded for 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins had been previously denatured, by incubation at 56°C for 20 min in the presence of 2% (w/v) SDS and 100 mM DTT, to separate the dimers of the aquaporins (Borgnia et al. 1999). The proteins were transferred to a PVDF membrane, for 1 h at 100 V. The membrane was blocked for 1 h at room temperature, in 0.1% (w/v) casein in Tris-buffered saline (TBS). After that, the membrane was incubated in TTBS (TBS with 0.05% Tween 20) in the presence of an anti-peptide antibody raised against the 42 N-terminal residues of *Arabidopsis* PIP1;1 (Kammerloher et al. 1994) (dilution 1:3,000; kindly provided by Prof. Dr. Schäffner), for 1 h at room temperature. Goat anti-rabbit IgG, coupled to horseradish peroxidase, was used as the secondary antibody (1:20,000). The signal was developed using DAB as the colorimetric substrate. For relative quantification, we measured the intensity of each plot using the Quantity one software (BioRad) and considered the control pepper intensity as 100%.

PIP1 aquaporin tissue printing on nitrocellulose paper

The nitrocellulose paper was soaked in 0.2 M $CaCl_2$ for 30 min, and then dried on paper towels. Cross-sections of approximately 1 mm thickness were prepared using a clean double-edged razor blade to cut roots (approximately 8–10 cm from the tip root). Freshly cut tissue was washed in distilled H_2O for 3 s, dried on kimwipes and blotted onto the nitrocellulose paper for 30 s, by application of a gentle

pressure. The tissue print was immediately dried with warm air, incubated for 1 h with anti-AtPIP1 antiserum and subsequently treated with alkaline phosphatase-conjugated secondary antibody for detection. The experiment was repeated twice.

Loading of Fluo-4/AM into root cells

The acetoxymethyl ester (AM) of Fluo-4, a Ca^{2+}-sensitive fluorescent dye was purchased from Molecular Probes (Eugene, OR, USA). The intact roots were incubated in a solution containing 20 μM Fluo-4/AM, 50 mM sorbitol and 0.2 mM $CaCl_2$ (pH 4.2), at 4°C for 2 h in the dark, followed by a 2-h incubation in aerated 0.2 mM $CaCl_2$, at 20°C in the dark. Fluo-4/AM was added from a stock solution of 1 mM Fluo-4/AM in DMSO. The final DMSO concentration in the incubation solution was approximately 1.8% (v/v). No negative effects of DMSO were observed at this concentration. The measurements were done in three independent root tips form tree different plants.

Confocal scanning laser microscopy

Root sections loaded with Fluo-4/AM were mounted in a chamber with a clean cover slip attached to the bottom. Fluorescence from the roots loaded with Fluo-4/Am was detected using a Leica TC SP2 confocal microscope. Excitation was at 488 nm and detection was between 510 and 525 nm.

Statistical analysis

The data were analysed statistically, using the SPSS 13.0 software package, by analysis of variance (ANOVA) and by Tukey's test. Significant differences were determined at $P < 0.05$.

Results

P_f measurements in root protoplasts

Protoplasts isolated from roots of control plants or plants treated with NaCl or $CaCl_2$, alone or in combination, were used to study how NaCl affects water transport and the role of calcium under salt stress. To measure water transport in isolated protoplasts, their bathing medium was diluted to half-strength with distilled water, which caused an increase in protoplast volume as a consequence of water passing into the protoplasts (Fig. 1a). The initial changes in protoplast volume with time (0.30–5 s) were followed using a microscope and used for the determination of the fitted swelling rate constant, k, and the osmotic water permeability coefficient, P_f, of individual protoplasts. Figure 1b shows that, although control protoplasts showed P_f values in the range of 50 μm s^{-1}, the NaCl treatment of plants reduced markedly protoplast P_f to values ~20 μm s^{-1}. When $CaCl_2$ was added to the plants, prior to NaCl treatment, the P_f value was partially recovered. Treatment of plants by calcium alone had no significant effect on protoplast P_f. In addition to measuring protoplast P_f in standard conditions, the functionality of the aquaporins was analysed by adding to the protoplast solution 50 μM $HgCl_2$ as blocking agent, alone or with the subsequent addition of 2 mM DTT. In protoplasts from control plants, P_f declined rapidly after a 2-min treatment with 50 μM $HgCl_2$ (Fig. 1a). A similar effect was observed for the P_f of $CaCl_2$- and $CaCl_2$ + NaCl-treated plants. However, NaCl-treated plants were only slightly affected by the mercurial ions. The addition of DTT (2 mM) returned P_f to

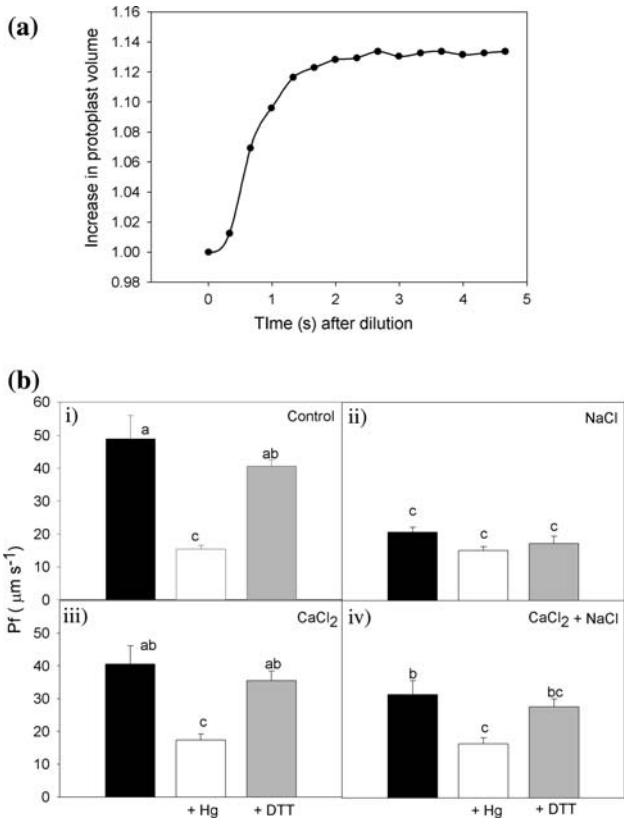

Fig. 1 **a** Representative protoplast swelling curve showing an increase in volume versus time, in a control protoplast (V_0 = 5986.75 μm^3) following dilution of the medium from 785 to 340 mOsmol kg^{-1}. **b** Osmotic water permeability (P_f) of pepper protoplasts isolated from roots of (i) control plants; (ii) 50 mM NaCl-treated plants; (iii)10 mM $CaCl_2$-treated plants; and (iv)10 mM $CaCl_2$-treated plants for 3 days prior to 50 mM NaCl addition. Measurements were performed before and after the addition of $HgCl_2$ (50 μM) and DTT (2 mM) (n = 100, ±SE). *Columns with the same letters* are not significantly different ($P < 0.05$, Tukey's test)

its initial value in all treatments except NaCl, where there was no significant effect.

Stopped-flow light scattering measurements

Osmotic water transport in plasma membrane vesicles purified from plants grown under the above saline treatments was determined by stopped-flow light scattering. Representative time course of scattered light intensity at 20°C following imposition of a 270 mOsmol kg^{-1} H_2O inwardly directed osmotic gradient are shown in Fig. 2a. Measurements were performed with vesicles resuspended in solutions at pH 6 or pH 8.3. The effect of a Ca^{2+} addition was also studied at pH 8.3 (Fig. 2b). Because of the blocking effects of acidic pH and calcium on plasma membrane aquaporins (Gerbeau et al. 2002; Alleva et al. 2006), it was assumed that maximal aquaporin functionality was maintained at pH 8.3, whereas aquaporins were at least partially blocked in the other measuring conditions. Under the same reference measuring conditions (pH 8.3), an effect of the different saline treatments on vesicle P_f was observed. P_f had the highest value ($\sim$30 μm s^{-1}) in plasma membrane vesicles from plants grown in control conditions and was decreased by about twofold in membranes from NaCl-treated plants. The other saline treatments (Ca^{2+} and $Na^+ + Ca^{2+}$) also decreased vesicle P_f with regard to the vesicles from control plants, but to a lesser extent. As compared to reference measurements at pH 8.3, measurements at pH 6 or with the addition of calcium at pH 8.3 both showed significant and similar reductions in P_f in plasma membrane vesicles from control plants, or plants treated by Ca^{2+} or NaCl + Ca^{2+}. By contrast, neither pH 6 nor calcium addition had an effect on P_f of vesicles from NaCl-treated plants.

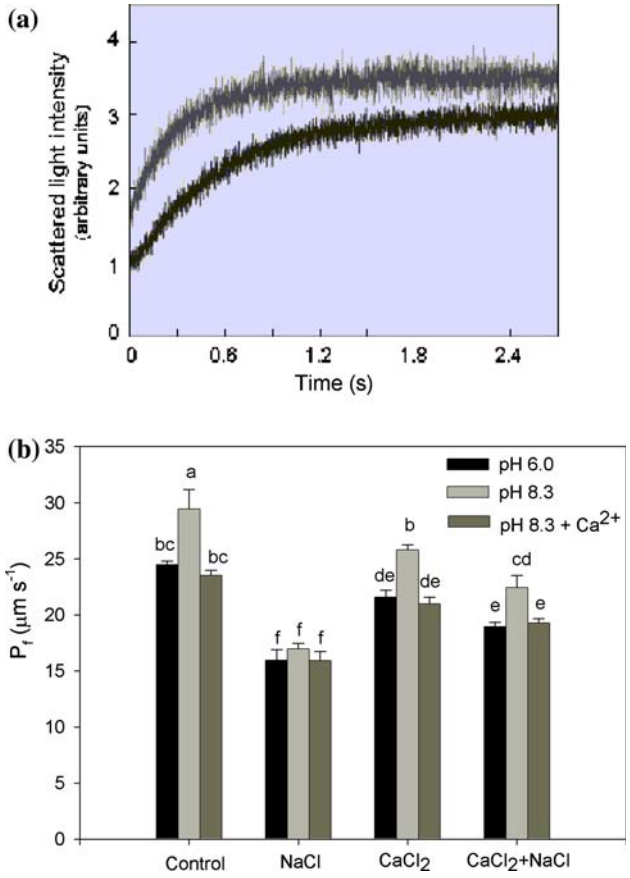

Fig. 2 **a** Representative stopped-flow recordings showing the kinetics of light scattering by plasma membrane vesicles in response to an inwardly directed osmotic gradient (see Materials and methods), at pH = 8.3, in the absence (*upper trace*) or in the presence (*lower trace*) of 0.3 mM $CaCl_2$. Fitted exponential rate constants: $k = 3.22$ s^{-1} (*upper curve*), $k = 1.90$ s^{-1} (*lower curve*). **b** Osmotic water permeability (P_f) at pH 6.0 and 8.3 of pepper plasma membrane vesicles isolated from roots of control plants, 50 mM NaCl-treated plants; 10 mM $CaCl_2$-treated plants and 10 mM $CaCl_2$-treated plants prior to 50 mM NaCl addition. Exogenous 0.3 mM $CaCl_2$ was also added to the purified vesicles at pH 8.3 and the P_f measured ($n = 5$, ±SE). *Columns with the same letters* are not significantly different ($P < 0.05$, Tukey's test)

Effect of salinity and calcium on aquaporin protein abundance

The abundance of at least some pepper PIP proteins was studied by Western blot (Fig. 3). The aim was to investigate whether the abundance of these aquaporins was correlated with P_f responses in protoplasts or in plasma membrane vesicles under salt stress. For this, we used an antibody raised against a 42 amino acid N-terminal peptide of *Arabidopsis thaliana* PIP1;1 (At3g61430). Based on the PIP amino acid sequences available, this domain is highly conserved between members of the PIP1 subfamily and between plant species. Here, we found that the antiserum recognises pepper proteins of similar molecular weight as PIP1;1 (and other PIP1s) recognised in an *Arabidopsis* extract. Therefore, the signal in pepper was interpreted due

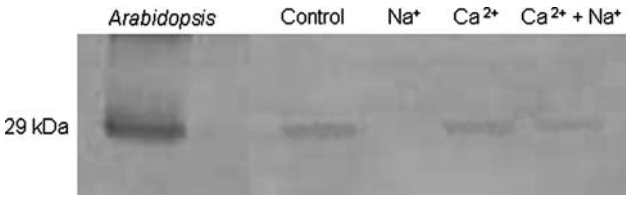

Fig. 3 Immunodetection of PIP1 homologues in the plasma membrane (PM) of *Arabidopsis thaliana* roots and roots of control pepper plants, 50 mM NaCl-treated plants, 10 mM $CaCl_2$-treated plants and 10 mM $CaCl_2$-treated plants for 3 days prior to 50 mM NaCl addition. Total PM was separated by SDS-PAGE and probed with antibody raised against AtPIP1;1. Equal amounts of proteins (12 μg) were loaded on to each lane

to PIP1 homologues. As a result of a 3-day NaCl treatment of the pepper plants, the abundance of PIP1 proteins in the root plasma membrane, as measured by immunoreactivity, significantly decreased. More specifically, the intensity of the signal with regard to control pepper was diminished by 87%). In experiments where plants were treated by $CaCl_2$ alone or prior to the NaCl treatment, the signal on the immunoblot as compared to that of pepper control plants was reduced by 13 and 29%, respectively. Nevertheless, the signal remained stronger than that for the NaCl treatment alone.

PIP1 tissue printing

Tissue printing was developed to investigate the localisation of PIP aquaporins in root sections of pepper plants (Fig. 4). We examined in particular the tissue distribution of PIP1s in roots under different saline treatments. We previously showed that a pre-immune serum did not reveal any signal in pepper roots (Cabañero et al. 2006). By contrast, the antibody raised against *A. thaliana* PIP1;1 showed a signal in all root sections, mainly in the epidermis and the outer cortical cells. PIP1 tissue printing also showed a decrease in the PIP1 abundance after NaCl treatment, with respect to control root sections. By contrast, the intensity of the signal in the plants treated with $CaCl_2$ was similar to that of control plants. Addition of $CaCl_2$ before the NaCl treatment increased the abundance of PIP1 aquaporins with regard to the addition of NaCl alone. Although semi-quantitative, the results confirm the above Western-blot analyses.

Measurement of Ca^{2+} with Fluo-4

To determine the effect of NaCl on the cytosolic abundance of free Ca^{2+}, representative images of cytosolic Ca^{2+} in the apical part of the root were analysed in control plants and in plants treated with 50 mM NaCl for 3 days (Fig. 5). These images showed the relative concentration of calcium in the epidermal cells of the root tips. Cortical cells could not be analysed in whole root tips due to the thickness of the samples. With regard to control plants (Fig. 5a), the NaCl treatment sharply reduced the concentration of cytoplasmic Ca^{2+} in the epidermal cells of the root tips (Fig. 5c).

Discussion

To characterise water transport in both intracellular and plasma membranes of plant cells, osmotic water permeability values (P_f) were measured on isolated protoplasts by standard light microscopy (Kaldenhoff et al. 1995; Ramahaleo et al. 1999; Martínez-Ballesta et al. 2000) and on purified plasma membrane vesicles using stopped-flow light scattering (Gerbeau et al. 2002; Trofimova et al. 2003). Although changes in membrane permeability may have occurred due to the stress associated with protoplast preparation, the moderately high P_f values (~ 50 μm s^{-1}) obtained in protoplasts of roots of control plants together with a marked inhibition by mercury support the idea that aquaporins can account for most of the water permeability in the cortical cells of these plants (Fig. 1). The reduction in the P_f of protoplasts from NaCl-treated plants, compared with protoplasts from control plants, and the fact that there was practically no mercury inhibition, led us to consider that inactivation of aquaporins or a reduction in their abundance had occurred following a salt stress. It is necessary to bear in mind that mercurials are poor pharmacological agents that have numerous secondary effects (Schütz and Tyerman 1997). Nevertheless, most of aquaporins are known to be characteristically mercury-sensitive, either by a direct blockade of the channel or by a secondary effect. It has been reported that $HgCl_2$ did not reduce cell respiration significantly during the time of treatment in aspen and *Arabidopsis*, suggesting that at least in these species the mercuric inhibition of root water flow

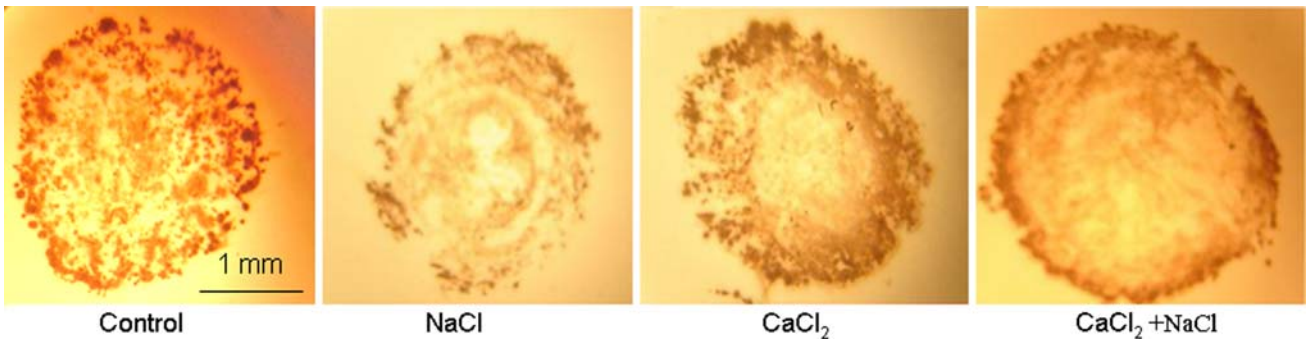

Fig. 4 Tissue printing with anti-AtPIP1;1 antiserum of the apical root sections of pepper plants. **a** Control plants, **b** plants growth with 50 mM NaCl, **c** plants growth with 10 mM $CaCl_2$, **d** 10 mM $CaCl_2$-treated plants for 3 days prior to 50 mM NaCl addition

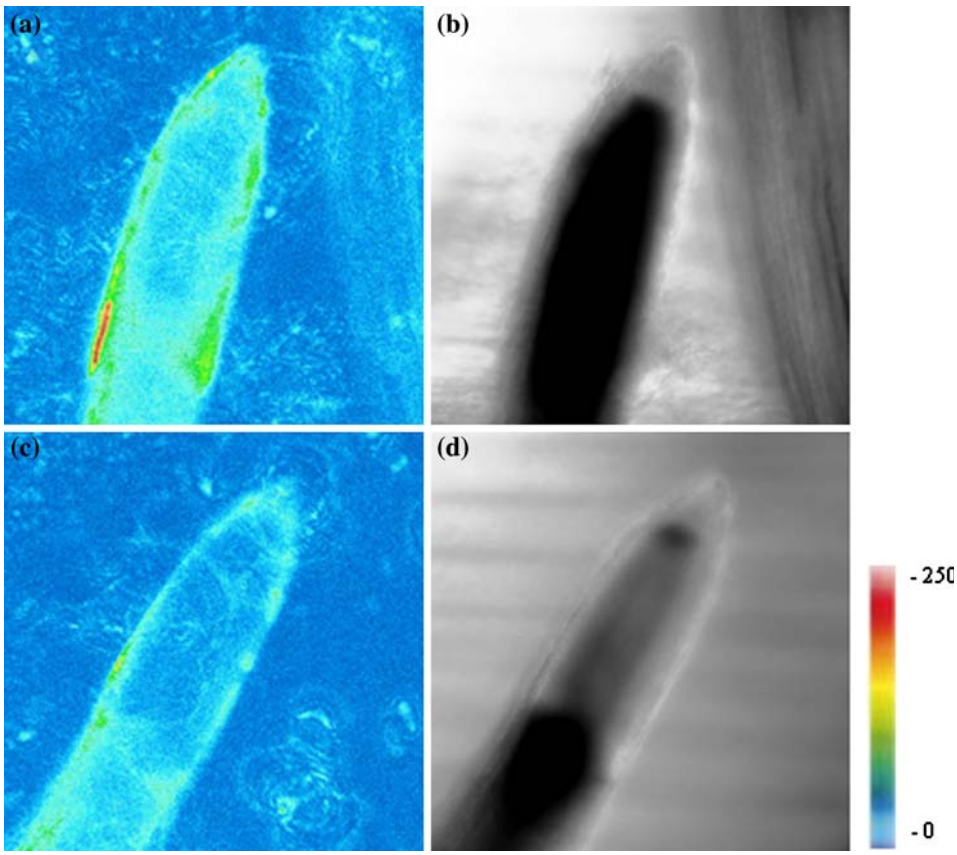

Fig. 5 Imaging of cytoplasmic Ca^{2+} within a root of control pepper plants (**a**, **b**) and within a root of 50 mM NaCl-treated plant (**c**, **d**). The plants were incubated with the fluorescent dye Fluo-4/AM and the images were monitored by confocal microscope (**a**, **c**). The Normasky images (**b**, **d**) illustrated the root tip position. Ca^{2+} concentrations, coded by heights and pseudocolors ranging from *blue* (low Ca^{2+}) to *red* (high Ca^{2+}), are depicted as pixel relative values

was not due to metabolic inhibition (Wan and Zwiazek 1999; Martínez-Ballesta et al. 2003).

While studies on purified membrane vesicles indicate a constitutively high P_f in the tonoplast of all plants investigated, these studies point to a lower P_f in the plasma membrane (reviewed in Maurel et al. 2002). It has been suggested that, in most living plant cells, the plasma membrane must be the limiting barrier to water uptake and that water channels in this membrane must be tightly regulated. Also, aquaporins in plasma membrane vesicles might have been partially inactivated due to the isolation procedure (Gerbeau et al. 2002). As a matter of fact, in control plants, P_f value and the presumed contribution of aquaporins were lower in isolated membrane vesicles than in protoplasts. A similar conclusion was raised in wheat roots, after comparison of water transport measurements in situ, using a cell pressure probe, and in purified plasma membrane and tonoplast vesicles (Zhang and Tyerman 1999). Based on a quantitative modelling of wheat root cells, the authors proposed that aquaporins at the plasma membrane had surely been inactivated during membrane purification.

Nevertheless, P_f values in purified plasma membrane vesicles were significantly decreased in NaCl-treated plants as compared with control plants (Fig. 2). The overall P_f results obtained from membrane vesicles of NaCl-treated plants showed the same tendencies as those obtained from protoplasts and both were in agreement with the PIP1 abundance. In addition, the P_f variations in both protoplasts and vesicles with NaCl + Ca^{2+} addition were consistent with our previous results (Carvajal et al. 2000; Martínez-Ballesta et al. 2003). They showed that a pretreatement of plants by Ca^{2+} produced a restoration of P_f, as compared to the addition of NaCl alone.

Another, independent effect of calcium on water transport was observed in purified plasma membrane vesicles. Here, we found that an external Ca^{2+} supply to the vesicle medium decreased P_f, in all membrane preparations except in those from NaCl-treated plants. Similar effects on membrane P_f was observed at acidic pH (pH 6). P_f inhibition upon direct application of Ca^{2+} and after blockade by H^+ has been reported previously in *Arabidopsis* and *Beta vulgaris* plasma membrane vesicles (Gerbeau et al. 2002; Alleva et al. 2006). Structure–function analysis of *Arabidopsis At*PIP2;2 (Tournaire-Roux et al. 2003) and the determination of spinach *So*PIP2;1 structure at atomic resolution (Törnroth-Horsefield et al. 2006) have brought critical insights into the mechanisms of plant plasma membrane aquaporin inhibition by pH and calcium. In summary, it has been proposed that in the closed aquaporin conformation the second cytoplasmic loop (*D*) caps the channel from the cytoplasm occluding the pore, whereas in

the open conformation the loop *D* is displaced, and this movement opens a hydrophobic gate blocking the channel entrance from the cytoplasm (Törnroth-Horsefield et al. 2006). In addition, a binding site for a divalent cation (Cd^{2+} in the atomic model) was observed in the vicinity of loop *D*. This feature may explain how divalent cations (and Ca^{2+} in particular) can play an important role in the gating of PIP aquaporins, as the cation can serve to anchor loop *D* through a network involving ionic interactions and hydrogen bonds.

We suggest that the different responses to Ca^{2+}, applied either to whole plants or to purified membrane vesicles, reflect different types of regulation. When it is applied in the nutrient solution, Ca^{2+} would operate by maintaining a critical concentration in the apoplast before it is eventually transported through the membrane. Such effects have been described as a response to osmotic/hydrostatic pressure in the apoplast and likely operate through mecano-sensitive Ca^{2+} channels (Netting 2000). However, in the membrane vesicle experiments, externally supplied Ca^{2+} would act directly to block the aquaporin pore (Törnroth-Horsefield et al. 2006). Along the same lines, it has been proposed that in guard cells (Yang et al. 2006), calcium is involved in stomatal movement through at least two routes, as an elicitor (second messenger) or as an aquaporin blocker.

The mechanisms that lead to Ca^{2+}-dependent regulation of aquaporins in root protoplasts or whole plants are as yet unclear. A principal role for Ca^{2+} channels in the plasma membrane has been postulated, for cell signalling, but also to mediate nutritional Ca^{2+} fluxes (White and Broadley 2003). Gerbeau et al. (2002) first postulated that the effects of Ca^{2+} seen in isolated membranes could operate in vivo and that $[Ca^{2+}]_{cyt}$ could regulate the water-permeability of the plasma membrane in *Arabidopsis* cultured cells. The recent study by Alleva et al. (2006) supports these ideas because, with respect to the earlier study (Gerbeau et al. 2002), a higher affinity component for aquaporin blockade by Ca^{2+} was resolved, and it was shown that Ca^{2+} acts from the cytosolic side of the membrane.

In another type of approach using plants grown under varying Ca^{2+} supplies, Cabañero et al. (2006) suggested that the opening and closing of aquaporins (as determined by inhibition of P_f by mercury in protoplasts) were, in turn, directly related to transport of Ca^{2+} from and to the cytosol, and specifically linked to the opening and closing of verapamil-sensitive Ca^{2+}-channels. Here, another mechanism of aquaporin gating, that is, reversible phosphorylation of the channel proteins (Johansson et al. 1998; Vera-Estrella et al. 2004) was invoked. Because it can be dependent on Ca^{2+}, this mechanism would link aquaporin regulation to Ca^{2+} signalling. More generally, the relationship between intracellular and extracellular Ca^{2+} and the possible involvement of secondary messengers, such as inositol-3 phosphate, point to the complexity of regulatory mechanisms involving Ca^{2+}.

In relation to the present study, we also have to consider the interacting effects of salt stress with calcium homeostasis and signalling. For instance, it has been reported that NaCl stress can affect membrane polarity and Ca^{2+} uptake in plant cells (Babourina et al. 2000). Also, NaCl caused reductions of the cytosolic Ca^{2+} gradient in *Arabidopsis* (Halperin and Lynch 2003). The confocal microscope studies performed in this paper showed that extended NaCl stress can reduce intracellular Ca^{2+}. This supports the idea that NaCl affects cell Ca^{2+} homeostasis which in turn would result in aquaporin inhibition. Other effects on water transport may still be mediated through salt-induced cytosolic acidification as was reported in *Nitellopsis obtusa* cells (Katsuhara et al. 1989). However, measurements in excised *Arabidopsis* roots, by means of in vivo ^{31}P-NMR (R. Bligny, Université J. Fourier, Grenoble, France, and C.Maurel, SubAgro, Montpellier, France, personal communication), or in root hairs with a pH-sensitive microelectrode (Halperin and Lynch 2003) failed to reveal any salt-induced change in cytosolic pH.

Many environmental factors, such as salinity, also critically interfere with aquaporin abundance. Thus, it has been observed that salt stress increased the level of RsPIP2-1 protein in the roots of radish seedlings, whereas the abundance of three RsPIP1s remained constant (Suga et al. 2002). The amount of MIP-C protein increased in *M. crystallinum* roots treated with NaCl, whereas MIP-F abundance was lower in the stressed tissues (Kirch et al. 2000). A recent report showed that, after NaCl treatment, the protein amounts of PIP2;1, PIP2;2 and PIP2;3 in *Arabidopsis* suspension cells increased several fold, whereas the abundance of PIP1 homologues did not change (Kobae et al. 2006). By contrast, Boursiac et al. (2005) observed in *Arabidopsis* plants a significant decrease in the abundance of PIP1 proteins in whole-cell extracts as soon as 30 min after salt exposure. This pointed to a rapid response to salt through dynamic control of PIP1 aquaporin translation and/or degradation. In this and another study by Martínez-Ballesta et al. (2003), long-term regulation of root water transport was explained mainly in terms of aquaporin transcriptional regulation, and abundance of PIP1 was reduced by about twofold. In the present study, the amounts of PIP1 in pepper root plasma membrane also decreased with salt stress.

In the present study, the abundance and tissue localisation of PIP1s was also investigated by tissue-print immunoblotting. Although the method provided a low resolution to determine the tissue localisation of PIP1s in detail, it could be appreciated that this sub-class of proteins was not prominent inside the vascular cylinder, whereas the greatest abundance was detected in the epidermis and the

outer layer of the cortex (Fig. 4). This suggests that PIP1s were more probably involved in water uptake into the symplast, rather than in water loading into the xylem. In addition, the PIP1 abundance was in agreement with the P_f results, indicating the role of Ca^{2+} in the restoration of PIP1 abundance under saline conditions.

In conclusion, several combined mechanisms may operate over different time windows in the regulation of aquaporins by Ca^{2+} in pepper roots under normal or salt stress conditions. These mechanisms, including Ca^{2+}-dependent blockade of PIPs and Ca^{2+}-dependent phosphorylation (activation), may overall contribute to a "fine adjustment" of aquaporin function at the plasma membrane. On the one hand, calcium waves associated with exposure to externally supplied Ca^{2+} or to the initial perception of salt stress may lead to aquaporin closure. On the other hand, an optimal apoplastic concentration of Ca^{2+} is required for transport through the plasma membrane. Accordingly, cytosolic Ca^{2+} was reduced after long-term exposure to salinity stress and seems to be associated to an overall inhibition of aquaporins. Externally supplied Ca^{2+} was able to counteract these effects. Finally, the abundance and diversity of plant aquaporins present in roots has to be borne in mind. Each isoform surely plays specific roles in various cell types and is regulated in specific physiological contexts. Thus, the next steps should be to quantify the involvement of each aquaporin isoform in root water uptake and to study their different responses to salinity and calcium.

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