

Changes in plasma membrane lipids, aquaporins and proton pump of broccoli roots, as an adaptation mechanism to salinity

Luis López-Pérez^a, María del Carmen Martínez-Ballesta^a, Christophe Maurel^b, Micaela Carvajal^{a,*}

^a Departamento de Nutrición Vegetal, Centro de Edafología y Biología Aplicada del Segura (CEBAS-CSIC), Campus Universitario de Espinardo, Ap. de Correos 164, 30100 Murcia, Spain

^b Biochimie et Physiologie Moléculaire des Plantes, Institut de Biologie Intégrative des Plantes, UMR 5004 CNRS/UMR 0386 INRA/Montpellier SupAgro/Université Montpellier 2, F-34060 Montpellier Cedex 1, France

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ABSTRACT

Salinity stress is known to modify the plasma membrane lipid and protein composition of plant cells. In this work, we determined the effects of salt stress on the lipid composition of broccoli root plasma membrane vesicles and investigated how these changes could affect water transport via aquaporins. *Brassica oleracea* L. var. *Italica* plants treated with different levels of NaCl (0, 40 or 80 mM) showed significant differences in sterol and fatty acid levels. Salinity increased linoleic (18:2) and linolenic (18:3) acids and stigmasterol, but decreased palmitoleic (16:1) and oleic (18:1) acids and sitosterol. Also, the unsaturation index increased with salinity. Salinity increased the expression of aquaporins of the PIP1 and PIP2 sub-families and the activity of the plasma membrane H⁺-ATPase. However, there was no effect of NaCl on water permeability (P_f) values of root plasma membrane vesicles, as determined by stopped-flow light scattering. The counteracting changes in lipid composition and aquaporin expression observed in NaCl-treated plants could allow to maintain the membrane permeability to water and a higher H⁺-ATPase activity, thereby helping to reduce partially the Na⁺ concentration in the cytoplasm of the cell while maintaining water uptake via cell-to-cell pathways. We propose that the modification of lipid composition could affect membrane stability and the abundance or activity of plasma membrane proteins such as aquaporins or H⁺-ATPase. This would provide a mechanism for controlling water permeability and for acclimation to salinity stress.

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1. Introduction

Many plants have developed different mechanisms to reduce the effect of salt stress, including stomatal closure, the uptake and biosynthesis of compatible solutes, compartmentation and exclusion of Na⁺ and Cl[−], cell structural changes and regulation of membrane permeability (Mansour and Salama, 2004; Zang and Komatsu, 2007). As the plasma membrane is one of the cell parts that salt reaches first, membrane lipids and transport proteins play a fundamental role in regulating permeability of this membrane and triggering primary responses to salinity (Cooke and Burden, 1990). Also, the control of water and nutrient distribution in the whole plant is an important factor in the acclimation of plants to a saline environment (Hasegawa et al., 2000). In this case also, the role of membrane proteins, such as aquaporins and H⁺-ATPases, is crucial. In many plants, changes in plasma membrane lipids, such as sterols and fatty acids, have been observed as a result of salt stress and may contribute to the control of membrane fluidity and permeability, as a stress-adaptation mechanism

(Elkhoui et al., 2004; Wu et al., 1998). In these respects, a decrease in the unsaturation of fatty acids is usually reported (Zhang et al., 2002), together with an increase of sitosterol (Navari-Izzo et al., 1989; Mansour et al., 1994). Bearing in mind that sitosterol is very efficient in reducing the water permeability of membranes (Schuler et al., 1990) a decrease in this lipid content can be anticipated in salt-responsive plants.

Aquaporins are transmembrane proteins that function as channels, to facilitate and regulate the permeation of water molecules across biological membranes (Maurel et al., 2008). In non-stressed plants, aquaporins may represent the predominant pathway for water flow across cell membranes (reviewed in Carvajal et al. (1998) and Maurel et al. (2008)). However, a decrease in water permeability of melon root protoplasts by a reduced activity of Hg-sensitive aquaporins has been observed in salinity stressed plants (Carvajal et al., 2000). Different regulations of plant aquaporins in response to salt stress have been observed, at the expression level in particular. Thus, it has been reported that the expression of plasma membrane aquaporin genes can be down-regulated (Katsuhara et al. 2003; Martínez-Ballesta et al., 2003; Boursiac et al., 2005), up-regulated (Liu et al., 1994; Uno et al., 1998; Yamada et al., 1997; Seki et al., 2002) or not significantly altered by salt

* Corresponding author. Tel.: +34 968 396310; fax: +34 968 396213.

E-mail address: mcarvaja@cebas.csic.es (M. Carvajal).

stress (Grote et al., 1998; Suga et al., 2002), depending on the NaCl concentration or plant tissue considered (Zhu et al., 2006). In addition to transcriptional regulation, post-translational modifications of aquaporins such as phosphorylation can also directly affect channel activity (Maurel et al., 1995; Prak et al., 2008). However, it is still undetermined whether water channel function can be affected by lipid composition via lipid/protein interactions, in a manner similar to other membrane-bound proteins/enzymes, or by changes in membrane fluidity.

The plasma membrane H⁺-ATPase is a functionally important protein that is involved in many physiological processes in plants, including those related to environmental stress (Arango et al., 2003). Control of the movement of ions across the plasma membrane, to maintain a low Na⁺ concentration in the cytoplasm, helps confer salinity tolerance (Wu and Seliskar, 1998) and this control is achieved in part by generation of an H⁺-ATPase-dependent electrochemical gradient (Serrano, 1989). Therefore, regulation of the plasma membrane H⁺-ATPase activity is expected to play an important role in plant salinity tolerance (Zhao and Qin, 2004; Janicka-Russak and Klobus, 2007). Accordingly, previous studies have indicated that salt stress can enhance plasma membrane H⁺-ATPase activity in herbaceous plants (Kerkeb et al., 2001; Sibole et al., 2005), whereas other reports have shown an inhibitory effect (Martinez-Ballesta et al., 2003). It was also shown that membrane composition is implicated in the modulation of H⁺-ATPase activity (see Cooke and Burden, 1990 for review).

Broccoli (*Brassica oleracea* L. var. *marathon*) plants are known to be moderately tolerant to salinity. In a previous work, we observed a biphasic growth inhibition of broccoli plants in response to salinity (López-Berenguer et al., 2006). During the first phase, growth reduction was high and probably related to water stress. In the second phase, growth reduction resulted from internal injury due to Na⁺ or Cl⁻, since osmotic adjustment was achieved and water relations were re-established. However, the effects of salinity on the plasma membrane, in relation to changes in the lipid composition and water permeability, have not been studied. The aim of this work was to determine the effects of salt stress on the membrane lipid composition of broccoli plants and evaluate how these changes could affect water transport and the membrane electrochemical gradient, through alterations in aquaporin and H⁺-ATPase activities, respectively.

2. Results and discussion

2.1. NaCl effect on the plasma membrane lipid composition

The lipid composition of root plasma membranes was analysed in control broccoli plants and plants grown with up to 80 mM NaCl for up to 15 d. As no change was observed between control plants and plants treated with 40 or 80 mM NaCl for 1 or 7 d, Table 1 shows only the results with plants exposed for 15 d to 80 mM NaCl. In these conditions, statistically significant differences in the amounts of fatty acids were observed between control and NaCl-treated plants. Palmitoleic acid (16:1) was the predominant unsaturated fatty acid in the plasma membrane of treated and untreated plants and a 25% reduction in its level was observed after NaCl addition. Also, a decrease by 27% in the oleic acid (18:1) content was detected, with regard to untreated plants, in plants grown under the saline treatment. By contrast, salinity increased the linoleic (18:2) and linolenic (18:3) acid levels, by 61% and 98%, respectively. Chaffai et al. (2007) showed that an increase of linolenic (18:3) acid indicates enhanced desaturase activity. In conditions of drought, a decrease in 18:3 reflects membrane damage, while an increase in the level of this fatty acid, as observed here, may represent a defence component (Zhang et al., 2005). To determine

Table 1

Fatty acid composition and sterol content in vesicles isolated of plasma membrane from broccoli roots treated with 0 (control) and 80 mM NaCl. Measurements were carried out after 15 d of NaCl addition ($n = 5 \pm \text{SE}$).

Parameter	Control (15 d)	80 mM NaCl (15 d)
Plasma membrane total protein (mg ml ⁻¹)	1.21 (±0.04) b	1.65 (±0.22) a
Fatty acids (%)		
Palmitoleic (C16:1)	40.6 (±1.08) a	30.6 (±1.96) b
Oleic (C18:1)	36.2 (±0.86) a	26.4 (±2.01) b
Linoleic (C18:2)	9.2 (±1.14) b	14.9 (±2.42) a
Linolenic (C18:3)	14.1 (±0.90) b	28.0 (±1.53) a
RUFA	0.6 (±0.06) b	1.7 (±0.28) a
DBI	137 (±2.79) b	171 (±5.48) a
Sterols (μg mg protein ⁻¹)		
Brassicasterol	1.22 (±0.08) a	1.12 (±0.06) a
Campesterol	10.6 (±2.09) a	10.7 (±1.54) a
Stigmasterol	6.06 (±1.84) b	10.49 (±1.35) a
Sitosterol	35.6 (±1.36) a	25.4 (±2.19) b
Stigmasterol/sitosterol	0.17 (±0.01) b	0.41 (±0.04) a

RUFA, ratio unsaturated fatty acid = (18:2 + 18:3/18:1); DBI, double bond index = $\Sigma(\text{unsaturated fatty acid} \times \text{number of double bond})$. Means with different letters in columns are statistically different ($P < 0.05$).

the unsaturation grade of the plasma membrane, the RUFA (ratio of unsaturated fatty acids) and DBI (double bond index) were calculated. Both indices increased significantly in NaCl-treated plants relative to control plants. Fatty acids, as a main component of membrane lipids, are considered to be important in salt tolerance of plants and other organisms (Malkit et al., 2002). Also, a high level of membrane lipid unsaturation is thought to maintain the membrane fluidity necessary for proper membrane functions which, in turn, can contribute to cell expansion and plant growth. Lin and Wu (1996) observed in a salt-tolerant clone of buffalograss an increase of unsaturated fatty acids, which was closely related to an increase of membrane permeability. Therefore, the differences in the fatty acid composition observed in this work between control and salinity-treated broccoli plants could be related to the necessity of maintaining a high degree of unsaturation in the root plasma membrane, to control membrane physico-chemical properties and cope with salt stress.

The effects of salinity on free sterol concentration of the root plasma membrane of broccoli plants were also investigated. Sitosterol showed the highest concentration in both control and 80 mM NaCl-treated plants and represented more than 50% of the total free sterols. A significant increase in the stigmasterol concentration was observed with NaCl addition, whereas sitosterol was decreased with regard to the control. Brassicasterol exhibited a low and similar content in the plasma membrane of roots of control and salinised plants. The contribution of other sterols (not shown) such as cholesterol was very low in control and NaCl-treated plants. Effects of salt on membrane sterols, similar to those described above, have been reported in plasma membrane fractions prepared from barley roots (Brown and Dupont, 1989). In addition, Douglas (1985) observed in different citrus varieties a general decrease in sitosterol and an increase in stigmasterol with salinity. However, the ratio of sitosterol to stigmasterol could depend on the plant species, salt stress intensity (Douglas, 1985) or tissue age (Geuns, 1973). While sitosterol was found to be very efficient in regulating membrane fluidity and water permeability, stigmasterol appeared to be ineffective for both functions (Schuler et al., 1990; Krajewski-Bertrand et al., 1992).

Although some results remain controversial, it has been proposed that free sterols may play a definitive role in ion exclusion through their effects on membrane fluidity and membrane permeability to ions (Hartmann, 1998). A major membrane rigidity would avoid Na⁺ and Cl⁻ fluxes into cell. Also, sterols are able to modulate the activity of transport proteins such as H⁺-ATPase

and Na^+/H^+ -antiporters, which are known to be modulated by salt stress (Tester and Davenport, 2003) and involved in ion homeostasis (Zhu, 2003). In agreement with this, Douglas (1985) found that, among citrus varieties, the major genotypic difference in plasma membrane sterol composition was a higher sitosterol to stigmasterol ratio in the better Cl^- excluder (Douglas, 1985). Therefore, in our experiment, the altered sterol composition of the plasma membrane of NaCl-treated plants (i.e. an increase of stigmasterol and a decrease in sitosterol) may reflect a response of broccoli root cells to adjust both water and ion transport during the acclimation of the plants to the saline environment.

2.2. NaCl effect on aquaporin abundance

The kinetic effects of different NaCl concentrations (40 and 80 mM) on the abundance of PIPs were determined by SDS-PAGE electrophoresis and Western blots (Fig. 1), using two specific antibodies developed against PIP homologues of *Arabidopsis thaliana*. The epitopes recognised by the antibodies are the first 42 amino acids of AtPIP1;1 and the last 17 amino acids of AtPIP2;2, respectively. Based on the PIP amino acid sequences available, the epitopes are conserved between plant species and the antisera are supposed to recognise members of the PIP1 and PIP2 subfamilies in broccoli plants, with molecular weights similar to their *Arabidopsis* counterparts. Control plants showed a strong immunoblot signal for PIP1s and a further and gradual increase over time in this signal, that is an increase in PIP1 abundance, was observed under both saline treatments, with respect to control plants (Fig. 1a). By contrast, in control plants or plants exposed for 1 d to salt, immunoblots showed a very faint anti-PIP2 signal. Yet, in a same way as for PIP1s, the anti-PIP2 antiserum revealed an increase in the intensity of the signal during prolonged (7 and 15 d) salt treatments (Fig. 1b). Various kinds of salt-dependent regulation profiles have been described previously for PIP1 and PIP2 aquaporins. Thus, Suga et al. (2002) showed that salinity increased the RsPIP2-1 protein content in radish seedlings whereas RsPIP1-1, RsPIP1-2 and RsPIP1-3 were not affected. In *Arabidopsis* roots, the abundance of PIP1 and PIP2 proteins was also found to be distinctly altered during the early phase (up to 2 h) of a salt treatment (Boursiac et al., 2005) while on a longer term (24 h), the two sub-classes showed a similarly reduced abundance. More generally, Jang et al. (2004) showed that, under various abiotic stress conditions including high salinity, modulation of *Arabidopsis* PIP genes is a complex process. Thus, a marked up- or down-regulation of PIP expression was observed under drought stress, whereas PIP genes were less-severely modulated by high salinity. In barley, different NaCl concentrations also had differing, gene-specific effects on the expression of MIP

genes (Zhu et al., 2006). The increased abundance of PIP1 and PIP2 aquaporins, as observed in broccoli plants during the course of a long-term salt exposure could reflect a mechanism to promote cellular water transport across the plasma membrane and avoid NaCl toxicity in the plant. However, the decrease in PIP2 abundance after short time of salt exposure did not result in a parallel decrease in root hydraulic conductivity, probably because of the multiplicity of aquaporin isoforms and expression patterns in the broccoli root. More specifically, this decrease in the PIP2 protein could be related to the response of water permeability previously described in leaf protoplasts (López-Berenguer et al., 2006) and whole roots (López-Pérez et al., 2007). In addition, López-Pérez et al. (2007) previously showed that broccoli plants developed after 14 d of a 80 mM NaCl treatment a phi thickening in roots, which was shown to act as a barrier to apoplastic flow. This indicates that, in these conditions, water was probably transported mainly via the cell-to-cell pathway. The contribution of aquaporins to this route provides not only higher water permeability but also a “fine” control and regulation of water flow (Maurel et al., 1997; Tyerman et al., 2002). Thus, aquaporin activity or abundance may efficiently control the overall water flow across tissues of broccoli plants under salinity, to increase their selectivity for water over salt, provided that the contribution of the apoplastic component is relatively low in these conditions.

2.3. NaCl effect on water permeability (P_f)

To characterise water transport in the plasma membrane of broccoli roots, plasma membrane vesicles were purified from plants grown under control or salt stress conditions and their osmotic water permeability value (P_f) was measured using stopped-flow light scattering (Gerbeau et al., 2002; Trofimova et al., 2003). Previous work in *Arabidopsis* and *Beta vulgaris* has shown that incubation of plasma membrane vesicles at acidic pH or in the presence of divalent cations leads to an inhibition of water channels (Gerbeau et al., 2002; Allewaert et al., 2006). The structural bases of the inhibitory effects of protons and divalent cations on plants PIPs have recently been elucidated (Tournaire-Roux et al., 2003; Törnroth-Horsefield et al., 2006). More specifically, it was 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.

In the present work, P_f measurements were performed at pH 8.3 or 6.0, in the absence of calcium, or at pH 8.3 in the presence of 0.3 mM CaCl_2 . Fig. 2 shows that P_f values of membrane vesicles purified from control plants were maximal at $\sim 45 \mu\text{m s}^{-1}$ in the first measuring conditions and reduced to $\sim 30 \mu\text{m s}^{-1}$ in the two latter conditions. This was interpreted to mean that water channels (PIP aquaporins) contributed to $\sim 1/3$ of membrane P_f , the residual P_f being accounted for by water transport across the lipid membrane. Fig. 2 also shows that treatment of plants by NaCl for 15 d had no effect, as compared to control plants, on the P_f values determined in either one of the three measuring conditions. Similar results were found for P_f values of plants treated by salt during 1 d (data not shown). Therefore, the salt treatment did not alter water transport through aquaporins or the lipid membrane in the purified membrane vesicles. The results suggest that, although significant, the salt-induced-changes in the lipid membrane composition

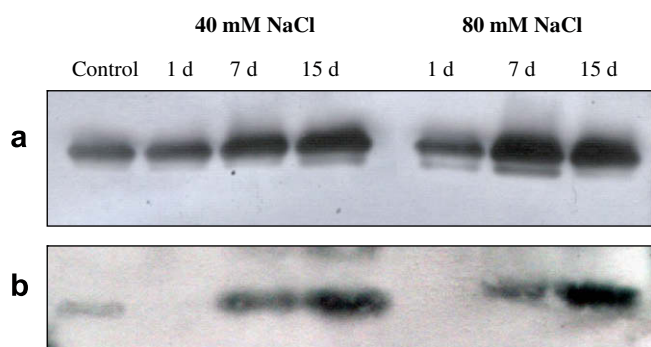


Fig. 1. Immunodetection of PIP1 and PIP2 homologues in the root plasma membrane (PM) of control broccoli plants and plants treated with 40 and 80 mM NaCl for 1, 7 and 15 d. Total PM was separated by SDS-PAGE and probed with antibody against AtPIP1;1 (a) and AtPIP2;2 (b).

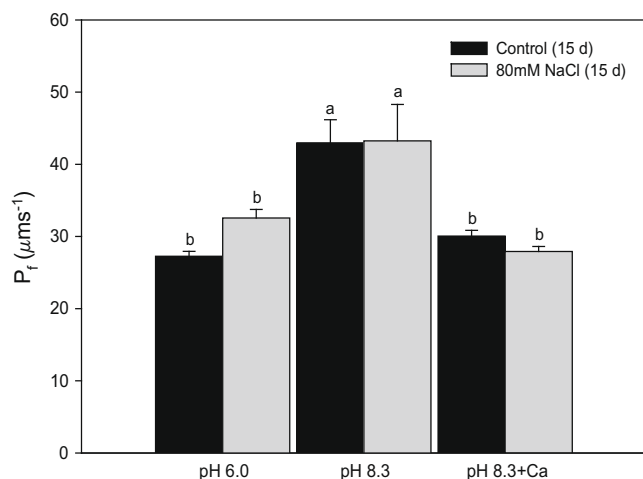


Fig. 2. Osmotic water permeability (P_f) at pH 6.0 and 8.3 of broccoli plasma membrane vesicles isolated from roots of control plants and plants treated with 80 mM NaCl for 15 d. Exogenous 0.3 mM CaCl_2 was also added to the purified vesicles at pH 8.3 and the P_f measured. Columns with different letters are statistically different ($P < 0.05$) ($n = 4 \pm \text{SE}$).

did not alter their intrinsic water transport properties. PIP2 proteins have been reported to show a higher water channel activity than PIP1 proteins after functional expression in *Xenopus* oocytes (Daniels et al., 1994; Chaumont et al., 2000; Moshelion et al., 2002) or yeast cells (Sakurai et al., 2005; Suga and Maeshima, 2004). Therefore, it was surprising that the marked increase in PIP2 abundance induced by long-term salt exposure was not associated to any change in P_f . We imagine, however, that P_f values in plasma membrane vesicles purified from control and 80 mM NaCl-treated plants could reflect inhibitory effects of changes in lipid composition on aquaporin activity that would be compensated by higher expression. In addition, the marked decrease in PIP2 abundance observed after a 1 d saline treatment was not associated to any decrease in P_f . Yet, expression of PIP1 aquaporins was constant in these conditions and, during the different phases of salt

exposure, interactions attributable to the formation of heteromers containing both PIP1 and PIP2 isoforms, could have occurred and may have contributed to membrane P_f (Fetter et al., 2004; Zelazny et al., 2007). We are also aware that the measured P_f values remained rather low. Despite a specific experimental procedure, aquaporins might, therefore, have been inhibited in part during plasma membrane vesicle purification. Thus, the possible links between PIP abundance and overall water permeability must be interpreted carefully, since a high diversity of PIP isoforms and a high diversity of regulation mechanisms under different stress conditions have to be taken into account.

2.4. NaCl effects on H^+ -ATPase activity

The H^+ -ATPase activity of the plasma membrane vesicles was altered depending on the length and strength of the NaCl treatments (Fig. 3). In plants grown with 40 mM NaCl, the activity was increased, with respect to control membranes, by 3.3-fold at 7 d, and 2-fold at 15 d. In plants treated with 80 mM NaCl, the H^+ -ATPase activity was increased, with respect to control plants, by 1.8-fold at 7 d. There were no statistical differences at 1 d with 40 mM and 1 and 15 d with 80 mM NaCl (Fig. 3a). In parallel immunoblot experiments, the signal for H^+ -ATPase increased in membranes from plants treated for 7 and 15 d with 40 mM NaCl and after a 7 d-treatment with 80 mM NaCl. These changes in H^+ -ATPase expression were in agreement with the observed changes in activity (Fig. 3b). It has been well-documented that salt treatment induces the activities of the plasma membrane and tonoplast proton pumps (Binzel, 1995; Klobus and Janicka-Russak, 2004). This increase in activity does not necessarily reflect an adaptive response and could be a consequence of the disruption of membrane integrity, causing an enhanced ATPase activity because proton gradient dissipation due to membrane leakage (Mansour and Salama, 2004). Yet, in our work, the higher H^+ -ATPase activity in NaCl-treated plants, relative to control plants, could have contributed to a partial reduction in cytoplasmic Na^+ concentration and is in consonance with the increase in the amount of proteins. Previous results showed no significant differences in Na^+ concentrations in broccoli roots after 7 and 15 d of treatment with

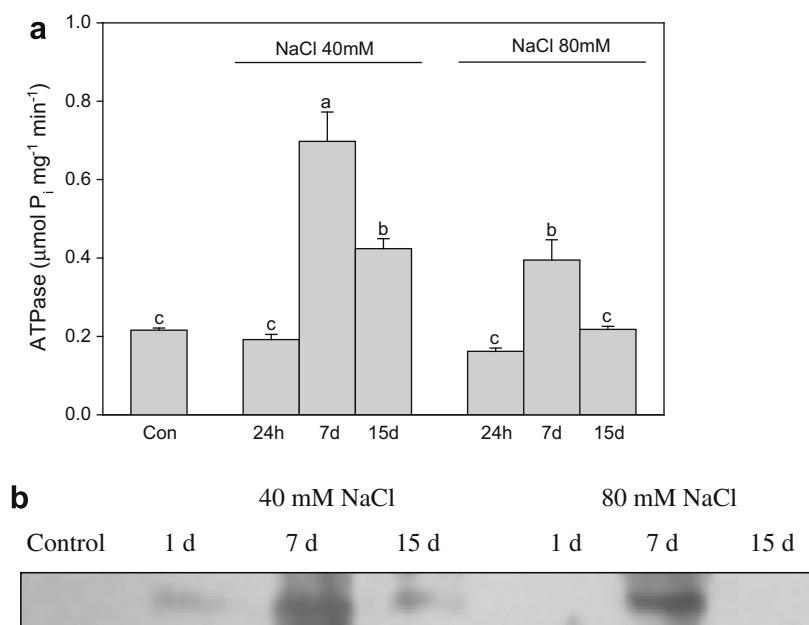


Fig. 3. H^+ -ATPase activity (a) and immunodetection (b) in the root plasma membrane of control broccoli plants and plants treated with 40 and 80 mM NaCl for 1, 7 and 15 d. Columns with different letters are statistically different ($P < 0.05$) ($n = 8 \pm \text{SE}$).

80 mM NaCl (López-Berenguer et al., 2006). Thus, the relative decrease in the H^+ -ATPase activity and abundance in 80 mM NaCl-treated plants 15 d after salt addition could indicate an adaptive response of broccoli plants to long-term salinity exposure. Furthermore, there is evidence that modifications of the composition of the lipid membrane can alter the kinetic properties of integral membrane proteins (Gronwald et al., 1990; Hartmann, 1998; Rodríguez-Rosales et al., 1999). Grandmougin-Ferjani et al. (1997) showed that the activity of the plasma membrane H^+ -ATPase appears to be very sensitive to its sternal environment. In particular, cholesterol and stigmasterol were found to stimulate proton pumping, whereas sitosterol behaved as an inhibitor (Hartmann, 1998). Zhao and Qin (2004) reported that the application of linoleic acid increased by 56% the root tonoplast H^+ -ATPase activity of barley seedlings treated with 200 mM NaCl. Our data show an increase in the stigmasterol concentration and a decrease in the sitosterol content in 80 mM NaCl-treated plants, with regard to control plants. In addition, the linoleic fatty acid increased with the NaCl application. Thus, the changes in H^+ -ATPase activity observed after 80 mM NaCl treatment are in agreement, not only with protein expression levels, but also with the salt-induced changes in membrane lipids. The increased plasma membrane H^+ -ATPase activity in plants treated with NaCl stresses the central role of the H^+ -ATPase in the transmembrane transport of protons, as a mechanism to avoid salt toxicity.

2.5. NaCl effect on electrolyte leakage (EL)

In order to characterise further the effects of salt stress on the plasma membrane of broccoli roots, we investigated the damaging effects of the stress and determined electrolyte leakage (EL) in the whole root (Fig. 4). Whereas a 40 mM NaCl treatment did not produce any change in EL, the application of 80 mM NaCl progressively increased EL by up to 2-fold. These results suggest that 80 mM NaCl changed the plasma membrane integrity of broccoli plants. An increase in EL with salinity has been shown previously in the plasma membrane of barley roots (Yu et al., 1998). In addition, it has been reported that electrolytes were altered markedly in salt-sensitive cultivars upon salt exposure (Mansour and Salama, 2004). Previously, it has been observed that EL may result from the appearance of membrane domains having different configurations, as can be seen after cold-induced damage to membrane lipids (Campos et al., 2003). Both membrane fluidity and changes in plasma membrane lipid composition directly affect transmembrane transport. Thus, in this work, we suggest that the EL was re-

lated to the changes in the lipid composition which resulted in an apparent increase in ion diffusion (Mansour et al., 1994). It has been demonstrated recently that electrolyte leakage measurements may be correlated with several physiological and biochemical parameters conditioning the plant responses to environmental conditions such as membrane acyl lipid concentrations (Lauriano et al., 2000). However, it is still unclear whether the observed increase in EL reflected a physiologically significant regulation of ion membrane permeability or a drawback of the salt-induced alteration in lipid membrane composition. It is of note that these effects did not result in any change in membrane P_f .

3. Concluding remarks

The results show that broccoli plants under high salinity stress (80 mM NaCl) markedly modified the lipid composition of the plasma membrane. Also, the apparent aquaporin activity of purified plasma membranes was not affected by salinity, as shown by the similar values of water permeability for 80 mM NaCl-treated and control plants. By contrast, salt exposure induced a significant accumulation of PIP1 and PIP2 homologues. This apparent paradox is as yet unsolved. It is possible that aquaporin activities were not properly preserved during membrane isolation or that changes in the lipid composition at 80 mM NaCl decreased the intrinsic activity of aquaporins and therefore counteracted their increased abundance. All these hypotheses will have to be investigated. At 40 mM NaCl, there was no change in lipid composition and the over-expression of aquaporins could possibly enhance membrane water transport. This effect could represent a mechanism of acclimation to salinity stress. Indeed, after the development of the phi thickening (López-Pérez et al., 2007) which acts as a barrier to apoplastic flow, water and nutrients must probably be transported via the cell-to-cell pathway. An up-regulation of aquaporins in the plasma membrane would confer on root cells novel possibilities for controlling and regulating the transport of water and nutrients.

4. Experimental

4.1. Plant culture

Seeds of broccoli (*Brassica oleracea* L. var. marathon) were pre-hydrated with de-ionised water and continuously-aerated for 12 h. After this, the seeds were germinated in vermiculite, in the dark at 28 °C, for two days. They were then transferred to a controlled-environmental chamber, with a 16-h light and 8-h dark cycle with temperatures of 25 and 20 °C and relative humidities of 60% and 80%, respectively. A photosynthetically-active radiation (PAR) of $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ was provided by a combination of fluorescent tubes (Philips TLD 36 W/83, Iena, Germany and Sylvania F36 W/GRO, Manchester, NH, USA) and metal halide lamps (Osram HQI, T 400 W, Berlin, Germany). After three days, the seedlings were placed in 15-l containers with continuously-aerated Hoagland nutrient solution (Epstein, 1972). After two weeks of growth, different saline treatments (40 and 80 mM NaCl) were applied for 1, 7 or 15 d. Photograph of plants grown during 15 d is shown in Fig. 5.

4.2. Lipid analysis

Sterol and fatty acids were determined as described by Mas et al. (1994). A mixture of chloroform-methanol (1:2, 0.75 ml) was added in an Eppendorf tube to membranes obtained with method I (0.5 ml), along with β -cholesterol (20 μl , 0.1 mg ml^{-1}) used here as an internal standard for sterol analysis. Chloroform (CHCl_3 ; 0.25 ml) was added and the mixture was shaken and

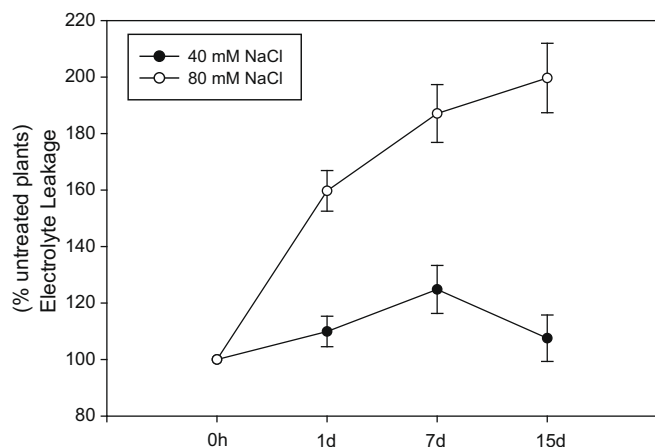


Fig. 4. Electrolyte leakage of root plasma membrane vesicles of control broccoli plants and plants treated with 40 and 80 mM NaCl for 1, 7 and 15 d ($n = 4 \pm \text{SE}$).

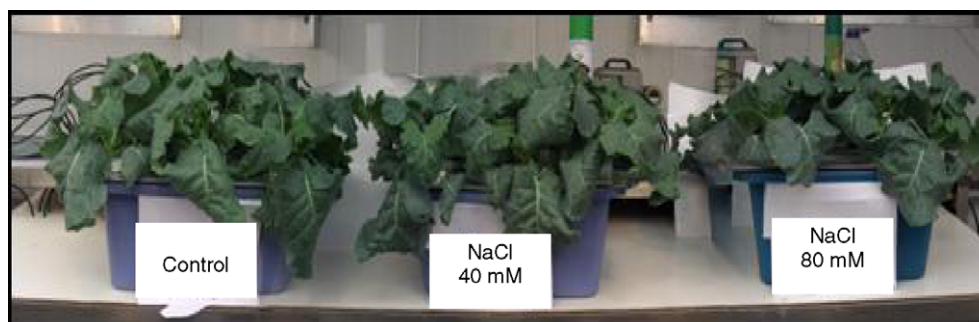


Fig. 5. Control broccoli plants and plants treated with 40 and 80 mM NaCl after two weeks of growth and two weeks of saline treatments. The plants were grown in controlled-environmental chamber with continuously-aerated Hoagland nutrient solution.

centrifuged at 10000g for 6 min. The CHCl_3 layer was retained, evaporated to dryness under N_2 and made up to 100 μl with CHCl_3 . For sterol analysis, 20 μl of the CHCl_3 extract was placed in a glass vial (2 ml), evaporated to dryness under N_2 and acetylated using pyridine (50 μl) and Ac_2O (100 μl). After 2 h, the solvents were evaporated under N_2 , ethyl acetate (20 μl) was added and the sterol analysed by GC using an HP5-bonded capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm) coupled to a flame ionisation detector (FID), with H_2 as carrier (1 ml min^{-1}) and a temperature programme of 120–260 $^\circ\text{C}$ at 5 $^\circ\text{C min}^{-1}$, then 260–280 $^\circ\text{C}$ at 2 $^\circ\text{C min}^{-1}$ and finally 280–300 $^\circ\text{C}$ at 6 $^\circ\text{C min}^{-1}$. The injector and detector temperatures were 150 and 320 $^\circ\text{C}$, respectively. Bound fatty acids were determined by using 20- μl portions of the CHCl_3 extract; evaporating them to dryness under N_2 , transmethylation with sodium methoxide (0.5 N) in methanol (0.5 ml) and heating at 30 $^\circ\text{C}$ for 7 min. The resultant fatty acids methyl esters were extracted with hexane (1 ml), evaporated under N_2 , dissolved in ethyl acetate (20 μl) and analysed by GC using an HP5-bonded capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μm), with FID, He as carrier (1 ml min^{-1}) and a temperature programme of 150–195 $^\circ\text{C}$ at 3 $^\circ\text{C min}^{-1}$, then 195–220 $^\circ\text{C}$ at 2 $^\circ\text{C min}^{-1}$ and finally 220–300 $^\circ\text{C}$ at 6 $^\circ\text{C min}^{-1}$. The injector and detector temperatures were 280 and 300 $^\circ\text{C}$, respectively.

4.3. Plasma membrane purification

Plasma membranes were isolated, using the two-phase aqueous polymer technique, according to Larsson et al. (1987) (I) or Gerbeau et al. (2002) (II); all procedures were performed at 4 $^\circ\text{C}$. Approximately 40 g of fresh root material were chopped finely and vacuum-infiltrated with 40 ml of 50 mM HEPES and 500 mM 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-phenanthroline, 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 10000g for 15 min and the supernatant further centrifuged at 55000g for 35 min, at 4 $^\circ\text{C}$, to yield a microsomal pellet, which was resuspended in 1 ml of 330 mM sucrose in 5 mM phosphate buffer pH 7.8 (method I) or 330 mM 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, 330 mM sucrose and 5 mM phosphate buffer (pH 7.8). The phase-system was centrifuged for 5 min at 4000g. 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 55000g for 35 min; the resulting pellet was resuspended in 1 ml of 250 mM sucrose, 5 mM Mes–Tris, 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. The isolation was carried out one day, one week and two weeks after applying NaCl. The Mg^{2+} -dependent ATPase activities sensitive to vanadate, oligomycin and KNO_3 were used as enzymatic markers of PM, mitochondria and tonoplast, respectively. These activities amounted to 91% (vanadate), 2.8% (oligomycin) and 6.8% (KNO_3) of the total ATPase activity ($n=3$). The contamination by Golgi membranes was assessed by the IDPase activity which represented 4.6% ($n=3$) of the total ATPase activity. These results are similar to those obtained for various other PM-enriched fractions prepared by phase partitioning (Widell and Larsson, 1990; Santoni et al., 2003).

4.4. Protein determination

Protein concentration was determined by the RC DC protein assay kit (Bio-Rad) following the recommendations of the manufacturer.

4.5. Gel electrophoresis and immunoblotting

Plasma membrane from root tissues of broccoli plants was isolated with method II as described previously. Equal protein amounts (10 μg per lane) were loaded for 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE). The proteins had been previously denatured, by incubating them at 56 $^\circ\text{C}$ for 20 min in the presence of 2% (w/v) SDS and 100 mM DTT, to dissociate 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 TBS (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 1:3000) (kindly provided by Schäffner) for 1 h at room temperature, an anti-peptide antibody raised against a 17 amino acid C-terminal peptide of *Arabidopsis* PIP2;2 (Santoni et al., 2003) (dilution 1:2000) for overnight at 4 $^\circ\text{C}$, or a polyclonal antibody raised against *Arabidopsis* H^+ -ATPase (kindly provided by Serrano) (dilution 1:1000) for 1 h at room temperature. Goat anti-rabbit IgG, coupled to horseradish peroxidase, was used as the secondary antibody (1:20000). The signal was developed using a chemiluminescent substrate (West-Pico, Super Signal; Pierce, Rockford, IL).

4.6. ATPase assay

ATPase activity was measured in root plasma membrane vesicles, by hydrolysis of ATP and the subsequent release of inorganic phosphate, using the method described by Coupland et al. (1991). To determine the 'basal' activity, the assay medium contained 2 mM MgCl_2 , 2 mM Na-ATP, 0.1 mM sodium molybdate, 0.1 mM sodium azide and 0.01% Triton X-100, made up to a final volume of 0.5 ml with 40 mM Tris-MES, pH 6.5. To determine the K^+ -stimulated activity, KCl (50 mM) was included in the above medium. The reaction was started by the addition of plasma membrane suspension (enough to provide 5–10 mg of protein) and the mixture was incubated for 30 min at 37 °C. The latency was considered as the ability of Triton X-100 to enhance the enzyme activity due to vesicle disruption, and the inhibition by sodium vanadate (22 mM) was assayed for the enzyme characterisation (data not shown). The reaction was stopped by the addition of 1 ml of "stopping reagent" made from (A) ammonium molybdate (4% w/v) plus 16 mM EDTA and (B) PVP-40 (4% w/v), 87.5 mM sulphuric acid and 172 mM hydroxylamine monohydrochloride: solutions A and B and water were combined in the ratio 3:2:1. Two minutes after adding the "stopping reagent", the colour was developed by adding 100 ml of a mixture of 6.47 M sodium hydroxide and 50 mM sodium carbonate. The absorbance was read at 720 nm.

4.7. Electrolyte leakage

The whole root from eight plants per treatment was placed in a tube containing 50 ml of distilled water and soaked for 3 h at room temperature; after this, the conductivity of the solution was measured and referred to as C_0 . Then, the tubes were kept at –80 °C for 1 h and soaked again at room temperature for 2 h, before measurement of the solution conductivity, this time referred to as C_T . The conductivity of the distilled water before the root immersion (C_w) was also measured. The percentage electrolyte leakage (EL) was calculated as: $\text{EL} = [(C_0 - C_w)/(C_T - C_w)] \times 100$.

4.8. Stopped-flow light scattering

The kinetics of the volume adjustment of the plasma membrane vesicles were followed by 90° light scattering at $\lambda_{\text{ex}} = 515$ nm. Measurements were carried out at 20 °C in an SFM3 stopped-flow spectrophotometer (Biologic, Claix, France), as described previously (Maurel et al., 1997). Purified membrane vesicles obtained with method II were diluted 100-fold in 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 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). 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: $P_f = k_{\text{exp}} \cdot V_0/A_v \cdot V_w \cdot C_{\text{out}}$. 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 osmolarity (Maurel et al., 1997).

4.9. Size of vesicles

The size of the membrane vesicles was estimated by electronic microscopy. For this, purified plasma membranes (1.0 mg membrane protein ml^{-1} , obtained with method II) were fixed by addi-

tion 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 100000g 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 in the same buffer for 2 h, the pellets were again washed twice with 0.15 M potassium phosphate buffer. Dehydration was carried out stepwise in 50%, 75% and 96% absolute ethanol (10 min twice for each concentration). Then, the pellets were washed twice in propylene oxide for 20 min and incubated in Spurr resin and propylene oxide, at a volume ratio of 1:1, for 1 h. The samples were then immersed in Spurr resin overnight, at 4 °C. Finally, the samples were embedded in Spurr resin. Ultra-thin sections were post-stained in 2% (w/v) uranyl acetate and Pb nitrate and studied in a Philip technique 12 electronic microscopy. The mean vesicle diameters were 271 and 243 nm for control and NaCl-treated plants, respectively. These values are in agreement with other values reported in the literature (Alleva et al., 2006).

4.10. Data analysis

Variance analysis and Tukey's HSD test were carried out to determine differences among treatments, using SYSTAT® 9.0 software for Windows.

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