

# Plant Aquaporins: New Perspectives on Water and Nutrient Uptake in Saline Environment

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**Abstract:** The mechanisms of salt stress and tolerance have been targets for genetic engineering, focusing on ion transport and compartmentation, synthesis of compatible solutes (osmolytes and osmoprotectants) and oxidative protection. In this review, we consider the integrated response to salinity with respect to water uptake, involving aquaporin functionality. Therefore, we have concentrated on how salinity can be alleviated, in part, if a perfect knowledge of water uptake and transport for each particular crop and set of conditions is available.

**Key words:** Aquaporins, salinity, nutrient uptake, water uptake, calcium.

## Introduction

Plant performance, usually expressed as crop yield, plant biomass, or crop quality, is affected adversely by salinity. Salinity is a major environmental stress and one of the most severe abiotic factors limiting agricultural production, since it alters the availability of water and nutrients. It affects plant growth through the development of osmotic stress and the disruption of ion homeostasis, which, in turn, causes metabolic dysfunction (Cheeseman, 1988).

The relationships between salinity and mineral nutrients are extremely complex and the interactions involved depend on the salinity level, composition of the salts, crop species, the studied nutrient, and a number of environmental factors. It has been reported that salinity affects ion activities in solution by changing the ionic strength, by ion-pair formation, and by precipitation (Cramer et al., 1987), resulting in excessive uptake and transport of the salt ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) and/or an inadequate uptake and transport of essential elements, to produce changes in mineral nutrient uptake that affect plant growth (Grattan and Grieve, 1993, 1999; Bayuelo-Jiménez et al., 2003). Also, significant entry of  $\text{Na}^+$  or  $\text{Cl}^-$  results in severe growth reduction or death in salt-sensitive or glycophytic spe-

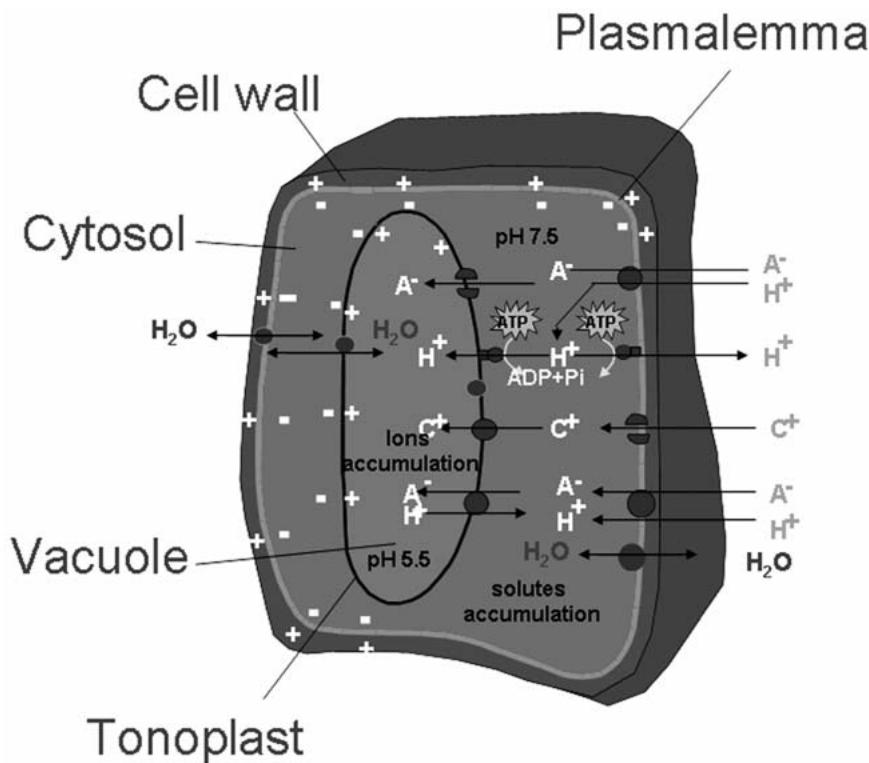
cies, whilst producing mild toxicity symptoms in salt-tolerant species (Maathuis and Amtmann, 1999).

Water movement within any system is governed by the driving force, which is described by gradients in water potential and the conductance of the flow path. The hydraulic conductance is considered an important factor in determining water supply to the plant (Munns and Passioura, 1984) and changes in hydraulic conductance can be caused by environmental conditions such as salinity (Peyrano et al., 1997). To maintain turgor, plants grown under salt stress undergo osmotic adjustment, which involves the net accumulation of solutes in cells in response to a fall in the water potential of the environment (Blum et al., 1996). Once the osmotic potential has changed, water uptake occurs (Fig. 1), but even so, other changes may take place as a consequence of salinity, such as a decrease in hydraulic conductivity of root membranes (Azaizeh and Steudle, 1991) and a decrease in stomatal conductance. So, studies of the influence of salinity on aquaporins are relevant to adaptation to saline conditions.

Recently, there has been a reasonable consensus on what to target for genetic engineering of salt tolerance. There has been a focus on ion transport and compartmentation, synthesis of compatible solutes (osmolytes and osmoprotectants) and oxidative protection. However, the processes that are commonly used as targets for intervention at the molecular level have not been very successful due to the multigenic nature of the response. Therefore, in this review, we have concentrated on the mechanisms of adaptation to salinity and how its effect can be alleviated by changing the  $\text{Ca}^{2+}$  concentration in the nutrient solution and by modifying plant water uptake, taking into account the response of aquaporins.

## Adaptation to Salinity Stress

The injurious effects of salinity on plant growth involve: (1) the low osmotic potential of the soil solution (water stress), (2) nutritional imbalance, (3) specific ion effects (salt stress), or (4) a combination of these factors (Marschner, 1995; Shannon, 1997). Salinity adversely affects phenotypic expression which affects plant growth and development at the physiological, biochemical (Munns, 2002), and molecular levels (Tester and Davenport, 2003; Mansour and Salama, 2004). The salt concentrations at which these effects occur differ according to the genotype, growth stage, environmental interactions,



**Fig. 1** Cellular homeostasis established after salt adaptation. Ions and solute organic accumulation are held to maintain the necessary turgor for water uptake. Ions, transport proteins and aquaporins are shown.

and ion species (Shannon et al., 1994). Adaptation to a sub-optimal environment depends on the possession of an optimum combination of characters that minimise deleterious effects and maximise advantageous effects (Jones and Jones, 1989). Thus, modifications are adaptive when they are finally expressed in terms of genotypes with improved growth and yield under salinity (Mansour et al., 2003). Salinity tolerance is more likely to be controlled by the complex interaction of several genes than by a single gene (Ashraf and Harris, 2004).

Despite a great deal of research into the salinity tolerance of plants, mainly on water relations, photosynthesis, and accumulation of various inorganic ions and organic metabolites (Munns, 2002), the metabolic sites at which salt stress damages plants and, conversely, the adaptive mechanisms developed by plants to survive salinity stress are still not well understood. The variation in salt tolerance mechanisms occurs not only among species, but, in many cases, also among cultivars within a single species (Ashraf, 2002).

Based on responses to high concentrations of salts, two widely different groups of plants have been characterised. Halophytes are native to saline soils and complete their life cycles in these environments (Taiz and Zeiger, 1991). For optimal growth, they require electrolyte concentrations (typically  $\text{Na}^+$  and  $\text{Cl}^-$ ) higher or much higher than those found in non-saline soils (Hasegawa et al., 2000). Glycophytes, or non-halophytes, are not resistant to salts in the same range as halophytes. A threshold concentration of salt, above which glycophytes show signs of growth inhibition, leaf discoloration, and loss of dry weight, has been described (Taiz and Zeiger, 1991).

#### Cellular adaptations to salinity

Ions are sequestered inside the vacuoles of leaf cells, with a subsequent increase in vacuolar osmotic potential. In addition, cells accumulate organic solutes in the cytoplasm in order to maintain the osmotic equilibrium between cytoplasm and vacuole (Fig. 1). This process has also been associated with succulence, due to an increase in the volume of the vacuole where the ions are stored. Since the cytosolic volume is smaller than the vacuolar volume, the carbon expenditure in synthesis of osmoprotectants is lower than in plants that exclude salts (Hasegawa et al., 2000).

In the process of intracellular compartmentation, ions enter leaf cells and are pumped into the vacuole before concentrations increase in the cytoplasm (for review see Munns, 2005). Vacuolar and plasma membrane  $\text{Na}^+/\text{H}^+$  antiporters, vacuolar and plasma membrane ATPases, and vacuolar pyrophosphatase are involved, the most extensively studied being the vacuolar and plasma membrane ATPases (Mansour et al., 2003). The pumping of  $\text{Na}^+$  into the vacuole is catalysed by a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter, and the  $\text{H}^+$  gradient is initially established by  $\text{H}^+$ -ATPase and pyrophosphatase proteins (Cheeseman, 1988; Blumwald et al., 2000). The activity of the  $\text{Na}^+/\text{H}^+$  antiporter has been shown to increase upon addition of  $\text{Na}^+$  (Fukuda et al., 2004a; Apse and Blumwald, 2002), this induction being much greater in salt-tolerant species than in salt-sensitive species (Wu et al., 2004; Ohta et al., 2002). The over-expression of the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter increased salt tolerance through vacuolar compartmentalisation of  $\text{Na}^+$  in different species, such as *Arabidopsis* (Apse et al., 1999), rice (*Oryza sativa*) (Fukuda et al., 2004b), and tomato (Zhang and Blumwald, 2001).

The activity of H<sup>+</sup> pumps could determine salt tolerance by affecting the membrane potential and proton gradient, since both affect the uptake, exclusion, and sequestration of different ions (Serrano and Gaxiola, 1994; Palmgren, 2001). Stimulation of plasma membrane ATPase activity has been found to occur as a response to salinity (Hasegawa et al., 2000; Maeshima, 2000), although other reports have shown an inhibitory effect (Martinez-Ballesta et al., 2003b; Wang et al., 2000). Mansour (2003) established that changes in pump activities in response to salt were not necessarily an adaptive response and could be a consequence of the disruption of membrane integrity that caused changes in protein activity. In addition, these changes were not always associated with increased capacity to pump Na<sup>+</sup> (Mansour et al., 2003). The mechanisms by which these transporters increase salinity tolerance are not yet clear. Different causes for modified activities of transport proteins under salinity have been described, such as changes in lipid-protein interactions, increased ATPase messages, changes in kinetic properties, alterations in intracellular calcium, and protein denaturation (Binzel and Ratajczak, 2002; Mansour et al., 2003; Mansour and Salama, 2004). So, the effects of salinity on the activities of plasma membrane and tonoplast ATPases depend on species, age, tissue, salt level, and experimental conditions, and the role of these proteins must be carefully studied.

Several reports have suggested that the interaction between K<sup>+</sup> and Na<sup>+</sup> uptake might represent a key factor in determining the Na<sup>+</sup> tolerance of plants and the effects of K<sup>+</sup> starvation on Na<sup>+</sup> and K<sup>+</sup> uptake mechanisms have been studied in wheat (Buschmann et al., 2000; Davenport and Tester, 2000) and *Arabidopsis* (Maathuis and Sanders, 1995; Demidchik et al., 2002). In this way, it has been shown that, in *Arabidopsis*, Na<sup>+</sup> translocation from root to shoot could be counteracted by the action of HKT-type K<sup>+</sup> transporters (Mäser et al., 2002), which help the plant to reduce Na<sup>+</sup> accumulation in the leaves. It has been observed that AtHKT1 mediates osmolality balance between xylem vessels and xylem parenchyma cells under saline conditions, removing the excess sodium from the xylem during osmotic and salinity stress and protecting the leaves from sodium toxicity (Sunarpi et al., 2005). In parallel with pumping into the vacuoles of Na<sup>+</sup>, an outflow into the cytoplasm, probably through ion channels, has been described. Thus, numerous non-selective cation channels in the vacuolar membrane of plant cells have been shown to be highly permeable to Na<sup>+</sup> as well as to other cations, such as K<sup>+</sup>. The opening of these channels in intact cells could compromise the ability of the cell to sequester Na<sup>+</sup> in the vacuole, but, given their relatively high permeability to cytotoxic cations (such as Na<sup>+</sup> and Ca<sup>2+</sup>), their activity was low (Demidchik and Tester, 2002). On the other hand, vacuolar channels in salt-tolerant and in salt-sensitive species did not show any important differences (Maathuis et al., 1992). An increased uptake of Na<sup>+</sup> by shoot vacuoles could facilitate osmotic adjustment, enhancing Na<sup>+</sup> compartmentation and reducing Na<sup>+</sup> accumulation in the cytosol, without any change in cellular Na<sup>+</sup> concentrations. This is possible because Na<sup>+</sup> transport across the tonoplast is bidirectional and dynamic (Tester and Davenport, 2003).

Accumulation of ions in the vacuole plays an important role in the response of plants to salinity. To accommodate the ionic balance in the vacuoles, cytoplasm accumulates low-molecular mass compounds that do not interfere with cellular metab-

olism (Shomerilan et al., 1991; Rathinasabapathi, 2000; Fernandez-Garcia et al., 2004b; Martinez-Ballesta et al., 2004). Such compatible solutes or osmoprotectants are highly soluble, and include secondary metabolites, such as quaternary ammonium compounds (glycine betaine), amino acids (proline), and polyols (sorbitol, mannitol), as well as sugars (sucrose, glucose, fructose, trehalose). Some studies with transgenic plants have demonstrated over-accumulation of these solutes (by stimulating synthesis, inhibiting breakdown or increasing transport to growing tissues). The over-expression of bacterial genes for trehalose synthesis in rice (Garg et al., 2002), or bacterial gene *mt1D* for mannitol synthesis in wheat (Abebe et al., 2003) induced tolerance to salinity. Other reports showed positive results for salt tolerance when plants are transformed with genes for osmoprotectants or protective proteins (Nuccio et al., 1999; Chen and Murata, 2002; Gorham and Wyn Jones, 2002; Rhodes et al., 2002; Flowers, 2004). However, transgenic manipulations do not always lead to osmotically significant over-accumulation of metabolites, as was the case for glycine betaine (Chen and Murata, 2002). Nevertheless, the production of osmoprotectants needs to be accompanied by a pleiotropic up-regulation of Na<sup>+</sup> pumps, since significant amounts of Na<sup>+</sup> were not compartmentalised in spite of the fact that an osmoprotectant alone could enable a plant to tolerate salinity (Tester and Davenport, 2003).

Another cellular response to salinity is the increased synthesis of a large number of proteins by plant cells. Accumulation of proteins in plants grown under saline conditions provides a storage form of nitrogen that can be re-utilised when the stress is removed and plays a role in osmotic adjustment (Singh et al., 1987). Proteins can be synthesised *de novo* in response to salt stress or are presented constitutively at low concentrations which decrease when plants are exposed to salt stress (Yan et al., 2005; Abbasi and Komatsu, 2004). A higher content of soluble proteins in salt-tolerant compared to salt-sensitive cultivars has been observed (Hamdia et al., 2004; Sibole et al., 2003). Synthesis of polyamines (such as putrescine and spermine) has been found to increase greatly in response to stresses (drought, salinity, etc.) (Mansour, 2000; Galston, 2001; Botella et al., 2000). In many cases, the increase of these compounds was higher in salt-tolerant than in salt-sensitive plants (Santa-Cruz et al., 1999; Kasinathan and Wingler, 2004). Although the function of these compounds is not clear, it is presumed to be protective, with a role in scavenging free radicals (Mansour, 2000). An ion-specific increase in the concentrations of polyamines has also been found (Lefevre et al., 2001).

Enhanced production of activated oxygen species in plants has been shown in response to different environmental stresses, such as salinity or drought (Mittova et al., 2004). Minimisation of the generation of reactive oxygen species (ROS) and maximisation of their removal are likely to be important components of plant responses to different stresses (Zhu, 2001; Vaidyanathan et al., 2003). Interestingly, plants containing high concentrations of antioxidants showed considerable resistance to oxidative damage caused by activated oxygen species (Badawi et al., 2004; Garratt et al., 2002; Turkan et al., 2005).

It has been proposed that the control of water transport is an important factor in relation to sensitivity to saline soils in some plants (Kerstiens et al., 2002; Very et al., 1998). Although both glycophytes and halophytes show reduced stomatal con-

ductance and root hydraulic conductivity under high-NaCl conditions (El Hendawy et al., 2005; James et al., 2002; Fernandez-Garcia et al., 2004a), there is evidence that water uptake and transport are the key for improving salinity resistance.

## Water Transport

### Root water uptake

Water is the single most abundant molecule in cells and organisms and is indispensable for life. In plants, water is transferred from the roots, in response to hydrostatic and osmotic gradients, to the aerial parts, where it evaporates in the transpiration stream through the stomatal pores. Although resistance to water flow exists mainly at the stomatal aperture, the root system can also represent a significant barrier (Steudle, 1994; Steudle and Peterson, 1998; Martre et al., 2001). The movement of water through plant tissues may occur by the following coexisting pathways: the apoplastic, the symplastic, and the transcellular pathway. The apoplastic pathway involves water flux around the cells, in the symplastic pathway the water is transported across plasmodesmata, and the transcellular pathway involves water transport across the plasma and vacuolar membranes. There are no approaches to separate the symplastic and transcellular pathways and they are summarised as the "cell-to-cell" pathway. In the root cortex, the flow of water is largely apoplastic but, at the endodermis, the Casparian band, a deposit of suberin and/or lignin, restricts radial water movement in the apoplast and cell-to-cell transport is carried out (Zimmermann and Steudle, 1998; Javot and Maurel, 2002). Thus, there are different pathways for water movement and they are used with different intensity depending on the species, the plasticity of the root, and the nature of the driving forces. For instance, in the presence of hydrostatic pressure gradients (such as in transpiring plants), water flow is largely apoplastic, since this route represents a low hydraulic resistance. However, under osmotic gradients (in the absence of transpiration, i.e., during the night when stomata are closed, in water-stress conditions or during an exudation phenomenon of an excised root), water flow is low and occurs mainly through membranes. Therefore, in older, suberised roots, where the apoplastic path is blocked, the cell-to-cell pathway predominates and fine regulation of water uptake is carried out at this level by a group of membrane proteins called water channels or aquaporins. In addition, depending on the environmental conditions and transpiration demand, plants can modify the apoplastic and "cell-to-cell" pathway contributions.

Long-distance water transport is carried out in the vascular tissues, where membrane barriers are, in most cases, non-existent. However, short-distance transport and transport in non-vascular tissues involve transport across membranes, which occurs by diffusion through the lipid bilayer, but is not enough to achieve the rapid transmembrane passage of water through the membrane, and several studies have demonstrated the involvement of proteinaceous water channels (Maurel et al., 1993; Kammerloher et al., 1994; Daniels et al., 1994). In addition, different studies on plant water relations, focused on the sensitivity of hydraulic conductivity to Hg, have demonstrated that their variability could be due to differences in the activity of aquaporins (Carvajal et al., 1999, 2000a, b; Clarkson et al.,

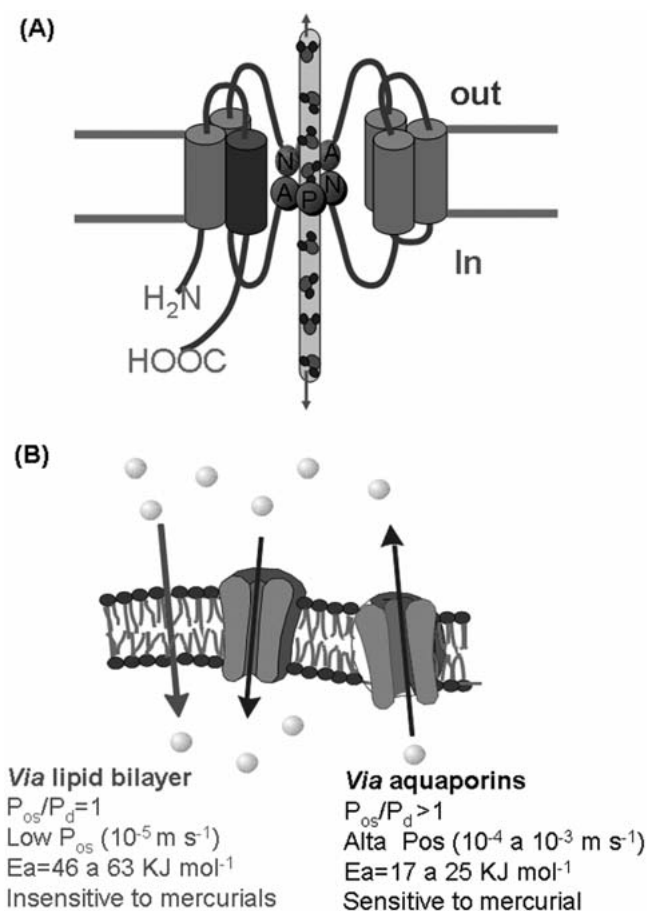
2000; Martínez-Ballesta et al., 2003 a, b). The inhibition of water flow across the root caused by HgCl<sub>2</sub> treatment was used to indicate the proportion of the total water flow which occurs via water channels, as they are characteristically Hg-sensitive; thus, the principal perturbation caused by Hg was through an effect on the radial movement of water across the root to the xylem (water channel-mediated pathway). It has been shown that HgCl<sub>2</sub> blocks the flow of water through aquaporins in plant roots and that the flow can be restored by reducing agents (Maggio and Joly, 1995; Carvajal et al., 1996). So, the rate of transmembrane water flux may be controlled by changes in the abundance or activity of aquaporins; in fact, the gating of aquaporins could play an important role in regulating water transport across cell membranes (Tyerman et al., 1999, 2002; Steudle, 2000, 2001). In addition, the response of plants to salinity involves water relations (Greenway and Munns, 1980). It has been reported that the long-term effects of salinity include the reduction of stomatal conductance (Chartzoulakis et al., 2000) and root hydraulic conductance,  $L_0$  (Evlagon et al., 1990; Navarro et al., 2000; Martínez-Ballesta et al., 2000), probably due to the hyperosmotic stress and ionic imbalance caused by high apoplastic concentrations of Na<sup>+</sup> and Cl<sup>-</sup>. Also, it has been suggested that the decrease in  $L_0$  by salinity could be closely related to the decrease in activity or concentration of aquaporins in the root plasma membrane (Carvajal et al., 1999, 2000a).

### Aquaporins

Aquaporins belong to the major intrinsic protein (MIP) family and facilitate the flow of water across cellular membranes (plasma and vacuolar membranes), following osmotic or hydrostatic pressure gradients (Chrispeels and Maurel, 1994). These proteins allow the organism to accelerate water movement through membranes, but diffusion will still occur in parallel (Fig. 2). The biological significance of aquaporins in plants is their ability to modulate transmembrane water transport in situations where adjustment of water flow is physiologically critical (for recent reviews see Maurel and Chrispeels, 2001; Baiges et al., 2002; Luu and Maurel, 2005). Furthermore, the ability to increase or decrease water permeability of a cell justifies the very high diversity of aquaporin homologues in plants: the complete genome of *Arabidopsis thaliana* has 35 full-length aquaporin genes (Johanson et al., 2001; Maurel et al., 2002) and similar diversity was found in other plant species such as maize (Chaumont et al., 2001) and rice (Sakurai et al., 2005). The two major subgroups are the plasma membrane intrinsic protein (PIP) and the tonoplast intrinsic protein (TIP), localised in the plasma membrane and tonoplast, respectively (Johnson et al., 1990; Daniels et al., 1994; Kammerloher et al., 1994). PIPs play an important role in controlling transcellular water transport, whereas TIPs seem to be involved in water exchange between the cytosolic and vacuolar compartments, which is involved in cell osmoregulation (Maurel et al., 1997; Tyerman et al., 1999). The highly regulated expression of aquaporins in plants suggests that transmembrane water transport may be important in many other processes in addition to those related to transpiration.

Salinity probably affects water channel function in plant roots via both gene expression and biochemical changes in the water channel protein, i.e., phosphorylation, which has been reported to be one of the mechanisms for molecular gating of aqua-





**Fig. 2** Water permeation through the lipid phase of cell membranes and through aquaporins. (A) The secondary structure of a monomer of AQP1, with water flows occurring through two NPA (single letter amino acid codes) motifs. (B) Summary of features associated with water flow across a lipid bilayer and water flow through a biological membrane studded with aquaporins. (Adapted from Tyerman et al., 1999.)

porins (Johansson et al., 1996). Dephosphorylation *in vivo* was increased in response to low apoplastic water potential (Johansson et al., 1998). In opposition to this observation, for a PIP homologue in spinach, Guenther et al. (2003) showed that under stress conditions, such as water deprivation and salinity, phosphorylation of Nodulin-26 was increased rather than decreased. In addition, NaCl has been shown to decrease the amount of mRNA encoding PIP aquaporin proteins in *Arabidopsis* (Martínez-Ballesta et al., 2003a; Boursiac et al., 2005). The expression of *HvPIP2;1* plasma membrane aquaporin was down-regulated after salt stress in barley (Katsuhara et al., 2002, 2003). 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 expression patterns of the MIP genes in leaves and cell suspensions of the halophyte sea aster (*Aster tripolium* L.) during a 24-h NaCl stress were studied by PCR (Uno et al., 1998). The study showed the inducible expression of 6 genes by salt stress, whereas no significant transcript accumulation was observed in salt-adapted cells. Similar results were observed by Yamada et al. (1997) in *Nicotiana excelsior*, for 3 MIP genes that were up-regulated under 24 h salt stress (Yamada et al., 1997). In addition, it has been observed that high salinity stress affected

the expression of aquaporin genes, differentially, depending on the type of PIP. Many PIP genes, including PIP1;1, PIP1;2, and PIP2;3, were up-regulated, whereas PIP1;5 and PIP2;6 were down-regulated by salt treatment (Jang et al., 2004). This initial down-regulation and the subsequent up-regulation of aquaporin gene expression have been observed in microarray analysis of two rice cultivars (Kawasaki et al., 2001) and in *Arabidopsis* (Maathuis et al., 2003). The up-regulation of certain aquaporin isoforms in *Arabidopsis* and in rice plants by salt stress was also observed in other studies (Liu et al., 1994; Seki et al., 2002). Comparison of the effects of two different NaCl concentrations on expression of maize MIP genes revealed differential and gene-specific effects (Zhu et al., 2005). After 2 h of 100 mM NaCl treatment, three highly expressed, specific isoforms, *ZmPIP1;1*, *ZmPIP1;5*, and *ZmPIP2;4*, were transiently induced and none of the *ZmTIP* genes were altered. By contrast, after the addition of 200 mM NaCl, these responses were lost. This emphasises isoform-specific functions in relation to tissue water permeation or growth, as well as differential responses to varying extents of the same abiotic stress. However, these findings may also mean that there are roles for aquaporins that differ from those related to the simple permeation of water or other uncharged molecules through cellular membranes: Hill et al. (2004) recently challenged the importance of a "simple permeation hypothesis", suggesting an additional function of aquaporins as osmotic or turgor sensors. The different down- or up-regulation of aquaporin gene expression during salt stress may play a role in limiting initial water loss during the early stages of salt stress and in assisting the subsequent uptake of water to maintain water homeostasis in high cellular salt conditions. However, it is too early to confirm the relationship between aquaporin expression and the beneficial or deleterious effect of each aquaporin on plants simply by comparing the expression patterns of PIP genes under stress conditions. Aharon et al. (2003) have shown that PIP1;2 over-expression had no beneficial effect under salt stress, and had a negative effect during drought stress. In transgenic rice plants, when over-expressing barley *HvPIP2;1* under salt stress, growth reduction was greater than in non-transgenic plants. In addition, a decrease in shoot water content and reduction in root mass or shoot mass were observed in transgenic plants maintained under salt stress for 2 weeks. These results indicate that over-expression of *HvPIP2;1* made rice plants more sensitive to NaCl. Therefore, both protein function and gene regulation are involved in the response of plants to salt stress. However, only under osmotic stress conditions (induced by mannitol) can *MtTIP1;2* be observed to be redistributed from the tonoplast to endosomal compartments in *Mesembryanthemum crystallinum* (Vera-Estrella et al., 2004), while no effect was observed with NaCl treatments. This suggests that aquaporin redistribution may be involved in restoring and maintaining cellular osmolarity under osmotic stress conditions. Nevertheless, the complexity of salinity has to be taken into account, since the ionic component of the salt stress initiates a separate and independent signalling pathway.

Down-regulation of root hydraulic conductivity following salt exposure has been observed in many plant species and represents one of the best-characterised examples of stress-induced regulation of water transport in plants. In addition, changes in root hydraulic conductivity, transpiration rates, and cellular osmotic water potential were observed after manipulation of plasma membrane intrinsic protein (PIP) transcript levels

through over-expression (Aharon et al., 2003), gene silencing by antisense suppression (Kaldenhoff et al., 1998; Martre et al., 2002; Siefritz et al., 2002), or T-DNA insertion (Javot et al., 2003). Although the hydraulic conductivity of tissues could be regulated by changing the levels of specific aquaporins, regulation could also occur by changing the activity of proteins. Thus, although the aquaporins are responsible for the transcellular movement of water across the cell membrane and their expression or abundance responded significantly and differently to environmental stress conditions, a deeper knowledge is necessary to explain the large variations in hydraulic conductivity at the level of tissues and organs in plants exposed to different environmental conditions. More complete analyses are required to dissect the contribution of the three transport pathways to overall water movement and to better understand the function of aquaporins in stress-related physiological processes.

In addition to water, aquaporins allow transport of small neutral solutes and/or gases, revealing the possibility that aquaporins may have multiple functions, including ammonia gas transport (Dean et al., 1999; Niemietz and Tyerman, 2000) and CO<sub>2</sub> diffusion (Terashima and Ono, 2002; Uehlein et al., 2003; Hanba et al., 2004). It has even been reported that *McTIP1;2*, an aquaporin from *Mesembryanthemum*, seems to be permeable to K<sup>+</sup> (Vera-Estrella et al., 2004), which may indicate a role of this water channel in osmotic adjustment of plant cells.

Recently, a model has been proposed that describes the gating of water channels in *Chara*, where osmolytes excluded by channels may create negative pressures in the interior of the channels, to produce a reversible mechanical collapse of the protein (Ye et al., 2004). The role of ABA in attenuating this process, to prevent protein deformation, was studied by Wan et al. (2004). A reversible gating of aquaporins by hydroxyl radicals (•OH) has also been described (Henzler et al., 2004). Although the mechanism by which •OH acts on aquaporins is not yet understood, it has been postulated that •OH could act either directly from inside the pore, when the molecule moves across, or indirectly by lipid peroxidation and attack by the resulting radicals outside. Other mechanisms for aquaporin molecular gating have also been described: it has been shown that cytosolic acidosis reversibly inhibited root hydraulic conductivity and closed aquaporins due probably to the protonation of a histidine residue in the plasma membrane water channel proteins (Tournaire-Roux et al., 2003). In addition, a co-operative interaction between PIP1 and PIP2 subgroups was suggested as a way to control plant plasma membrane water permeability (Martre et al., 2002; Fetter et al., 2004).

Maathuis et al. (2003) showed that aquaporins and V-type ATPases were the only classes of membrane transporters that responded to all cation stresses investigated. Co-ordinated down-regulation of aquaporin isoforms in plants was described after calcium deprivation or after exposure to a high salt concentration, suggesting a common response to stress conditions. However, in pepper plants, it has been observed that ATPase activity and aquaporins are not related, although both responded similarly to salt stress (Martínez-Ballesta et al., 2003b). Divalent cations can change the water permeability of *Arabidopsis* plasma membrane from a high- to a low-water permeability state (Gerbeau et al., 2002). These findings

point to a novel mechanism that allows short-term regulation of aquaporins by a direct cation blockade of the protein (Gerbeau et al., 2002).

Aquaporins dominate transmembrane water movement and can be regulated in the same way as other channels, which introduces the possibility of the control of water movement far beyond the general acceptance that it is controlled by passive water potential differences. Regarding aquaporin regulation, there is still much work to be done. It seems that the mechanisms of aquaporin regulation do not operate separately. Water stress and salinity act on aquaporin function at the transcription level, via protein relocalisation, and by effects on protein phosphorylation. A future aim could be to study how plants are able to integrate all these mechanisms and how they adjust water and solute transport to their needs.

### Ameliorative Effect of Calcium

#### *Interaction of Ca<sup>2+</sup> and Na<sup>+</sup>*

Calcium is an essential nutrient that is required for structural roles in the cell wall and membranes, as a counter-cation for inorganic and organic anions in the vacuole and as an intracellular messenger in the cytosol (Marschner, 1995; White and Broadley, 2003).

Under saline conditions, disorders may result from changes in Ca<sup>2+</sup> availability, competitive uptake, transport, or partitioning within the plant (Grattan and Grieve, 1999). Na<sup>+</sup> may impede calcium transport through the apoplast to the xylem vessels by a reduction in the activity of available Ca<sup>2+</sup> in solution and in competitive uptake from the soil solution (Lynch and Läuchli, 1985) or by suppression of membrane surface charge (Reid and Smith, 2000). Salinity has been shown to induce calcium deficiency in different species, such as *Arabidopsis*, pepper, or wheat (Cabañero et al., 2004; Lynch and Läuchli, 1988; Cramer and Jones, 1996). Furthermore, in salt-tolerant plants, under low-Ca<sup>2+</sup> conditions Na<sup>+</sup> could specifically induce desorption of Ca<sup>2+</sup> from the plasma membrane, consequently leading to growth inhibition (Murata et al., 2000). It has also been suggested that salinity can decrease the synthesis of cell wall polymers in favour of osmotic solute accumulation (Renault, 2005). This has a special relevance in woody species, where the damage could be serious. Salinity-induced growth inhibition was reduced or completely restored in many plants by supplemental Ca<sup>2+</sup> (Cramer, 2002; Reid and Smith, 2000; Husain et al., 2004; Shabala et al., 2003), probably by inhibition of Na<sup>+</sup> influx as shown in pepper (*Capsicum annuum*, L.) (Rubio et al., 2003). Therefore, the availability of Ca<sup>2+</sup> to cells seems to be fundamental in stress tolerance. In some cases, an increase of Na<sup>+</sup> concentration in the shoot was not proportional to the increase of external NaCl, suggesting that the maintenance of high K<sup>+</sup>/Na<sup>+</sup> and Ca<sup>2+</sup>/Na<sup>+</sup> ratios is critical for plant survival in saline environments (Renault, 2005).

#### *Ca<sup>2+</sup> as second messenger*

The (Ca<sup>2+</sup>)<sub>cyt</sub> of plant cells increases in response to many developmental signals and environmental changes, this being essential to produce a physiological response (White and Broadley, 2003). Stress-induced alterations in the cytosolic concentration of Ca<sup>2+</sup> occur as a result of the influx of Ca<sup>2+</sup> from out-

side the cell or release of  $\text{Ca}^{2+}$  from intracellular stores (Knight, 2000).

Calcium enters plant cells through  $\text{Ca}^{2+}$ -permeable ion channels in the plasma membrane (White, 2001).  $\text{Ca}^{2+}$  is taken up and released by cellular organelles and transported inward and outward across the plasma membrane (Sanders et al., 1999). When the external  $\text{Ca}^{2+}$  is high, the steep transmembrane gradient and the negative membrane potential strongly favour  $\text{Ca}^{2+}$  accumulation in the cytoplasm, and it has to be removed rapidly, either back to the external medium or into cellular organelles, in order to keep the signalling pathways functional (Webb, 1999). Since a high  $(\text{Ca}^{2+})_{\text{cyt}}$  is cytotoxic, a sub-micromolar level is maintained by  $\text{Ca}^{2+}$ -ATPases and  $\text{H}^+/\text{Ca}^{2+}$  antiporters (reviewed in White and Broadley, 2003). For *Arabidopsis thaliana* L. root hairs, high concentrations of NaCl reduced intracellular calcium levels (including in the vacuole, ER, and other organelles) (Halperin et al., 2003). In fact, the reaction to salinity involves a slow and brief spike of  $(\text{Ca}^{2+})_{\text{cyt}}$ , followed by a sustained elevation and, finally, a decrease in  $\text{Ca}^{2+}$  which can be maintained for days if the stress conditions persist (White and Broadley, 2003). Because of the spatial-temporal complexity of the  $\text{Ca}^{2+}$  signals generated by environmental factors, the diverse families of  $\text{Ca}^{2+}$  sensors identified in plants are expected to contribute to the conversion of  $\text{Ca}^{2+}$  signals into cellular responses (Perruc et al., 2004). Indeed, several lines of evidence suggest that calmodulin, calcineurin B-like proteins and calcium-dependent protein kinases (CDPK) are involved in signal transduction for stresses such as dehydration, low temperature, and high salinity (Luan et al., 2002; Perruc et al., 2004).

#### Water relations, aquaporins, and $\text{Ca}^{2+}$

Taking into account the low mobility and accumulation capacity of  $\text{Ca}^{2+}$ , its absorption and transport within the plant through water influx are obviously essential. The response of plants to salinity involves changes in water relations (Greenway and Munns, 1980). It has been reported that one of the long-term effects of salinity is reduction of stomatal conductance (Chartzoulakis and Klapaki, 2000; Bayuelo-Jiménez et al., 2003). Also, the effects of salinity seem to involve root hydraulic conductance (Carvajal et al., 1999, 2000a; Cabañero et al., 2004), reducing water uptake, and transport through the plant.

It has been demonstrated that the toxic effects of NaCl on diverse plant species can be ameliorated by addition of  $\text{Ca}^{2+}$  to the external solution (Ehret et al., 1990). Thus, the availability of  $\text{Ca}^{2+}$  to cells seems to be fundamental in a wide range of stress responses. An adequate supply of calcium maintains membrane integrity and selectivity (Grattan and Grieve, 1999). Kinraide (1999) has suggested that three mechanisms are involved in the ameliorative effect of calcium. The first mechanism is based on alleviation of toxicity by the electrostatic displacement of the toxicant. The second mechanism involves replacement of essential  $\text{Ca}^{2+}$  at the plasma membrane surface when a toxicant has displaced enough  $\text{Ca}^{2+}$  to limit growth. In the third mechanism,  $\text{Ca}^{2+}$  reduces the effectiveness of the toxicant when the blockade of channels, at the membrane surface or in the tissue, has allowed the entry of  $\text{Na}^+$  into the cells, or an elevated  $\text{Ca}^{2+}$  level enables more effective compartmentation of  $\text{Na}^+$  within the tissue (Kinraide, 1999). Related

to this, Cabañero et al. (2006, in press) observed that reduction of  $\text{Ca}^{2+}$  influx through the membrane (using verapamil as a  $\text{Ca}^{2+}$  channel blocker) seemed to be related to the reduction of aquaporin functionality in pepper protoplasts.

Calcium plays an important role in the water transport of plants growing under salt stress and, in addition, its concentration in the nutrient solution can determine the restoration of root hydraulic conductivity (Azaizeh and Steudle, 1991; Martínez-Ballesta et al., 2003b; Cabañero et al., 2004). Results with melon and pepper plants suggest that NaCl decreases the passage of water through the membrane and roots by reducing the activity of Hg-sensitive aquaporins, and that the ameliorative effect of  $\text{Ca}^{2+}$  on NaCl stress could be related to aquaporin function (Carvajal et al., 1999, 2000b; Cabañero et al., 2004). Nevertheless, in *Cornus stolonifera* Michx.,  $\text{Ca}^{2+}$  had no effect on the reduction of transpiration and stomatal conductance induced by  $\text{Na}^+$  (Renault, 2005), and supplemental  $\text{K}^+$  and  $\text{Ca}^{2+}$  did not ameliorate the water status in tomato and rice in the short term, but did optimise the nutritional status (Baba and Fujiyama, 2003).

In our work, it has been shown that nutritional calcium is involved in plasma membrane water channel regulation, but its effect is not due to alteration of the stability of the plasma membrane. However, the fact that results showing that  $\text{Ca}^{2+}$  blocks aquaporins exist in the literature (Gerbeau et al., 2002) indicates the need for deeper research into  $\text{Ca}^{2+}$  regulation of aquaporins and its physiological role in whole plant conditions, since the differing results suggest differences between nutritional calcium and intracellular  $\text{Ca}^{2+}$  signalling.

Although not much is known about the processes that allow perception of environmental changes by root cells and subsequent aquaporin regulation (Javot and Maurel, 2002), studies on this topic reveal that, besides regulation at the transcriptional level, a possible mechanism for control of aquaporin permeability could be a block or a shift from an activated to an inactivated, less-permeable state (Kaldenhoff and Eckert, 1999). It has been reported that aquaporin functionality may be controlled by  $\text{Ca}^{2+}$  (Johansson et al., 1996; Azad et al., 2004) and triggered by environmental factors (Steudle and Henzler, 1995).

Alterations in  $(\text{Ca}^{2+})_{\text{cyt}}$  may constitute a signal that is transduced via calmodulin, a calcium-dependent protein kinase, and other  $\text{Ca}^{2+}$ -controlled proteins, to effect a wide array of downstream responses involved in the protection of the plant and adaptation to new environmental conditions (Knight, 2000). Reversible phosphorylation could be considered as a potent mechanism for plant aquaporin regulation, during development and in the response of plants to environmental stimuli (Luu and Maurel, 2005), but changes in aquaporin expression have also to be taken into account (Martínez-Ballesta et al., 2003a)

#### Concluding Remarks

The mechanisms of salt stress response and tolerance have to integrate many aspects of plant metabolism and physiology. Classically, studies have given particular attention to the control of  $\text{Na}^+$  acquisition and allocation in plants. Therefore, investigations have centred on genetically modifying the trans-



porters involved in this. However, the complexity of the plant response to salinity, that implies a multigenic process, means that this strategy would only be successful in specific cases. The intrinsic logic of ion homeostasis is complex and based on adjustment of turgor and intracellular ion concentration. As has been stated, such complexity could lead to despair for those having to work in this area. Therefore, beside studies on the molecular biology of salt tolerance, physiological studies have revealed that the tolerance can be increased to some extent by modifying water uptake. For this, simple correction of the nutrient solution, together with a perfect knowledge of the water uptake and transport involving aquaporin regulation in each particular crop and conditions, could be the key for improving salinity tolerance.

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