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Review

# Regulation by salt of vacuolar $H^+$-ATPase and $H^+$-pyrophosphatase activities and $Na^+/H^+$ exchange

Paulo Silva[1,2] and Hernâni Gerós[1,2,*]

[1]Centro de Investigação e de Tecnologias Agro-Ambientais e Biológicas (CITAB); Portugal; [2]Departamento de Biologia; Universidade do Minho; Braga, Portugal

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**Over the last decades several efforts have been carried out to determine the mechanisms of salt homeostasis in plants and, more recently, to identify genes implicated in salt tolerance, with some plants being successfully genetically engineered to improve resistance to salt. It is well established that the efficient exclusion of $Na^+$ excess from the cytoplasm and vacuolar $Na^+$ accumulation are the most important steps towards the maintenance of ion homeostasis inside the cell. Therefore, the vacuole of plant cells plays a pivotal role in the storage of salt. After the identification of the vacuolar $Na^+/H^+$ antiporter *Nhx1* in *Saccharomyces cerevisiae*, the first plant $Na^+/H^+$ antiporter, *AtNHX1*, was isolated from Arabidopsis and its overexpression resulted in plants exhibiting increased salt tolerance. Also, the identification of the plasma membrane $Na^+/H^+$ exchanger SOS1 and how it is regulated by a protein kinase SOS2 and a calcium binding protein SOS3 were great achievements in the understanding of plant salt resistance. Both tonoplast and plasma membrane antiporters exclude $Na^+$ from the cytosol driven by the proton-motive force generated by the plasma membrane $H^+$-ATPase and by the vacuolar membrane $H^+$-ATPase and $H^+$-pyrophosphatase and it has been shown that the activity of these proteins responds to salinity. In this review we focus on the transcriptional and post-transcriptional regulation by salt of tonoplast proton pumps and $Na^+/H^+$ exchangers and on the signalling pathways involved in salt sensing.**

## Introduction

Approximately 20% of the world's cultivated land and nearly half of irrigated land are affected by salinity, which has become a serious threat to agricultural production limiting plant growth and productivity worldwide.[1,2] Excessive salinity imposes two stress factors on plants: an osmotic component that results from the reduced water availability caused by an increase in osmotic pressure in the soil, and an ionic stress resulting from a solute imbalance, causing changes in the $K^+/Na^+$ ratio and increasing the concentration of $Na^+$ and $Cl^-$ in the cytosol.[3] Sodium toxicity is caused mainly by the similarity of the $Na^+$ and $K^+$ ions to plant transporters and enzymes. Plant cells typically maintain a high $K^+/Na^+$ ratio in their cytosol with relatively high $K^+$, in the order of 100–200 mM, and low $Na^+$, of about 1–10 mM.[4]

Several efforts have been undertaken to enhance the salt tolerance of economically important plants by traditional plant breeding as well as by biotechnological approaches.[5,6] Traditional breeding programs trying to improve abiotic stress tolerance have had some success, but are limited by the multigenic nature of the trait. Arabidopsis also proved to be extremely important for assessing functions for individual stress-associated genes due to the availability of knock-out mutants and its amenability for genetic manipulation.[7] The in vitro culture approach has been proved effective in the selection of salt-tolerant cell lines and subsequent regeneration of whole plants with improved salt tolerance, such as alfalfa,[8] rice[9,10] and potato.[11]

Osmolytes like proline, glycine-betaine, trehalose and sugar alcohols such as mannitol and sorbitol that are abundantly produced and accumulated in salt treated cells represent a critical component of salt-stress responses. These compounds are expected to work through lowering the osmotic potential of cells or by protecting various cellular structures and proteins during stress.[2] The addition of NaCl to suspension cultured cells of *Olea europaea* enhanced the capacity of the polyol:$H^+$ symport system and the amount of *OeMaT1* (*Olea europaea* mannitol transporter 1) transcripts, whereas it strongly repressed mannitol dehydrogenase activity providing intracellular accumulation of mannitol.[12] Therefore, the improvement of salt tolerance in plants could be achieved by the increased production of osmolytes or stress proteins that protect or reduce damage caused by salt stress.[13] Thus, when *Nicotiana tabacum*, *Populus tomentosa* and other plants were genetically engineered to synthesize mannitol through introduction of an *Escherichia coli* mannitol-1-phosphate dehydrogenase (mtlD), which catalyzes the biosynthesis of mannitol from fructose, it resulted in more salt-tolerant plants.[14,15] Also, in Arabidopsis, mtlD gene transfer and expression enhanced seed

*Correspondence to: Hernâni Gerós; Departamento de Biologia; Universidade do Minho; Campus de Gualtar; Braga 4710-057 Portugal; Tel.: +351.253.604048; Fax: +351.253678980; Email: geros@bio.uminho.pt

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germination under salinity conditions.[16] Moreover, a relationship between antioxidant defence system and salt tolerance was demonstrated in cotton and sunflower calli lines grown under NaCl.[17,18] Gueta-Dahan and co-workers have also reported that salt tolerance acquisition in a citrus cell line was related with improved resistance to oxidative stress.[19] Concordantly, the exogenous application of mannitol was shown to protect wheat plants from the harmful effects of salt-induced oxidative stress by enhancing the activity of antioxidant enzymes.[20]

The ability to compartmentalise salt into the vacuoles is an important step towards the maintenance of ion homeostasis inside the cell. The first plant tonoplast $Na^+/H^+$ antiporter, *AtNHX1*, was isolated from Arabidopsis[21,22] and several studies have shown that the exposure to salt upregulates $Na^+/H^+$ antiport activity, suggesting a role of the exchanger in salt tolerance. The activity of this secondary transport system is driven by the proton-motive force generated by the vacuolar membrane $H^+$-ATPase and $H^+$-pyrophosphatase[23] that also respond to salt levels through transcriptional and post-transcriptional regulation mechanisms. The direct stimulation of the vacuolar $Na^+/H^+$ antiport system may be coordinated with the increased activity of the vacuolar $H^+$ pumps, which provide the driving force for the operation of the cation exchanger. Thus, the overexpression of $H^+$-pyrophosphatase Avp1 was reported to confer salt tolerance on transgenic plants.[24] In the present paper, the role of the tonoplast $Na^+/H^+$ exchanger and proton pumps V-$H^+$-ATPase and pyrophosphatase on plant response to high salinity are dissected in relation with their regulation by $Na^+$ and signalling pathways involved on salt sensing.

## Two Proton Pumps Energize the Vacuolar Membrane

The vacuoles of plant cells are widely diverse in form, size, content, functional dynamics and play central roles in plant growth, development and stress responses.[25,26] They have recognized functions in protein turnover, pH and ion homeostasis, turgor pressure maintenance, sequestration of toxic compounds and pigmentation. The central vacuole, which can occupy more than 80% of the total plant cell volume, is separated from the surrounding cytosol by the tonoplast membrane that controls the passage of inorganic and organic solutes to and from the cytoplasm through a wide range of pumps, carriers, ion channels and receptors,[27,28] but these proteins are generally less well known than the corresponding plasma membrane proteins. Proteomic methodologies can provide important insights into the potential functions of these proteins.[23,29]

The electrogenic $H^+$ pumps V-$H^+$-ATPase and V-$H^+$-PPase are major components of the vacuolar membrane of plant cells.[23,26] With the noticeable exception of lemon, where $H^+$-PPase can be ruled out as the primary proton pump,[30] all plant species from which vacuolar membranes were studied exhibit V-$H^+$-PPase activity in addition to V-$H^+$-ATPase activity. The V-$H^+$-ATPase is universally present in the membranes of different internal acidic organelles in eukaryotic cells and has an intricate structure: a peripheral $V_1$ sector which contains three copies of the A- and B-subunits, responsible for the catalytic activity, and the subunits C-H which form a central stalk linking the $V_1$ to the hydrophobic membrane-embedded $V_o$ sector. The $V_o$ sector contains the a-subunit and six copies of the c-subunit, which forms a proton-conducting channel. As in their F-type homologues, where ATP is regenerated by induced conformational changes due to a rotatory mechanism, parts of the V-$H^+$-ATPases have been shown to rotate when ATP is supplied, suggesting a very similar enzymatic mechanism for both proton pumps.[26] In contrast to the V-$H^+$-ATPase, the V-$H^+$-PPase consists of a single polypeptide and exists as a dimmer of subunits of 71–80 kDa. It is distributed among most land plants, but only some algae, protozoa, bacteria and archaebacteria, and uses $PP_i$ as its energy source.[31]

In several plant models the V-$H^+$-PPase seems to be able to generate and maintain across the vacuolar membrane a higher pH gradient than the V-$H^+$-ATPase, at $PP_i$ concentrations in the micromolar range.[32-35] Generally, V-$H^+$-PPase activity is high in young tissues, whereas V-$H^+$-ATPase activity is relatively constant during growth and maturation.[26] In pear fruit the ratio of V-$H^+$-PPase activity to V-$H^+$-ATPase activity indicated that V-$H^+$-PPase is a major $H^+$-pump of the vacuolar membranes of young fruit and that the contribution of V-$H^+$-ATPase increases with fruit development, finally, V-$H^+$-ATPase becomes the major $H^+$-pump during the later stages of fruit development.[32] In growing tissues and exponentially growing cultured cells, a large amount of $PP_i$ is produced as a by-product of several metabolic processes, such as DNA and RNA synthesis, sucrose and cellulose synthesis and more $PP_i$ is available to be used as a source of energy for active transport of protons into the vacuoles.[26] Other studies have shown that activity of the vacuolar V-$H^+$-PPase may allow the plant cell to conserve the free energy of $PP_i$ in a transmembrane pH gradient driving the synthesis of ATP.[36]

## Regulation of V-$H^+$-PPase and V-$H^+$-ATPase Activity by Salt

The regulation of both V-$H^+$-ATPase and V-$H^+$-PPase activity by salt is well reported in the literature; however to date, no clear correlative pattern has been found for activation or deactivation of both proton pumps in response to salinity. Evidence for a decreased activity of V-$H^+$-PPase with exposure to NaCl has been described several times,[37-42] but it has been shown that the activity of V-$H^+$-PPase increases in several plants grown within saline environments.[35,43-46] In salt adapted cell line of *Solanum tuberosum*, the activity of V-$H^+$-PPase increased about threefold over cells cultivated in the absence of salt.[35] In the halophyte *Suaeda salsa* only in the case of 0.1 M NaCl treatment was V-$H^+$-PPase markedly increased over the entire duration of the experiment, all other treatments only led to a small transient increase of V-$H^+$-PPase activity or to a decrease of activity compared to controls; thus, under salt stress and osmotic stress conditions in *S. salsa*, V-$H^+$-PPase activity seems to be less important physiologically than V-$H^+$-ATPase activity.[47] As discussed by these authors, NaCl responses of the V-$H^+$-PPase depend on plant species and type of treatment and cannot be generalized.

In same plants a clear correlation between the activity of V-$H^+$-PPase and protein amount has been detected, suggesting that increased or decreased protein levels may be at least partly

responsible for the stimulation and repression of V-H+-PPase activity, respectively. This is the case of *S. tuberosum* where immunoblot analysis showed that increased amounts of V-H+-PPase protein are present in the tonoplast of NaCl-tolerant calli. A control step enhancing transcription or protein translation rates and/or diminishing the turnover of the protein is most likely involved in the *S. tuberosum* cells in response to salt.[35] Similarly, an increased accumulation of the 68 kDa V-H+-PPase catalytic subunit in vacuolar membrane vesicles isolated from *Salicornia bigelovii* grown in 200 mM NaCl was observed.[46] In tonoplast vesicles from wheat (*Triticum aestivum*) roots exposed to severe NaCI stress (200 mmol/L) for 3 days the strong reduction in V-H+-PPase substrate hydrolysis activity correlated with lower amounts of V-H+-PPase protein.[40] However, the decreased proton transport and hydrolytic activities of V-H+-PPase in 3-day-old seedlings of *Vigna unguiculata* treated with 100 mmol/L NaCl did not show any correlation with V-H+-PPase protein levels, suggesting that regulation of the activity was due to a partial enzyme inactivation.[41] There is evidence that transcripts encoding V-H+-PPase are regulated by salt stress in maize and bean plants.[48] The physiological significance and the regulation of the gene expression of V-H+-PPase has been reviewed by Maeshima.[31]

Although in some plants a reduced activity of V-H+-PPase has been observed in response to salt, it is well documented that increased salt accumulation in the vacuole is likely the result, at least in part, of more driving force for $Na^+/H^+$ exchange provided by and V-H+-PPase or V-H+-ATPase activity, or both. Thus, the overexpression of the vacuolar H+-PPase AVP1 in *Arabidopsis thaliana* resulted in plants exhibiting a higher salt tolerance, which was probably a consequence of an increased proton gradient across the tonoplast.[24]

A general sodium-induced increase in V-H+-ATPase activity in plant response to salt has been reported.[35,37,38,41,42,44,45,47,49-56] In contrast, the activity of V-H+-ATPase in *Daucus carrota* was unaffected by salt treatment[43] and was even repressed in wheat roots under severe NaCl stress.[40]

In the halophyte *S. salsa*, the main strategy of salt-tolerance seems to be an upregulation of V-H+-ATPase.[47] The hydrolytic and H+ pumping activity of the V-H+-ATPase in tonoplast vesicles derived from leaves increased two-fold in salt-treated leaves (200 mM NaCl) compared with the control leaves.[56] In *Mesembryanthemum crystallinum*, where the tonoplast ATPase seems to be the main enzyme responsible for the energization of malate accumulation in Crassulacean acid metabolism (CAM),[38] both V-H+-ATPase H+-transport and ATP hydrolytic activity were twofold higher in vesicles isolated from leaves of plants treated with 200 mM NaCl when compared with the activity measured in control plants.[51] In *Populus euphratica*, studies showed that cell suspensions respond to salt stress by increasing both the V-H+-ATPase hydrolytic[42,55] and H+ pumping activities.[55] V-H+-ATPase H+-pumping was also stimulated in NaCl-adapted cells of tobacco,[50] in salt-stressed roots of barley,[49] mung bean[37] and sunflower,[45] in cowpea seedlings subjected to NaCl,[41] as well as in *S. tuberosum* calli adapted to 150 mM NaCl.[35]

Several reports have shown that the activity of V-H+-ATPase varies in parallel with protein amount. This is the case of cowpea seedlings subjected to NaCl treatment when western blot analysis of A- and B-subunits of V-H+-ATPase revealed that the protein content of the two subunits increased in parallel with the increase of proton transport and hydrolytic activities.[41] Also, in plants of *M. crystallinum* L. two subunits of the V-H+-ATPase with Mr of about 27 and 31 kDa showed particularly high intensities only in the CAM state, induced by salt treatment or aging, when the total ATP hydrolytic activity of the tonoplast ATPase was higher. Therefore, the increase in ATPase activity was accompanied by de-novo synthesis of tonoplast proteins.[38] In *S. salsa* the upregulation of V-H+-ATPase activity is not obtained by structural changes of the enzyme, but also by an increase in protein amount.[47]

Other studies have shown that in some plants salt-mediated increase of the V-H+-ATPase activity is not mediated by the increase in protein expression, as in the halophytes *M. crystallinum*[54] and *S. bigelovii*.[46,52] In tobacco,[50] the relative H+ transport capacity per unit of 69 kilodalton subunit of the tonoplast ATPase of vesicles isolated from NaCl adapted cells was fourfold greater than that observed for vesicles from unadapted cells. Such correlation between enzyme activity and protein content was also found for the tonoplast V-H+-ATPase in potato cell lines when western blotting analysis revealed that the relative amount of A subunit of the V-H+-ATPase remained constant in NaCl-tolerant calli despite the observed increase in both hydrolytic and H+-pumping activity in the salt-tolerant cell line.[35] Therefore, since the amount of the subunit A is likely to represent the protein level of V-H+-ATPase, and post-translational modifications such as phosphorylation/dephosphorylation, the assembly of other subunits or the action of regulatory proteins might be involved. Phosphorylation and dephosphorylation of proteins is a common example of a post-translational modification that has the potential to alter protein activity.[57] It was shown that V-H+-ATPases are potential targets of WNK kinases and their associated signaling pathways.[58] Recently, the Ser/Thr kinase SOS2 (see below) was implicated in the regulation of V-H+-ATPase activity in Arabidopsis, coordinating changes in ion transport during salt stress.[59] Proteolysis has also been show to regulate V-H+-ATPase. In wheat the proteolysis of subunit A of V-H+-ATPase was related to the observed decreased activity of the proton pump in response to salt stress.[40]

The ability to respond to salinity stress with changes in the gene expression of the vacuolar ATPase might be a prerequisite and a characteristic of salt tolerance in plants.[60,61] It has been shown that the transcript levels of some subunits are upregulated in response to salt stress. In fully expanded leaves of *M. crystallinum*, 8 h after salt treatment, there was an increase in the transcript levels of subunit c mRNA but not of subunit A or B,[62] which correlates well with the observed increase in activity of the V-H+-ATPase in vesicles from leaf mesophyll tissue from plants treated with salt,[51] whereas in roots and young leaves, mRNA levels for all the three subunits increased about 2-fold compared to control plants. The expression of vacuolar H+-ATPase genes does not always involve a fixed stoichiometry of mRNAs for the different subunits and the mRNA level for subunit c is particularly sensitive to developmental and environmental changes.[62] Also, the emerging knowledge on subunit isogenes in some species including Arabidopsis illustrates another level of complexity, the regulation of isogene expression and function of subunit isoforms.[61]

Moreover, other factors may account for the regulation of tonoplast transport proteins, such as changes in lipid-protein interactions, since alterations in membrane lipid composition and structure have been associated with salt stress,[63,64] and ATPase activity could be regulated by changes in the membrane lipids.[65,66]

## Regulation of $Na^+/H^+$ Antiport Activity by Salt

Vacuolar $Na^+/H^+$ antiporters have been investigated as the key to salt tolerance in plants.[3] The antiporter mediates transport of $Na^+$ into the vacuole. In 1985, Blumwald and Poole demonstrated the activity of the antiporter in tonoplast vesicles from red beet storage tissue[67] and in 1991, Barkla and Blumwald identified a 170-kDa protein associated with the vacuolar $Na^+/H^+$ antiport of *Beta vulgaris*.[68] In yeast, the $Na^+/H^+$ antiporter *Nhx1*, which contributes to cellular $Na^+$ homeostasis, was identified by Nass and co-worker.[69] The exchanger was localized to the late endosome/prevacuolar compartment and it was proposed that it may be involved in $Na^+$ transport, water movement and vesicle volume regulation,[70] as well as in osmotolerance following sudden exposure to hyperosmotic media.[71] The first plant $Na^+/H^+$ antiporter, *AtNHX1*, was isolated from Arabidopsis by functional genetic complementation of a yeast mutant defective for endosomal $Na^+/H^+$ activity,[21,22] and its overexpression suppressed some of the salt-sensitive phenotypes of the *nhx1* yeast strain.[22] Since then, several $Na^+/H^+$ antiporter genes have been characterized in plants such as rice,[72,73] *Atriplex gmelini*,[74] *B. vulgaris*,[75] *Brassica napus*,[76] cotton,[77] wheat[78-80] and grapevine.[81] Six AtNHX isoforms were found in Arabidopsis, and for five of them $Na^+/H^+$ transport activity has been demonstrated[82,83] (Fig. 1). AtNHX1 and AtNHX2 are the most highly expressed members of this family, and corresponding transcripts are widely distributed, while AtNHX3 and AtNHX4 transcripts are almost exclusively present in flowers and roots. Yamaguchi and co-workers reported that AtNHX1 comprises nine transmembrane domains, with the hydrophilic C-terminal domain facing the vacuolar lumen and the N terminus facing the cytosol. Three hydrophobic regions do not appear to span the tonoplast membrane, yet appear to be membrane associated.[84] However, Sato and Sakaguchi[85] place the C-terminal domain in the cytoplasm and confirm a structural analogy between AtNHX1 and the human NHE1, with both antiporters having 12 transmembrane domains and AtNHX1 lacking a N-terminal signal peptide (Fig. 2). These results agree well with the structure proposed for VvNHX1.[86]

Chloride channels have already been identified and cloned in plants[87,88] and, in yeasts, mutants lacking the gene *GEF1* encoding a chloride channel are more susceptible to cation toxicity.[89] More recently two tonoplast $Cl^-$ transporter genes from rice, *OsClC1* and *OsClC2*, were identified and functionally characterized in yeast.[90] The level of expression of *OsClC1*, but not of *OsClC2*, was increased by treatment with NaCl. In *P. euphratica*, an enhanced ability of the V-$H^+$-PPase to create a $H^+$ gradient in the presence of $Cl^-$ was demonstrated.[42] In fact, results by Chen and co-workers showed that in salt stressed *P. euphratica*, young root cortical cells accumulated $Cl^-$ in the vacuoles when compared with control plants,[91] and in suspension-cultured cells subjected to 200 mM NaCl, a higher amount of $Cl^-$ was found in the vacuole than in the cytoplasm and cell wall.[92] This may be due to an adaptation of salt-tolerant plants to NaCl stress, where a greater permeability of the tonoplast vesicles to $Cl^-$ can allow it to accumulate in the vacuole down its electrical gradient, dissipating an inside-positive membrane potential and thus stimulating the formation of a higher ΔpH through V-$H^+$-ATPase and V-$H^+$-PPase activity,[93] which can be used in sodium (and other cations) detoxification and in an increase in osmotic pressure by means of the accumulation of sodium in the vacuole.[22] Thus, it appears that this transporter protein could be the physiological counterpart to NHX for the accumulation of $Cl^-$. As discussed by Martinoia and co-workers,[26] it is not still clear if it works as a channel, as suggested by Nakamura and co-workers,[90] or as a $Cl^-/H^+$ antiporter.

Contrary to the notion that multiple traits introduced by breeding into crop plants are needed to obtain salt-tolerant plants, the overexpression of the vacuolar $Na^+/H^+$ antiport has shown to improve salinity tolerance in several plants. The first evidence showed that the overexpression of *AtNHX1* in Arabidopsis plants promoted sustained growth and development in soil watered with up to 200 mM NaCl,[21] although recent evidences report that transgenic Arabidopsis do not show a significantly improved salt tolerance above that of control plants.[94] In addition, transgenic tomato plants overexpressing *AtNHX1* were able to grow, flower and produce fruit in the presence of 200 mM NaCl, and sodium accumulated in leaves but not in the fruit.[95] Also, transgenic *B. napus* plants overexpressing the same gene from Arabidopsis, were able to grow, flower and produce seeds in the presence of 200 mM NaCl,[96] and transgenic tobacco plants overexpressing *GhNHX1* from cotton exhibited higher salt tolerance than the wild-type plants.[77] The overexpression of the $Na^+/H^+$ antiporter gene clone from *OsNHX1*, improved the salt tolerance of transgenic rice cells and plants.[73]

The role of tonoplast $Na^+/H^+$ antiporter in plant salt tolerance has been reinforced by several evidences showing that exposure to salt promotes the increase of $Na^+/H^+$ antiport activity[35,42,46,51,53,97,98] (Fig. 3). Some reports show upregulation of *NHX* genes,[22,56,72-77,79,80,99] increased protein abundance[56,74-76] or regulation at protein activity level.[46,100] Garbarino and co-workers[97,100] have shown that the inducible $Na^+/H^+$ antiporter activity observed in tonoplast from barley roots grown in the presence of NaCl was due to activation of an existing protein rather than to de novo protein synthesis, since the rapid induction was observed in the presence of inhibitors of protein synthesis. As shown below, there can be coordination of activity between the exchangers on the tonoplast and plasma membranes[101] and the C-terminus of AtNHX1, which may face the vacuolar lumen,[84] may have a key role in the regulation of the protein activity by binding calmodulin.[102] Moreover, in *A. gmelini*,[74] *B. vulgaris*,[75] *B. napus*[76] and *S. salsa*,[56] upregulation of the tonoplast $Na^+/H^+$ antiport activity is due to increase of both transcription and translation. A crosstalk between osmotic stress and vacuole accumulation of $Na^+$ has been demonstrated in Arabidopsis where osmotic stress activates the synthesis of abscisic acid (ABA), which upregulates the transcription of AtNHX1.[99]

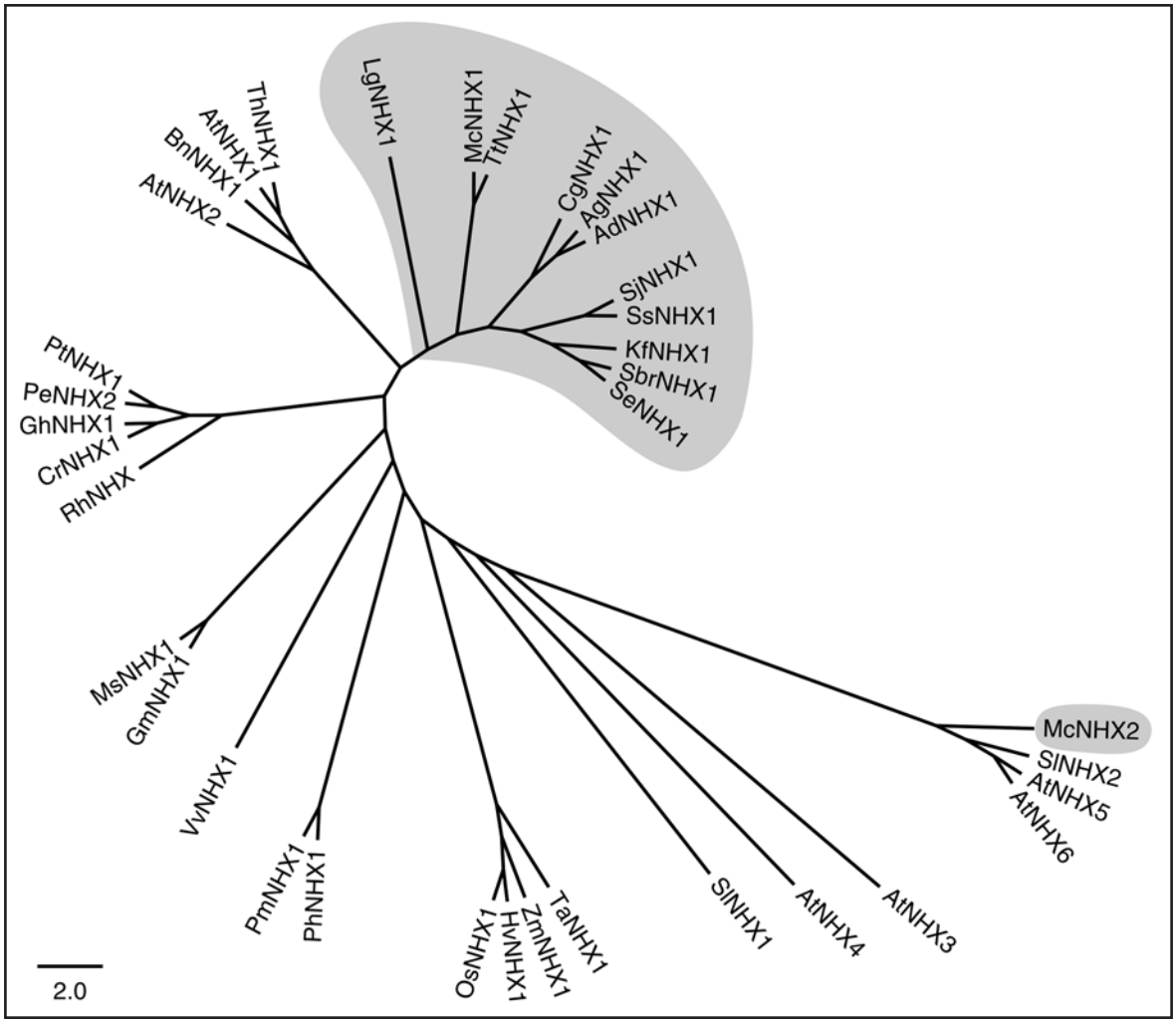


Figure 1. Phylogenetic tree of $Na^+/H^+$ antiporters. Sequence analysis was performed online using Mobyle (http://mobyle.pasteur.fr/). A multiple sequence alignment of several antiporter protein sequences was generated using ClustalW and the neighbour-joining method was used to calculate evolutionary distances. The unrooted phylogenetic tree was constructed using the FigTree software package (FigTree 1.2.2, http://tree.bio.ed.ac.uk/software/figtree/). Antiporter sequences from the following species were used in the construction of the tree: *Atriplex dimorphostegia* (*AdNHX1*, AY211397), *Atriplex gmelini* (*AgNHX1*, AB038492), *Arabidopsis thaliana* (*AtNHX1*, NM_122597; *AtNHX2*, NM_111375; *AtNHX3*, NM_124929; *AtNHX4*, NM_111512; *AtNHX5*, NM_104315; *AtNHX6*, NM_106609), *Brassica napus* (*BnNHX1*, AY189676), *Chenopodium glaucum* (*CgNHX1*, AY371319), *Citrus reticulata* (*CrNHX1*, AY607026), *Gossypium hirsutum* (*GhNHX1*,AF515632), *Glycine max* (*GmNHX1*, AY972078), *Hordeum vulgare* (*HvNHX1*, AB089197), *Kalidium foliatum* (*KfNHX1*, AY825250), *Limonium gmelinii* (*LgNHX1*, EU780457), *Mesembryanthemum crystallinum* (*McNHX1*, AM746985; *McNHX2*, AM748092), *Medicago sativa* (*MsNHX1*, AY456096), *Oryza sativa* (*OsNHX1*, AB021878), *Populus euphratica* (*PeNHX2*, EU382999), *Petunia hybrida* (*PhNHX1*, AB051817), *Plantago maritima* (*PmNHX1*, EU233808), *Populus tomentosa* (*PtNHX1*, AY660749), *Rosa hybrida* (*RhNHX1*, AB199912), *Salicornia brachiata* (*SbNHX1*, EU448383), *Salicornia europaea* (*SeNHX1*, AY131235), *Suaeda japonica* (*SjNHX1*, AB198178), *Solanum lycopersicum* (*SlNHX1*, AJ306630; *SlNHX2*, AJ306631), *Suaeda salsa* (*SsNHX1*, AY261806), *Tetragonia tetragonioides* (*TtNHX1*, AF527625), *Thellungiella halophila* (*ThNHX1*, FJ713100), *Triticum aestivum* (*TaNHX1*, AY461512), *Vitis vinifera* (*VvNHX1*, AY634283), *Zea mays* (*ZmNHX1*, NM_001111751). The shaded area represents halophytic species.

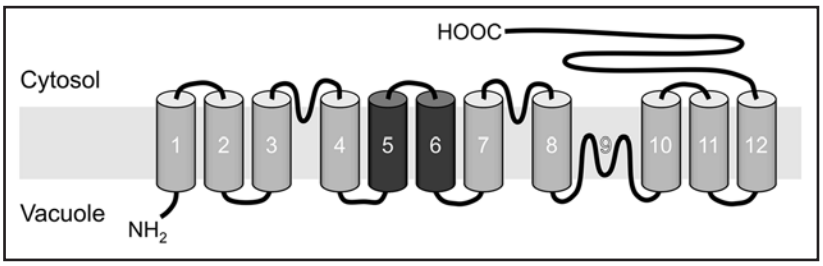


Figure 2. Topological model of the Arabidopsis $Na^+/H^+$ exchanger AtNHX1, showing 12 transmembrane domains, and with a hydrophobic, luminal N-terminal and a hydrophilic, cytosolic C-terminal. The model was constructed and adapted according to the work of Sato and co-workers.[85] The darker transmembrane domains represent the predicted active site.[84]

Overall, higher-than-normal levels of *NHX* transcripts, protein and vacuolar $Na^+/H^+$ antiport activity, have been reported in several plants in response to salt supporting the key role of $Na^+/H^+$ exchanger in plant salinity tolerance.

## $Na^+$ Sensing

To survive and develop normally, plants must constantly perceive changes in their environment and respond properly through a variety of molecular mechanisms. One of the most important abiotic stresses for crop productivity concerns plant dehydration. Plants suffer from dehydration under high salinity and drought, as well as low-temperature conditions, all of which cause hyperosmotic stress characterized by a decreased turgor pressure and water loss. Dehydration triggers the biosynthesis of the abscisic acid (ABA) hormone and it has been known for a long time that a significant set of genes, induced by drought, salt and cold stresses, are also activated by ABA.[103] The mechanisms involved in the sensing of osmotic and salt stress in plants remain poorly understood, and the majority of the available information comes from studies in microorganisms. In yeast, hyperosmotic stress is sensed by two types of osmosensors, SLN1 and SHO1, which feed finally into HOG (high-osmolarity glycerol) MAPK pathway.[7] In Arabidopsis, the SLN1 homologue ATHKl functions as an osmosensor and transmits the stress signal to a downstream MAPK cascade. The introduction of the ATHK1 cDNA into the yeast double mutant, which lacks SLN1, suppressed lethality in high-salinity media and activated the high osmolarity glycerol response 1 (HOG1) mitogen-activated protein kinase (MAPK).[104] Also, the activity of the plant histidine kinase cytokinin response 1 (Cre1) is regulated by changes in turgor pressure, in a manner identical to that of Sln1, being a probable candidate for sensing osmotic stress in plants.[105] The gene *NtC7* from tobacco codes for a receptor-like protein functioning in osmotic adjustment whose membrane location was confirmed in onion epidermis cells transiently expressing an NtC7-green fluorescent protein fusion protein. Its transcripts were found to accumulate rapidly and transiently within 1 h upon treatments with not only wounding but also salt and osmotic stresses.[106]

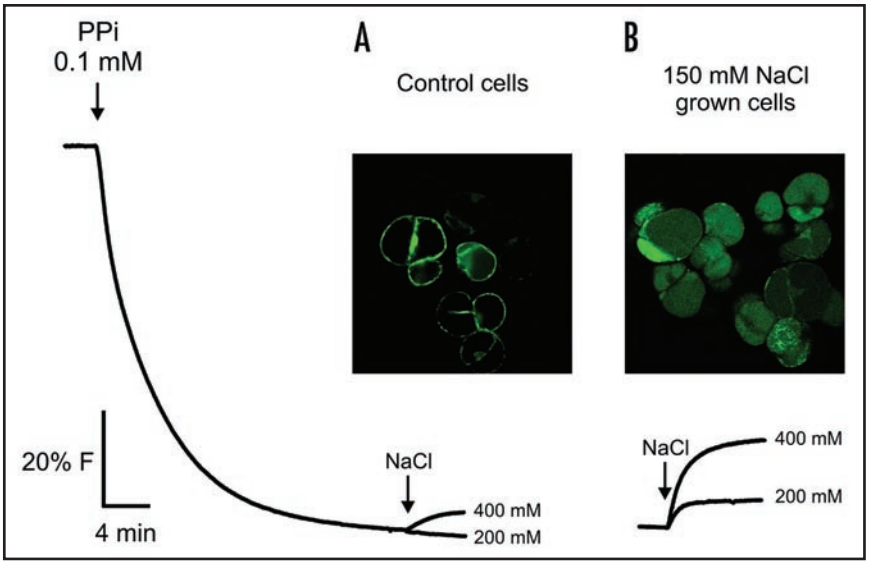


Figure 3. Dissipation of a $PP_i$-dependent $H^+$ gradient upon addition of 200 mM and 400 mM NaCl (final concentrations) to tonoplast vesicles isolated from *P. euphratica* suspension-cultured cells grown in the absence of salt (A) and in the presence of 150 mM NaCl (B). *Inserts:* Confocal imaging of $Na^+$ accumulation in *P. euphratica* suspension cells stained with Sodium Green (Adapted from Silva et al.[42] with kind permission from Springer).

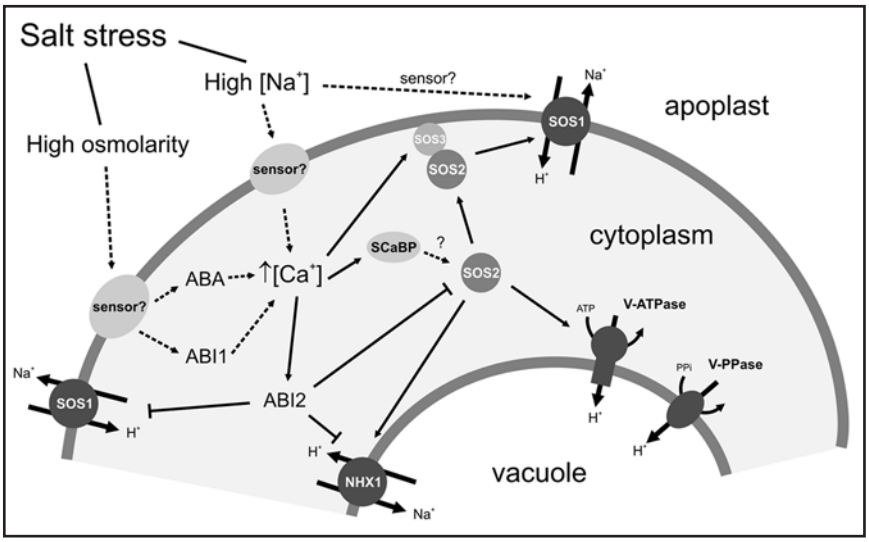


Figure 4. Signalling pathways responsible for sodium extrusion in Arabidopsis under salt stress. Excess $Na^+$ and high osmolarity are separately perceived by yet unidentified sensors at the plasma membrane level, which then induce an increase in cytosolic $Ca^{2+}$ concentration. This increase is then sensed by SOS3 which activates SOS2. The activated SOS3-SOS2 protein complex phosphorylates SOS1, the plasma membrane $Na^+/H^+$ antiporter, resulting in the efflux of $Na^+$ ions.[107] SOS2 has also been shown to regulate NHX1 antiport activity[101] and V-$H^+$-ATPase activity[59] in a SOS3-independent manner, possibly by SOS3-like $Ca^{2+}$-binding proteins (SCaBP) that target it to the tonoplast. Salt stress can also induce the accumulation of ABA, which, by means of ABI1 and ABI2, can negatively regulate SOS2 or SOS1 and NHX1.[115]

The knowledge on how $Na^+$ is sensed is still very limited in most cellular systems. Theoretically, $Na^+$ can be sensed either before or after entering the cell, or both (Fig. 4). Extracellular $Na^+$ may be sensed by a membrane receptor, whereas intracellular $Na^+$ may be sensed either by membrane proteins or by any of the many $Na^+$-sensitive enzymes in the cytoplasm.[107] In spite of the molecular identity of $Na^+$ sensor(s) remaining elusive, the plasma-membrane $Na^+/H^+$ antiporter SOS1 (SALT OVERLY SENSITIVE1) is a possible candidate.[108] The SOSl gene encodes a

transmembrane protein with similarities to plasma membrane $Na^+/H^+$ antiporters from bacteria and fungi and the steady-state level of transcript is upregulated by NaCl stress.[108] Transgenic plants showed substantial upregulation of *SOS1* transcript levels upon NaCl treatment, suggesting post-transcriptional control of *SOS1* transcript accumulation. Undifferentiated callus cultures regenerated from transgenic plants were also more tolerant of salt stress, which was correlated with reduced $Na^+$ content in the transgenic cells.[109] When expressed in a yeast mutant deficient in endogenous $Na^+$ transporters, *SOS1* was able to reduce $Na^+$ accumulation and improve salt tolerance of the mutant cells, and confocal imaging of a SOS1-green fluorescent protein fusion protein in transgenic Arabidopsis plants indicated that SOS1 is localized in the plasma membrane.[110]

The SOS pathway was discovered when three *salt-overly-sensitive* mutants (*sos1*, *sos2* and *sos3*) were characterized in a genetic screen designed to identify components of the cellular machinery that contributes to salt tolerance in Arabidopsis. SOS2 is predicted to encode a serine/threonine type protein kinase with an N terminal catalytic domain similar to that of the yeast SNF1 kinase[111] and *SOS3* encodes a $Ca^{2+}$ sensor protein that shares significant sequence similarity with the calcineurin B subunit from yeast and neuronal calcium sensors from animals.[112] SOS1 has been shown to be an output or target of the SOS pathway whose activity is controlled by SOS2/SOS3. *SOS1* expression was upregulated in response to NaCl stress and this upregulation is abated in sos3 or sos2 mutant plants.[108] SOS1 ion transporter, the SOS2 protein kinase, and its associated $Ca^+$ sensor SOS3 constitute a functional module being SOS1 the phosphorylation substrate for the SOS2/SOS3 kinase complex.[113]

Besides the implication of SOS2 in the regulation of V-$H^+$-ATPase activity in Arabidopsis,[59] recent evidences have also demonstrated that the tonoplast $Na^+/H^+$ exchanger is also a target of the SOS pathway, being regulated by the SOS2 kinase[101] and the autophosphorylation of Ser 228 of SOS2 seem to be important for its function under salt stress.[114] In *sos1* deletion mutants, $Na^+/H^+$ exchange activity is significantly higher, while in *sos2* deletion mutants this activity is strongly reduced. Activated SOS2 protein added in vitro increased tonoplast $Na^+/H^+$-exchange activity in vesicles isolated from mutants lacking SOS2 but did not have any effect on activity in vesicles isolated from wild-type, *sos1* or *sos3*.[101] There can be coordination of activity between the exchangers on the tonoplast and plasma membranes; when the activity of one exchanger is missing or reduced, the activity of the other may be enhanced to compensate for the lost activity. This compensation could provide an adaptive mechanism to enable the plant to maintain the low levels of intracellular $Na^+$ required for growth.[101] Yamaguchi and co-workers[84] have shown that the deletion of the C-terminus of AtNHX1 resulted in a dramatic increase in the relative rate of $Na^+/H^+$ transport. In a more recent work it was shown that C-terminus can interact with a vacuolar calmodulin-like protein (AtCaM15) in a $Ca^{2+}$- and pH dependent manner.[102] The pH-dependence of the interaction between AtCaM15 and AtNHX1 could suggest the presence of pH-dependent signaling components in the vacuole.

## Conclusion

Planet Earth is a highly saline environment, with a salt content of about 30 g of sodium chloride per liter of water.[5] Furthermore, around 20% of all irrigated land is adversely affected by salinity,[116] and thus the comprehension of plant defense mechanisms against salt stress has important implications in plant productivity. Many scientific advances have been achieved in understanding physiological, biochemical and molecular aspects of salt stress resistance, like the identification of key genes such as those encoding the plasma membrane SOS1 and the vacuolar NHX1 antiporters, and the recent progresses in the elucidation of the SOS signalling pathway.[117] Some of this knowledge has led to the successful improvement of plant salt tolerance through manipulation of one of those genes alone, such as the overexpression of *OsNHX1* in rice[73] and *AtNHX1* in tomato,[95] in spite of salt stress resistance being considered a multigenic trait. As stated by Flowers,[5] "transgenic technology will undoubtedly continue to aid the search for the cellular mechanisms that underlie tolerance, but the complexity of the trait is likely to mean that the road to engineering such tolerance into sensitive species will be long" and "experience suggests authors should avoid hyperbole in their titles and summaries, as this does little service to the long-term aim of improving the salt tolerance of crops in the field." This is a fascinating area of research that is still wide open. The elucidation of signalling pathways responsible for responses to salt, drought and other abiotic stresses, and the cross-talk between these different pathways could allow the treatment of plants with exogenous compounds—such as mannitol[20] and other osmoprotectants and antioxidants—without recurring to genetic manipulation, avoiding the introduction in Nature of genetic engineered plants.

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