

Differential expression and function of *Arabidopsis thaliana* NHX Na⁺/H⁺ antiporters in the salt stress response

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Summary

The *Arabidopsis thaliana* vacuolar Na⁺/H⁺ antiporter AtNHX1 is a salt tolerance determinant. Predicted amino acid sequence similarity, protein topology and the presence of functional domains conserved in AtNHX1 and prototypical mammalian NHE Na⁺/H⁺ exchangers led to the identification of five additional *AtNHX* genes (AtNHX2–6). The *AtNHX1* and *AtNHX2* mRNAs are the most prevalent transcripts among this family of genes in seedling shoots and roots. A lower-abundance *AtNHX5* mRNA is present in both shoots and roots, whereas *AtNHX3* transcript is expressed predominantly in roots. *AtNHX4* and *AtNHX6* mRNAs were detected only by RT-PCR. *AtNHX1*, 2 or 5 suppress, with differential efficacy, the Na⁺/Li⁺-sensitive phenotype of a yeast mutant that is deficient in the endosomal/vacuolar Na⁺/H⁺ antiporter ScNHX1. Ion accumulation data indicate that these AtNHX proteins function to facilitate Na⁺ ion compartmentalization and maintain intracellular K⁺ status. Seedling steady-state mRNA levels of *AtNHX1* and *AtNHX2* increase similarly after treatment with NaCl, an equi-osmolar concentration of sorbitol, or ABA, whereas *AtNHX5* transcript abundance increases only in response to salt treatment. Hyper-osmotic up-regulation of *AtNHX1*, 2 or 5 expression is not dependent on the SOS pathway that controls ion homeostasis. However, steady-state *AtNHX1*, 2 and 5 transcript abundance is greater in *sos1*, *sos2* and *sos3* plants growing in medium that is not supplemented with sorbitol or NaCl, providing evidence that transcription of these genes is negatively affected by the SOS pathway in the absence of stress. *AtNHX1* and *AtNHX2* transcripts accumulate in response to ABA but not to NaCl in the *aba2-1*, mutant indicating that the osmotic responsiveness of these genes is ABA-dependent. An as yet undefined stress signal pathway that is ABA- and SOS-independent apparently controls transcriptional up-regulation of *AtNHX5* expression by hyper-saline shock. Similar to AtNHX1, AtNHX2 is localized to the tonoplast of plant cells. Together, these results implicate AtNHX2 and 5, together with AtNHX1, as salt tolerance determinants, and indicate that AtNHX2 has a major function in vacuolar compartmentalization of Na⁺.

Keywords: endosomal transporter gene family, osmotic stress, stress signalling, abscisic acid, salt tolerance.

Introduction

Salinity is a major constraint of crop productivity because it reduces yield and limits expansion of agriculture onto previously uncultivated land (Flowers and Yeo, 1995). Plant salt stress survival and adaptation (resumption of growth after stress exposure) requires cellular ion homeostasis involving net intracellular Na⁺ and Cl⁻ uptake and subsequent vacuolar compartmentalization without cyto-

toxic ion accumulation (Blumwald *et al.*, 2000; Hasegawa *et al.*, 2000; Niu *et al.*, 1995). Vacuolar partitioning of Na⁺ and Cl⁻ also contributes to maintenance of cellular water status, together with organic solutes which are the principal osmolytes that accumulate in the cytosol and organelles to balance intracellular osmotic status of cells in salt grown plants (Rhodes and Hanson, 1993). Cellular

ion exclusion is not an adaptation of higher plants, presumably because cell enlargement is a part of development whereby cells increase their volume after division by as much as 100-fold (Lyndon, 1990). Vacuolar expansion is the primary mechanism for this massive cell enlargement. The capacity for vacuolar compartmentalization of Na^+ and Cl^- is a salt adaptation conserved in halophytes and glycophytes (Blumwald *et al.*, 2000; Hasegawa *et al.*, 2000).

Energy-dependent Na^+ transport across plant cell membranes is usually coupled to the proton (H^+) electrochemical potential established by H^+ -translocating pumps (Blumwald *et al.*, 2000; Hasegawa *et al.*, 2000). H^+ transport across these membranes increases with salt treatment and may be attributed both to pump activation and enhanced transcription (Hasegawa *et al.*, 2000). Plasma membrane and tonoplast transporters mediate the downhill transport of H^+ to the cytosol by energy-dependent Na^+ efflux from the cytosol. Both plasma membrane and tonoplast Na^+/H^+ antiporter activities increase in response to salt treatment, at least in halophytic species (Blumwald *et al.*, 2000).

The Na^+ -hypersensitive *Arabidopsis* mutant *sos1* is defective for a plasma membrane Na^+/H^+ antiporter (Shi *et al.*, 2000). SOS1 is a 127 kDa protein which, based on sequence alignment analysis, is most similar in the transmembrane region to the *Synechocystis* spp. and *Pseudomonas aeruginosa* Na^+/H^+ antiporters SynNhaP and NhaP, respectively (Hamada *et al.*, 2001; Utsugi *et al.*, 1998). SOS1 may be categorized phylogenetically together with *Saccharomyces cerevisiae* NHA1 and *Schizosaccharomyces pombe* SOD2 (Jia *et al.*, 1992; Prior *et al.*, 1996; Shi *et al.*, 2000).

Arabidopsis AtNHX1 has sequence similarity to mammalian NHE Na^+/H^+ exchangers (Counillon and Pouysségur, 2000; Nass *et al.*, 1997) and yeast NHX1, and contains the consensus FF(I/L)(Y/F)LFLPPI amiloride binding domain. Expression of *AtNHX1* suppresses the Na^+/Li^+ sensitivity of a yeast *nhx1* mutant, implying that it functions as an endosomal Na^+/H^+ antiporter (Gaxiola *et al.*, 1999; Quintero *et al.*, 2000). *AtNHX1* transcript is detected in both roots and

shoots, and salt treatment causes transcript accumulation predominantly in leaves (Gaxiola *et al.*, 1999; Quintero *et al.*, 2000). However, some reports indicate that salt induction of transcript abundance is minimal (Apse *et al.*, 1999; Chauhan *et al.*, 2000). Ectopic expression of *AtNHX1* mediates plant salt tolerance sufficiency (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001). A null mutation of the tonoplast Na^+/H^+ antiporter gene that is normally expressed in the corolla of *Ipomoea nil* flowers disrupts the vacuolar pH of these cells (Fukuda-Tanaka *et al.*, 2000; Yamaguchi *et al.*, 2001). Vacuolar pH modulates flower colour pigmentation, which is important for attracting pollinators.

In this study, we determined that five additional members of the *Arabidopsis* NHX Na^+/H^+ antiporter gene family produce transcripts that are detectable in seedlings. Expression of *AtNHX1*, 2 and 5 suppresses the salt-sensitive phenotype of a yeast mutant defective for the endosomal/vacuolar Na^+/H^+ antiporter NHX1. *AtNHX1* and *AtNHX2* transcripts are constitutive in shoots and roots, and their steady-state message level increases in response to high salt stress through an ABA-dependent process. *AtNHX5* expression is also salt-regulated, but independent of ABA.

Results

The Arabidopsis AtNHX family comprises six genes

Analysis of the recently completed *Arabidopsis* genome database (*Arabidopsis* Genome Initiative, 2000) indicated the presence of 16 genes annotated as encoding putative Na^+/H^+ antiporters. Predicted amino acid sequence similarity/identity and topological complementarity to AtNHX1, including 12 transmembrane spanning domains and the presence of a putative amiloride binding site, led to the categorization of six loci as *AtNHX* gene family members (Table 1 and Figure 1). The cladogram of Na^+/H^+ antiporters indicates that AtNHX2–6 have greater sequence similarity to AtNHX1 than to any orthologous proteins of non-

Table 1 Amino acid similarity comparison of the six *Arabidopsis thaliana* family members of AtNHX Na^+/H^+ antiporters.

	AtNHX1 (AF007271)	AtNHX2 (AC009465)	AtNHX3 (AC011623)	AtNHX4 (AB015479)	AtNHX5 (AC005287)	AtNHX6 (AC010793)
AtNHX1 (AF007271)	–	87.5%	68.6%	56.0%	23.4%	22.9%
AtNHX2 (AC009465)		–	68.9%	56.1%	23.2%	22.1%
AtNHX3 (AC011623)			–	55.4%	23.4%	21.7%
AtNHX4 (AB015479)				–	23.8%	21.9%
AtNHX5 (AC005287)					–	78.7%
AtNHX6 (AC010793)						–

The family members are listed in order of sequence similarity beginning with the prototype AtNHX1. Accession numbers corresponding to the *Arabidopsis* genome sequencing project database are given in parentheses

plant origin or SOS1 (Figure 2). The *Arabidopsis* NHX Na⁺/H⁺ antiporter family members are herein designated as AtNHX1–6 in decreasing order of phylogenetic relatedness to the prototype AtNHX1 (Table 1 and Figure 2) and

categorized into subgroups (AtNHX1–4 and AtNHX5 and 6). AtNHX2–4 are 56.0–87.5% similar to AtNHX1, whereas AtNHX5 and 6 are 78.7% similar to each other but only 21–23% similar to the AtNHX1–4 isoforms.

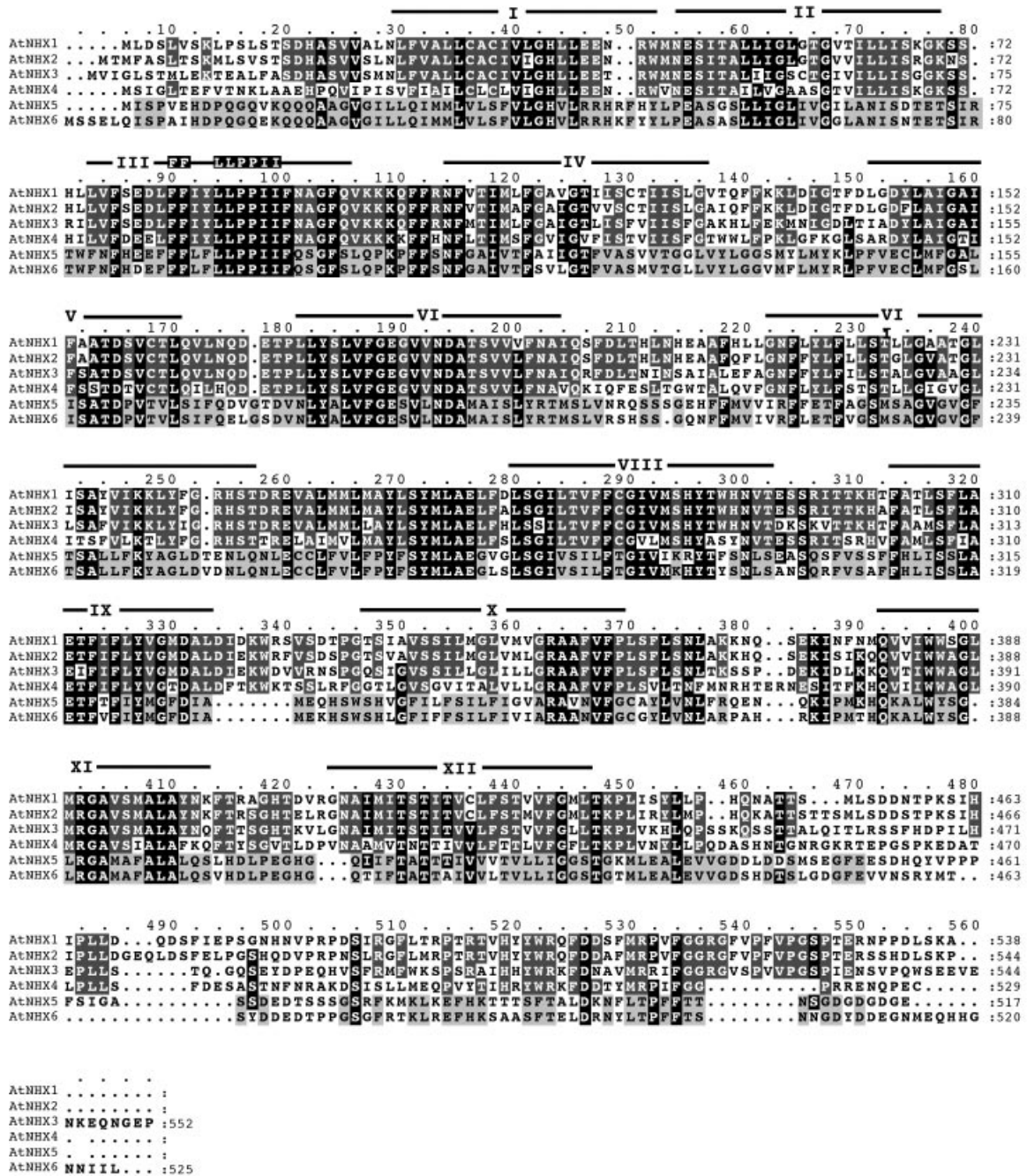


Figure 1. Six *Arabidopsis thaliana* isogenes (*AtNHX*) encode proteins that have domains with sequence similarity to metazoan NHE Na⁺/H⁺ exchangers. The predicted peptides from *AtNHX1* (AF007271), *AtNHX2* (AC009465), *AtNHX3* (AC0011623), *AtNHX4* (AB015479), *AtNHX5* (AC005287) and *AtNHX6* (AC010793) are aligned based on analysis using the CLUSTAL V method (Higgins and Sharp, 1989). Residues that are identical in all *AtNHX* family members are highlighted in black, those identical in subgroup 1 family members (*AtNHX1–4*) are shaded in dark grey, and those residues specific to the subgroup 2 family members (*AtNHX5* and *6*) are highlighted in light grey. Putative transmembrane domains are indicated by Roman numerals. The consensus amidolipid binding motif is indicated in transmembrane domain III.

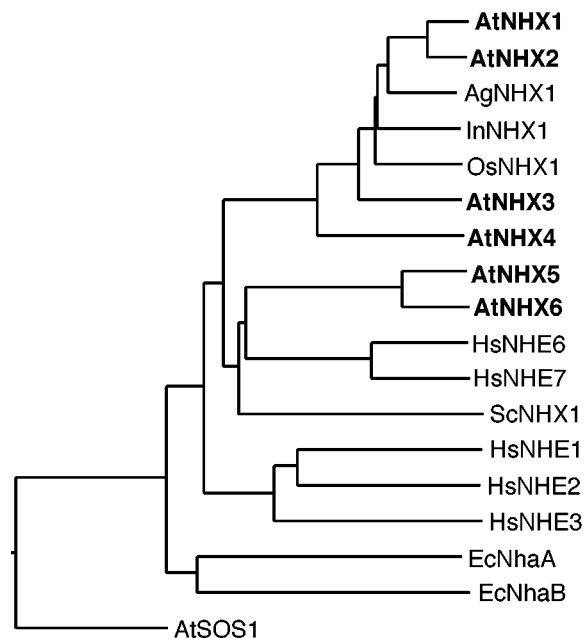


Figure 2. Cladogram indicating that AtNHX isoforms can be paired into two phylogenetic subgroups.

Alignment analysis was performed using the CLUSTAL V method (Higgins and Sharp, 1989). The accession numbers and sources of the Na⁺/H⁺ antiporters are as follows: *AtSOS1* (AAF76139), *Arabidopsis thaliana*; *InNHX1* (BAB60899), *Ipomoea nil*; *OsNHX1* (BAA83337), *Oryza sativa*; *AgNHX1* (BAB11940), *Atriplex gmelini*; *ScNHX1* (NP 010744), *Saccharomyces cerevisiae*; *HsNHE1* (P19634), *Homo sapiens*; *HsNHE2* (AAD41635), *H. sapiens*; *HsNHE3* (P48764), *H. sapiens*; *HsNHE6* (Q92581), *H. sapiens*; *HsNHE7* (NP 115980), *H. sapiens*; *EcNhaA* (P13738), *Escherichia coli*; *EcNhaB* (P27377), *E. coli*. The AtNHX family members are highlighted in bold.

Arabidopsis AtNHX1, 2 and 5 are functional Na⁺/H⁺ antiporters

To gain information about functional conservation among the distinct AtNHX isoforms, full-length cDNAs corresponding to *AtNHX2* and *5* were isolated. These representatives of the two AtNHX protein subgroups were compared to *AtNHX1* for capacity to mediate salt tolerance in yeast. Expression of both *AtNHX2* and *5* suppressed the alkali cation-sensitive phenotype of an *nhx1* mutant, indicating that these are orthologous to yeast *ScNHX1* and plant *AtNHX1* (Figure 3). *AtNHX1* expression enhances Na⁺/Li⁺ tolerance equivalently to endogenous *ScNHX1*, but cannot substitute for the plasma membrane-localized Na⁺/H⁺ exchanger *ScNHA1* (Quintero *et al.*, 2000). *AtNHX2* mediated greater Na⁺/Li⁺ tolerance than *AtNHX1*, providing functional evidence that this antiporter is also an important *Arabidopsis* salt tolerance determinant. Interestingly, *AtNHX5* increased Na⁺ tolerance to a similar extent as *AtNHX1*, but was less proficient in conferring tolerance to Li⁺. Both *AtNHX2* and *5* suppressed the characteristic hygromycin sensitivity of *nhx1* cells, indicat-

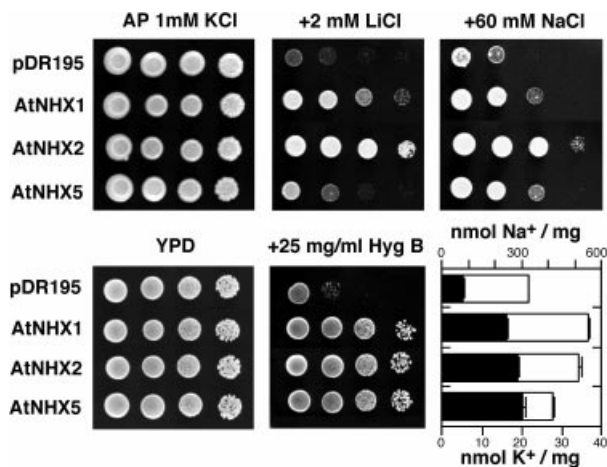


Figure 3. AtNHX1, 2 and 5 are functional Na⁺/H⁺ antiporters based on suppression of salt- and hygromycin-sensitive phenotypes of a yeast *nhx1* mutant.

AtNHX1, 2 and 5 cDNAs were subcloned into the yeast expression vector pDR195 (Rentsch *et al.*, 1995) and transformed into strain AXT3K (*ena1-4 nha1 nhx1*). Aliquots (10 μ l) from saturated yeast cultures or 10-fold serial dilutions were spotted onto AP plates containing 1 mM KCl without or with NaCl or LiCl, or YPD plates with hygromycin B (Hyg B). Also shown is the intracellular Na⁺ (white bars) or K⁺ (black bars) content determined by atomic emission spectrophotometry of cells expressing *AtNHX1*, 2 or 5, or transformed with an empty pDR195 vector. These cells were grown in liquid AP medium with 1 mM KCl and 50 mM NaCl until mid-log phase and recovered by filtration. Values are the average of three replicate samples.

ing that these proteins function in endosomal compartments, as do *ScNHX1* and *AtNHX1* (Gaxiola *et al.*, 1999; Quintero *et al.*, 2000). These results suggest that NHX proteins may perform similar cellular functions yet have distinct transport capacities.

AtNHX1 localizes to the yeast vacuolar membrane and mediates ion compartmentalization (Quintero *et al.*, 2000). To determine whether *AtNHX2* and *5* imparted NaCl tolerance by a mechanism similar to that of *AtNHX1*, the intracellular ion content of *nhx1* cells grown in AP medium with 50 mM NaCl and 1 mM KCl was measured. As shown in Figure 3, the NaCl tolerance conferred by AtNHX proteins correlated with greater Na⁺ content, indicative of ion compartmentalization rather than extrusion. The activity of AtNHX proteins also enhanced intracellular K⁺ status.

Differential expression of AtNHX genes

Gene-specific PCR primers corresponding to *AtNHX1-6* were synthesized based on the sequence information obtained from BAC clones AF007271, AC009465, AC011623, AB015479, AC005287 and AC010793, respectively. Probes were synthesized by RT-PCR and correspond to unique sequence regions of *AtNHX1-6* starting at a residue upstream of the translation stop codon and extending into the 3' untranslated region. Each probe

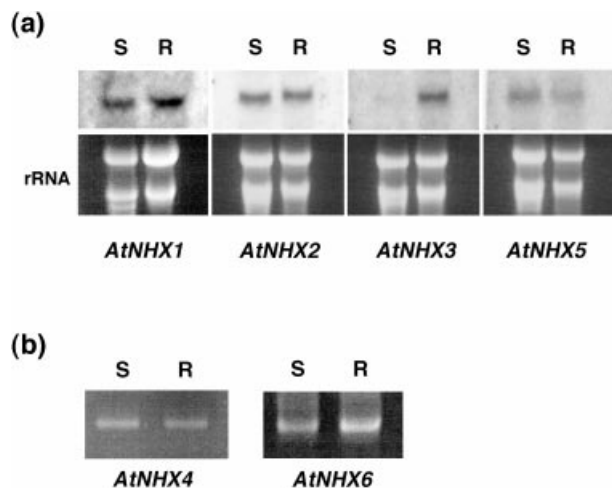


Figure 4. *AtNHX* genes are regulated differentially in *Arabidopsis* shoots and roots.

Total RNA was isolated from 3-week-old Col-0 *g1* plants. (a) Northern blot illustrating relative *AtNHX1–3* and *5* transcript abundance in shoots (S) and roots (R) after electrophoretic separation of total RNA (10 µg). Ribosomal RNA (rRNA) was visualized (bottom) after ethidium bromide staining. (b) *AtNHX4* and *6* transcripts detected by RT-PCR analysis using gene-specific primers.

detected unique restriction fragments on Southern blots of *Arabidopsis thaliana* ecotype Col-0 *g1* genomic DNA, indicating their gene specificity (data not shown).

Northern analysis using total RNA from 21-day-old Col-0 *g1* seedlings indicated that *AtNHX1* and *2* transcripts are the most abundant *AtNHX* mRNA products in shoots and roots (Figure 4a). The *AtNHX3* transcript was apparent only in roots and a low-abundance *AtNHX5* mRNA was detected in shoots or roots. *AtNHX4* and *AtNHX6* messages were not apparent on Northern blots (data not shown) but were detected in shoots and roots using RT-PCR (Figure 4b). These results establish that all six *AtNHX* genes are transcriptionally active in seedlings, and indicate that their function is not restricted to a later developmental stage.

AtNHX1 and *2* transcripts were more abundant in Col-0 *g1* seedlings subjected to iso-osmotic NaCl and sorbitol treatments (Figure 5). Moreover, *AtNHX1* and *2* transcripts accumulated to a greater extent in seedlings subjected to exogenous ABA application (Figures 5, 6 and 7), indicating that the osmotic responsiveness of these genes is ABA-dependent. Most probably *AtNHX1* and *2* are responsive to hyper-osmotic stress rather than to Na⁺ toxicity. Conversely, *AtNHX5* transcript abundance in seedlings was induced by NaCl but was not affected by sorbitol treatment or exogenous ABA (Figure 5), suggesting that this gene is more directly responsive to ionic stress. However, *AtNHX5* expression was unresponsive to a growth inhibitory concentration of LiCl. Li⁺ is a more

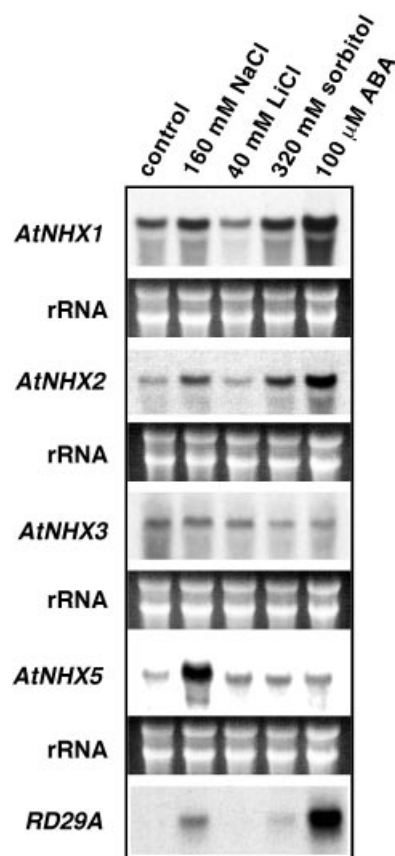


Figure 5. Hypertonic stress and ABA induce *AtNHX1* and *AtNHX2* expression but *AtNHX5* is responsive only to NaCl.

Fourteen-day-old *Arabidopsis thaliana* Col-0 *g1* seedlings were inoculated into fresh medium without (control) or with 160 mM NaCl, 40 mM LiCl, 320 mM sorbitol or 100 µM ABA, and maintained for 5 h. Total RNA (10 µg) from each group of seedlings was separated electrophoretically and then blotted onto nylon membrane. The membranes were hybridized with DIG-labelled DNA probes (*AtNHX1–3*, *AtNHX5* or *RD29A*). The ribosomal RNA (rRNA) was detected after ethidium bromide staining.

toxic analogue of Na⁺ that reduces plant growth at low millimolar concentrations. No appreciable difference in *AtNHX3* transcript level was detected in seedlings as a consequence of hyper-osmotic stress or exogenous ABA treatment. Osmotic stress- and ABA-responsive gene expression was monitored in these experiments by detecting the abundance of transcripts *RD29A* (responsive to dehydration) (Figures 5 and 6) and *RD22* (not shown) (Shinozaki and Yamaguchi-Shinozaki, 2000).

Signalling pathways governing *AtNHX* expression

Differential expression of *AtNHX* genes appears to be regulated by distinct signalling processes elicited by osmotic and ionic cues. Many plant responses to dehydration or hyper-osmotic stress are regulated by ABA (Shinozaki and Yamaguchi-Shinozaki, 2000), whereas ion

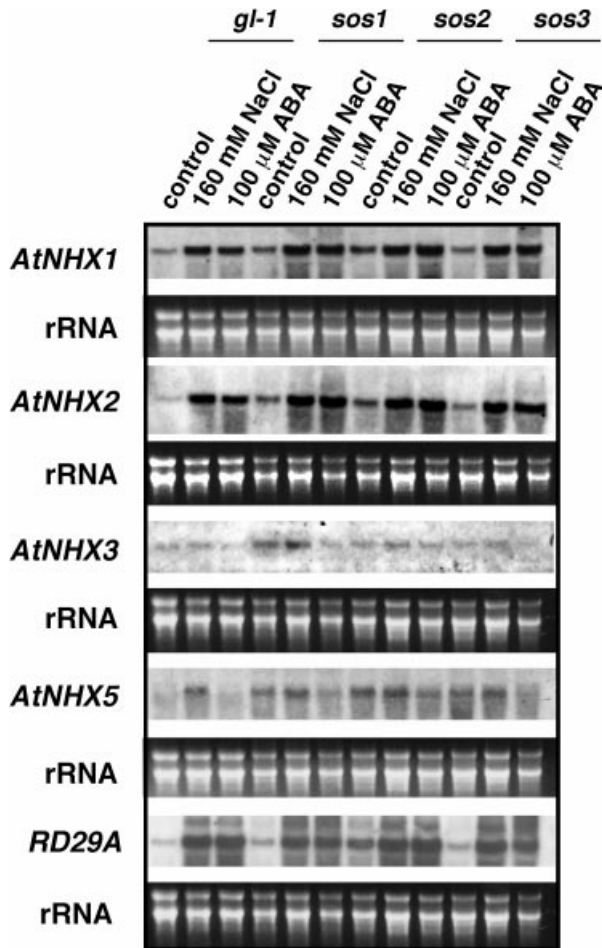


Figure 6. NaCl stress induction of *AtNHX1*, 2 or 5 expression is not dependent on the SOS signal pathway. Total RNA was isolated from 14-day-old seedlings (Col-0 *gl1* or *sos1-3*) that were cultured into fresh medium without (control) or supplemented with 160 mM NaCl or 100 μ M ABA for 5 h. Northern blot analysis was conducted as indicated in Experimental procedures using *AtNHX1-3* or 5 or *RD29A* probes; rRNA was detected after staining with ethidium bromide.

homeostasis is controlled by the SOS2/SOS3 pathway which is Ca^{2+} -activated but ABA-independent (Zhu, 2000; Zhu, 2001a; Zhu, 2001b). *sos* and *aba* mutants were used to determine differences in the ion- and ABA-responsiveness of *AtNHX* genes.

SOS3 is an EF hand Ca^{2+} -binding protein that is required for the activation of the SNF-like kinase SOS2. SOS2 in turn transcriptionally and post-transcriptionally activates the plasma membrane Na^+/H^+ antiporter SOS1 (Shi *et al.*, 2000; Zhu, 2000; Zhu, 2001a; Zhu, 2001b). The constitutive abundance of *AtNHX1* and 2 transcripts was moderately but consistently greater in untreated seedlings of *sos* mutants compared to those of wild-type (Figure 6). However, salt or ABA treatment induced accumulation of *AtNHX1* and 2 transcripts to similar extent in seedlings of

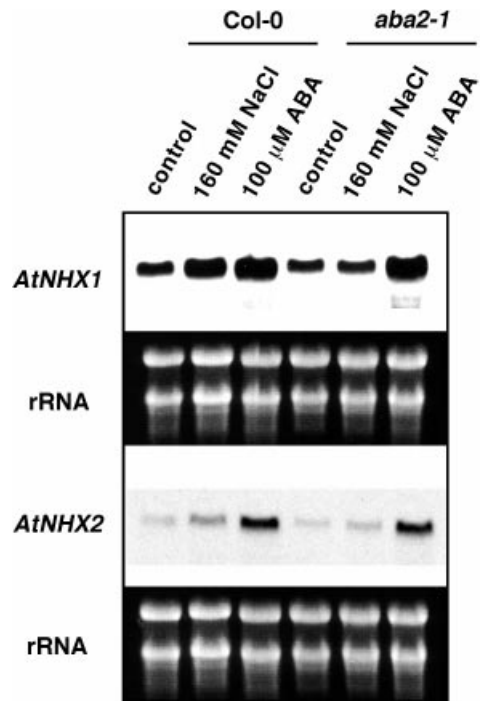


Figure 7. NaCl induction of *AtNHX1* or 2 expression requires ABA. Fourteen-day-old seedlings of wild-type (Col-0 *gl1*) or *aba2-1* were cultured into fresh medium without (control) or supplemented with 160 mM NaCl or 100 μ M ABA and maintained for 5 h. Northern blot analysis was conducted as described in Experimental Procedures. Ribosomal RNA (rRNA) was detected after ethidium bromide staining.

both wild-type and *sos* mutants. Constitutive *AtNHX5* transcript abundance was significantly higher in untreated *sos* mutants and matched the NaCl-specific induction level. *AtNHX3* mRNA levels were fairly constant regardless of treatments and genotypes. NaCl- or ABA-induced *RD29A* expression is also similar in the wild-type or *sos* mutant plants (Ishitani *et al.*, 1998). These results suggest that transcriptional control of the *AtNHX* Na^+/H^+ antiporters may be under negative regulation by the SOS pathway. However, as *AtNHX1*, 2 and 5 up-regulation by stress treatments was largely independent of SOS2/SOS3, the greater basal gene expression in *sos* mutants, including *sos1*, might reflect permanent stress elicited by salts in the growth medium, thereby leading to *AtNHX* gene up-regulation.

NaCl treatment did not increase the transcript abundance of *AtNHX1* or 2 in the *aba2-1* mutant as it did in wild-type seedlings (Figure 7). The *aba2-1* mutant is ABA-deficient, with little capacity to accumulate the hormone even after dehydration, but is responsive to ABA applied exogenously (Léon-Kloosterziel *et al.*, 1996). Exogenous ABA application caused *AtNHX1* and *AtNHX2* mRNA accumulation in *aba2-1* to a similar level as in wild-type after treatment with salt or ABA. Thus, salt induction of

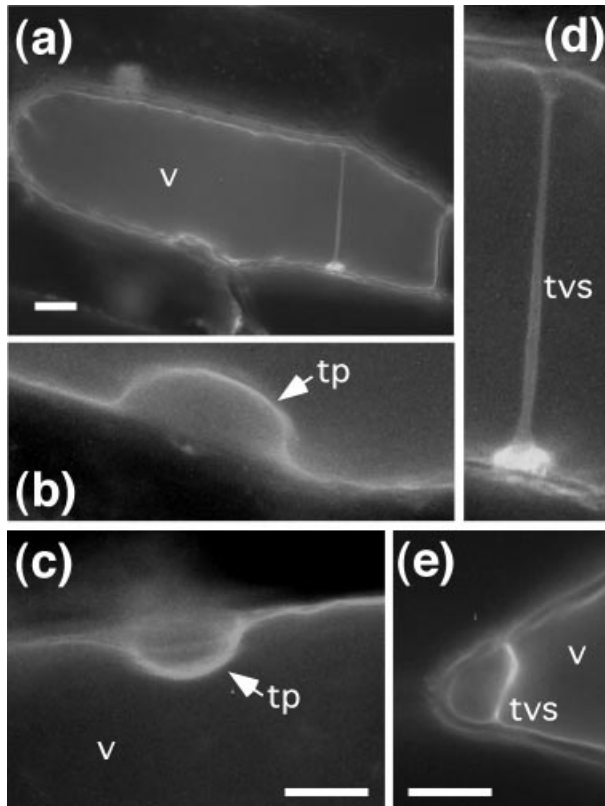


Figure 8. Tonoplast localization of AtNHX2:GFP. Transient expression in onion epidermal cells of an AtNHX2:GFP translational fusion product was visualized by epifluorescence microscopy. GFP fluorescence is concentrated to the vacuolar membrane. (a) The large vacuole (v) of onion epidermal cells occupies most of the cell volume. (b,c) The tonoplast (tp) follows the cell contour except in the nuclear region where the tonoplast detaches from the cell surface. (d,e) Transvacuolar strands of cytoplasm (tvs) spanning the cell body. Note that the transvacuolar strand depicted in (d) is lined by two tonoplast membranes. Bar = 50 μm .

AtNHX1 and *AtNHX2* is probably dependent on ABA signalling.

Tonoplast localization of AtNHX2

AtNHX1 localizes to the vacuole of plant and yeast cells (Apse *et al.*, 1999; Quintero *et al.*, 2000). As *AtNHX1* and *AtNHX2* expression was induced similarly in plants by osmotic challenge, we determined whether *AtNHX2* is also localized to the tonoplast where it contributes to vacuolar ion sequestration and osmotic adjustment. A translational fusion was constructed in which GFP was added to the C-terminus of full-length *AtNHX2* polypeptide. The chimeric construct was delivered into onion epidermal cells by particle bombardment, and transient expression of *AtNHX2:GFP* was monitored by epifluorescence. As shown in Figure 8, *AtNHX2:GFP* localized to the tonoplast.

The large vacuole of epidermal cells confined most of the cytoplasm to a thin peripheral layer and circumscribed *AtNHX2:GFP* fluorescence to the contour of the cell, except in places where the tonoplast was separated from the cell periphery by nuclei. Membranes lining transvacuolar strands of cytoplasm spanning the cell body also produced fluorescence that upon close-up observation clearly depicted two vacuolar membranes. These results indicate that both *AtNHX1* and 2 are tonoplast antiporters that probably function in the vacuolar ion compartmentalization necessary for cell volume regulation and cytosolic Na⁺ detoxification.

Discussion

Six genes encode members of the NHX-like Na⁺/H⁺ antiporter family

Phylogenetically, the *Arabidopsis* NHX family is categorized into groups comprising *AtNHX1*–4 and *AtNHX5* and 6. *AtNHX1*, 2 and 5 have functional Na⁺/H⁺ antiporter activity based on yeast complementation data (Figure 3). *AtNHX1* can substitute functionally for *ScNHX1*, the endosomal Na⁺/H⁺ antiporter, and requires endosomal/vacuolar V-type ATPase activity to mediate vacuolar ion compartmentalization in yeast (Quintero *et al.*, 2000). Expression of *AtNHX2* or *AtNHX5* also suppresses salt- and hygromycin-sensitive phenotypes of *nhx1* cells, implying that their products are native tonoplast or endosomal Na⁺/H⁺ antiporters. Accordingly, *AtNHX2* and 5 promoted ion (Na⁺ and K⁺) accumulation in yeast (Figure 3), and the *AtNHX2:GFP* translational fusion was targeted to the tonoplast of onion cells (Figure 8). *AtNHX2* conferred greater salt resistance to yeast cells than *AtNHX1*, which is functionally sufficient for salt tolerance when over-expressed in plants (Apse *et al.*, 1999; Zhang and Blumwald, 2001; Zhang *et al.*, 2001). *AtNHX5*, which together with *AtNHX6* is in the phylogenetic subgroup most diverged from *AtNHX1*, also suppressed the salt and hygromycin sensitivities of *nhx1* cells but marginally increased tolerance to Li⁺, suggesting that these distinct family members may have different substrate specificity. Perhaps specific plant cells or organelles have unique antiporter transport capacity in order to mediate vacuolar cation compartmentalization or pH control.

Regulation of AtNHX family member expression in salt stress signalling

Osmotic- and/or NaCl-responsive expression of *AtNHX1*, 2 and 5 in *Arabidopsis* indicates that transcriptional regulation of these genes is a component of the plant salt stress response. The requirement for vacuolar Na⁺ compartmentalization in cells of plants growing in salt makes it likely

that coordinated regulation of these antiporter genes is necessary for salt adaptation. Recent models used to describe the current understanding of osmotically regulated gene expression delineate ABA-dependent or -independent responses (Shinozaki and Yamaguchi-Shinozaki, 2000; Zhu, 2001a). Two osmotic- and ABA-dependent signal pathways have been proposed to regulate stress tolerance determinant genes; one functions through activation by MYC/MYB and the other via bZip transcription factors (Shinozaki and Yamaguchi-Shinozaki, 2000). Analysis of the *AtNHX1* and 2 promoters (up to 1.5 kb upstream of the translation start codon) identified no ABA-responsive elements (ABRE). However, the promoters of each gene contained consensus AACNG/CACGTG motifs of MYC/MYB-interacting elements, implying that *AtNHX1* and 2 are outputs of the ABA-dependent pathway that is activated by these transcription factors, as is *RD22* (Ishitani *et al.*, 1998). The salt responsiveness of *AtNHX5* is not ABA-dependent, similar to the expression of *SOS1* which encodes a plasma membrane Na^+/H^+ antiporter responsible for cellular efflux of the cation (Shi *et al.*, 2000). However, in contrast to *SOS1* expression, salt-induced transcriptional regulation of *AtNHX5* is not signalled through the SOS pathway that mediates Ca^{2+} -dependent Na^+ homeostasis in plants (Zhu, 2001a). Basal expression of *AtNHX1*, 2 and 5 was moderately but consistently higher in the *sos* mutants. As up-regulation of salt-responsive *AtNHX* genes also occurred in a mutant deficient for the *SOS1* transporter that has no known function as a signalling intermediate, *AtNHX* gene expression might be enhanced because of the permanent ionic stress status of the *sos* mutants. Apparently, at least three different signalling pathways are responsible for NaCl tolerance of plants (Figure 9), providing an indication that the Na^+/H^+ antiporters function in different physiological processes or cell types.

The existence of six functional *AtNHX* genes that encode Na^+/H^+ antiporters indicates that plants have a critical need to regulate Na^+ homeostasis through vacuolar compartmentalization, despite the fact that this cation is not judged universally to be an essential mineral element (Salisbury and Ross, 1992). Recently, *AtHKT1* was confirmed by *in planta* evidence to be an Na^+ influx system, further illustrating that uptake of the cation is an important component of plant cellular physiology (Rus *et al.*, 2001). Alternatively, perhaps the antiporters have other essential functions. Our results with purified protein reconstituted into artificial lipid vesicles indicate that *AtNHX1* transports Na^+ and K^+ with similar affinity (Venema *et al.*, 2002). The endomembrane NHE antiporters of mammalian cells also transport K^+ (Numata and Orłowski, 2001). Thus, the function of endosomal transporters under physiological growth conditions may be to control the pH of organelles and/or the cytosol. An *Ipomoea nil* (Japanese morning

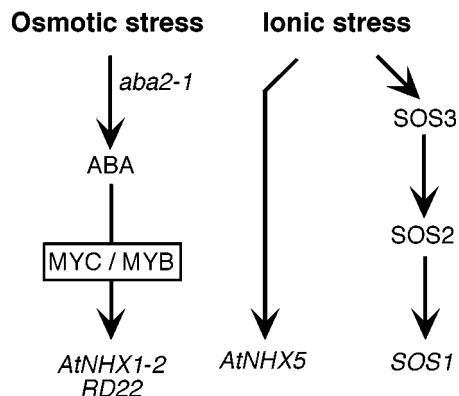


Figure 9. Hyperosmotic and ionic stress signalling pathways that regulate *AtNHX1*, 2 and 5 expression.

glory) mutation abrogates the capacity to increase vacuolar pH required for production of the anthocyanins necessary for corolla colour transition from purple to blue (Fukuda-Tanaka *et al.*, 2000, Yamaguchi *et al.*, 2001). The mutant contains a transposon (*Tpn4*) insertion upstream of the translation start codon in a gene encoding proteins most similar to *AtNHX1* and 2 (Figure 2), implying another essential function for a tonoplast $\text{Na}^+/\text{K}^+/\text{H}^+$ antiporter in controlling vacuolar pH.

Nevertheless, the results presented here clearly indicate that *AtNHX2* and 5, together with *AtNHX1* (Apse *et al.*, 1999; Gaxiola *et al.*, 1999; Quintero *et al.*, 2000) are plant salt tolerance determinants. *AtNHX1* and 2 transcripts are prevalent in shoots and roots suggesting that these antiporters have an important function in cell types that are major ion sinks in these organs. The coordinated regulation of *AtNHX1* and 2 may indicate that these antiporters have some redundant or compensatory functions. Dissection of *AtNHX* family member localization will identify cell types in which antiporter function is most essential for salt tolerance of plants, and perhaps provide insight into how salt stress adaptation, which requires multiple effectors, is coordinated within tissues and organs. However, *AtNHX3*, 4 and 6 expression may not be associated with salt adaptation, at least in seedlings. Only thorough genetic dissection will establish unequivocally the function(s) of the *AtNHX* family member products.

Experimental procedures

Plant materials

Arabidopsis thaliana (ecotype Columbia-0) *gl1*, *sos1*, *sos2*, *sos3* and *aba2-1* seedlings or plants were used in the experiments. Seedling cultures were initiated in liquid medium (McKinney *et al.*, 1995) by inoculating seeds into one-half strength MS

medium contained in 100 ml flasks. Cultures were placed onto a gyratory shaker (30 rev min⁻¹) for 14 days under continuous fluorescent light at 25°C. Treatments were administered by transferring seedlings to fresh MS medium without or with 160 mM NaCl, 40 mM LiCl, 320 mM sorbitol or 100 µM ABA for 5 h. Seedlings were then rinsed with distilled water, blotted to remove excess moisture and immediately frozen at -80°C.

Seeds were sown in soil and germinated in a greenhouse. One-week-old seedlings were transferred into Turface (Bancroft Bag Inc., West Monroe, Louisiana, USA) and maintained for 2 weeks under a 16 h:8 h (light:dark) regime in the greenhouse. Greenhouse-grown plants were separated into shoots and roots, rinsed with water, blotted and frozen at -80°C.

Functional expression of AtNHX2 and 5 in yeast

The complete *AtNHX2* coding region was amplified by PCR using the high-fidelity Pwo polymerase (Roche, Mannheim, Germany), template from a cDNA library (Minet *et al.*, 1992), and specific primers that annealed to sequences that included the start and stop codons (underlined): 5'-AACTCGAGAAAGAAAGATGACA-ATGTTTCG-3' and 5'-ATGCGGCCGCTCAAGGTTTACTAAGATCA-3', respectively. A full-length *AtNHX5* cDNA was isolated from the cDNA library using the PCR-screening method (Amaravadi and King, 1994) with the following primers which annealed to positions +273 and +599, respectively: 5'-GAGTTCTTCTTCTGTTTTG-3' and 5'-CACAGCATCATTCAAGAACTGA-3'. The nucleotide sequence of both cDNAs was determined to establish that there were no mutations. For yeast complementation, *AtNHX2* and 5 cDNAs were subcloned into the yeast expression vector pDR195 (Rentsch *et al.*, 1995). Plasmids were transformed into the yeast strain AXT3K (*ena1-4::HIS3, nha1::LEU2, nhx1::KanMX*) using the PEG lithium acetate protocol (Elble, 1992). For cation tolerance testing, 10 µl aliquots from saturated yeast cultures or 10-fold serial dilutions were spotted onto AP plates (8 mM phosphoric acid, 10 mM L-arginine, 2 mM MgSO₄, 0.2 mM CaCl₂, 2% glucose, plus vitamins and trace elements) supplemented with 1 mM KCl with or without NaCl or LiCl as indicated. Resistance to hygromycin B was assayed in YPD medium (1% yeast extract, 2% peptone and 2% glucose). To measure intracellular Na⁺ and K⁺ contents, yeast cells that were grown in AP medium with 1 mM KCl and 50 mM NaCl to an A₅₅₀ of approximately 0.3, harvested by filtration through 0.8 µm Millipore membrane, washed with 20 mM MgCl₂, and extracted with acid. Ion contents of the samples were determined by atomic emission spectrophotometry.

Cloning of gene-specific AtNHX cDNA fragments

RNA was isolated from *Arabidopsis thaliana* ecotype Col-0 *g11* plants using the RNeasy total RNA isolation kit (Qiagen, Valencia, California, USA). First-strand cDNA was synthesized using the Superscript II kit (Gibco BRL, Rockville, Maryland, USA) from total RNA (2 µg), and used as template for PCR amplification. PCR amplification was performed using ExTaq DNA polymerase (TaKaRa, Shiga, Japan), a pair of nested forward gene-specific primers for *AtNHX1-6*, as follows (1st and 2nd primers, respectively): *AtNHX1*, 5'-CAACACCCCAAATCCATAC-3' and 5'-ATATCCCTTTGTTGGACCAA-3'; *AtNHX2*, 5'-CACTGAGCCAGGTTCTCCGA-3' and 5'-AAGAAGATGCGACACTTCCT-3'; *AtNHX3*, 5'-CACTAAGATCTTCTTCCAC-3' and 5'-GATCCGATCCTCA-TGAGCC-3'; *AtNHX4*, 5'-TAGCACTCCGAAATCAATCC-3' and 5'-ACATCCGCTCCTCGATGGT-3'; *AtNHX5*, 5'-CTTTGAAGAGA-

GCGATCATC-3' and 5'-AGTATGTCCCTCCTCTTTT-3'; and *AtNHX6*, 5'-GAGGTGGTGAACAGTCGTTA-3' and 5'-GCCAGG-AAGTGGATTCAGGA-3', and oligo-dT (21-mer) as the reverse primer. The PCR reaction mixture contained 5 µl of 10× ExTaq buffer, 5 µl of 2.5 mM dNTP mix and 1 µM of each amplification primer (100 µM stock). After 5 min at 94°C, ExTaq polymerase was added to initiate the reaction and the PCR programme cycle: 30 sec at 94°C, 30 sec at 55°C, 90 sec at 72°C for 20 cycles, and a final extension at 72°C for 5 min. The PCR product was separated by agarose gel electrophoresis and cloned into pBluescript SK(-) (Stratagene, La Jolla, California, USA). The nucleotide sequence of each amplified DNA fragment was determined.

RNA blots

RNA was isolated from 14-day-old seedlings cultured *in vitro* or 3-week-old greenhouse grown plants using the RNeasy total RNA isolation kit (Qiagen). RNA aliquots (10 µg) were resolved electrophoretically in denaturing formaldehyde-agarose gels and transferred onto nylon membranes (Schleicher & Schuell, Keene, New Hampshire, USA). RNA was cross-linked to the membrane (XL-1000, Spectronics Corporation, Westbury, New York, USA) and hybridized with DIG-labelled DNA probes at 65°C using standard protocols (Roche, Indianapolis, Indiana, USA). The blots were washed twice in 2× SSC and 0.1% w/v SDS at 25°C and twice in 0.5× SSC and 0.1% w/v SDS at 65°C. Under these hybridization conditions, each *AtNHX* probe hybridized to unique genomic fragments based on Southern blot analysis of Col-0 *g11* genomic DNA.

RT-PCR amplification of AtNHX4 and 6 transcripts

Protocols for total RNA extraction and synthesis of cDNA (from 3 µg of RNA) were as described above. The cDNA template was amplified by PCR using ExTaq DNA polymerase (TaKaRa) and gene-specific primers for *AtNHX4* or 6.

Localization of AtNHX2:GFP

Intracellular localization assays were performed by monitoring the transient expression of an *AtNHX2:GFP* translational fusion product in onion epidermal cells after DNA particle bombardment. To prevent expression in bacterial cells, the *AtNHX2* cDNA was modified to include the first intron of the gene. The coding region of GFP (Sheen *et al.*, 1995) was fused, in-frame, to the C-terminus of *AtNHX2*, and this translational fusion product was cloned into the vector pGreen-35S (John Innes Centre; <http://www.pgreen.ac.uk>). The *AtNHX2:GFP* cDNA was coated onto gold particles (1 µm) and delivered into onion cells using a Biolistic PDS-1000 He apparatus (Bio-Rad). The bombardment parameters were: rupture disk bursting pressure, 900 psi; distance to macro-carrier, 8 mm; distance to stopping screen, 6 mm; distance to target tissue, 6 cm. Onion epidermal cells were placed into MS medium with 2% sucrose prior to bombardment and incubated in the dark at 28°C for 24–36 h after particle delivery. GFP fluorescence was visualized under a Zeiss Axioskop microscope equipped with an FITC filter set (BP 450–490 nm, FT 510 nm, LP 515 nm).

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References

- Amaravadi, L. and King, M.W. (1994) A rapid and efficient, nonradioactive method for screening recombinant DNA libraries. *Biotechniques*, **16**, 98–103.
- Apse, M.P., Aharon, G.S., Snedden, W.A. and Blumwald, E. (1999) Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiporter in *Arabidopsis*. *Science*, **285**, 1256–1258.
- Arabidopsis Genome Initiative (2000) Sequence and analysis of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796–815.
- Blumwald, E., Aharon, G.S. and Apse, M.P. (2000) Sodium transport in plant cells. *Biochim. Biophys. Acta*, **1465**, 140–151.
- Chauhan, S., Forsthoefel, M., Ran, Y., Quigley, F., Nelson, D.E. and Bohnert, H.J. (2000) Na⁺/myo-inositol symporters and Na⁺/H⁺ antiporter in *Mesembryanthemum crystallinum*. *Plant J.* **24**, 511–522.
- Counillon, L. and Pouysseur, J. (2000) The expanding family of eucaryotic Na⁺/H⁺ exchangers. *J. Biol. Chem.* **275**, 1–4.
- Elble, R. (1992) A simple and efficient procedure for transformation of yeast. *Biotechniques*, **13**, 18–20.
- Flowers, T.J. and Yeo, A.R. (1995) Breeding for salinity resistance in crop plants: where next? *Aust. J. Plant Physiol.* **22**, 875–884.
- Fukuda-Tanaka, S., Inagaki, Y., Yamaguchi, T., Saito, N. and Iida, S. (2000) Colour-enhancing protein in blue petals. *Nature*, **407**, 581.
- Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L. and Fink, G.R. (1999) The *Arabidopsis thaliana* proton transporters, AtNhx1 and Avp1, can function in cation detoxification in yeast. *Proc. Natl Acad. Sci. USA*, **96**, 1480–1485.
- Hamada, A., Hibino, T., Nakamura, T. and Takabe, T. (2001) Na⁺/H⁺ antiporter from *Synechocystis* species PCC 6803, homologous to SOS1, contains an aspartic residue and long C-terminal tail important for the carrier activity. *Plant Physiol.* **125**, 437–446.
- Hasegawa, P.M., Bressan, R.A., Zhu, J.-K. and Bohnert, H.J. (2000) Plant cellular and molecular responses to high salinity. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **51**, 463–499.
- Higgins, D.G. and Sharp, P.M. (1989) Fast and sensitive multiple sequence alignments on a microcomputer. *CABIOS*, **5**, 151–153.
- Ishitani, M., Xiong, L., Lee, H., Stevenson, B. and Zhu, J.-K. (1998) *HOS1*, a genetic locus involved in cold-responsive gene expression in *Arabidopsis*. *Plant Cell*, **10**, 1151–1161.
- Jia, Z., McCullough, N., Martel, R., Hemmingsen, S. and Young, P. (1992) Gene amplification at a locus encoding a putative Na⁺/H⁺ antiporter confers sodium and lithium tolerance in fission yeast. *EMBO J.* **11**, 1631–1640.
- Léon-Kloosterziel, K.M., Gil, M.A., Ruijs, G.J., Jacobsen, S.E., Olszewski, N.E., Schwartz, S.H., Zeevaart, J.A.D. and Koornneef, M. (1996) Isolation and characterization of abscisic acid-deficient *Arabidopsis* mutants at two new loci. *Plant J.* **10**, 655–661.
- Lyndon, R.F. (1990) Plant development: the cellular basis. In: *Topics in Plant Physiology* (Black, M. and Chapman, J., eds). London: Unwin-Hyman Ltd., pp. 165–189.
- McKinney, E.C., Ali, N., Traut, A., Feldmann, K.A., Belostotsky, D.A., McDowell, J.M. and Meagher, R.B. (1995) Sequence-based identification of T-DNA insertion mutants in *Arabidopsis*: actin mutants *act2-1* and *act4-1*. *Plant J.* **8**, 613–622.
- Minet, M., Dufour, M.E. and Lacroute, F. (1992) Complementation of *Saccharomyces cerevisiae* auxotrophic mutants by *Arabidopsis thaliana* cDNAs. *Plant J.* **2**, 417–422.
- Nass, R., Cunningham, K.W. and Rao, R. (1997) Intracellular sequestration of sodium by a novel Na⁺/H⁺ exchanger in yeast is enhanced by mutations in the plasma membrane H⁺-ATPase. *J. Biol. Chem.* **272**, 26145–26152.
- Niu, X., Bressan, R.A., Hasegawa, P.M. and Pardo, J.M. (1995) Ion homeostasis in NaCl stress environments. *Plant Physiol.* **109**, 735–742.
- Numata, M. and Orłowski, J. (2001) Molecular cloning and characterization of a novel (Na⁺, K⁺)/H⁺ exchanger localized to the trans-Golgi network. *J. Biol. Chem.* **276**, 17387–17394.
- Prior, C., Potier, S., Souciet, J.L. and Sychrova, H. (1996) Characterization of the *NHA1* gene encoding a Na⁺/H⁺ antiporter of the yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **387**, 89–93.
- Quintero, F.J., Blatt, M.R. and Pardo, J.M. (2000) Functional conservation between yeast and plant endosomal Na⁺/H⁺ antiporters. *FEBS Lett.* **471**, 224–228.
- Rentsch, D., Laloi, M., Rouhara, I., Schmelzer, E., Delrot, S. and Frommer, W.B. (1995) *NTR1* encodes a high affinity oligopeptide transporter in *Arabidopsis*. *FEBS Lett.* **370**, 264–268.
- Rhodes, D. and Hanson, A.D. (1993) Quaternary ammonium and tertiary sulfonium compounds in higher plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 357–384.
- Rus, A., Yokoi, S., Sharkhuu, A., Reddy, M., Lee, B.H., Matsumoto, T.K., Koiwa, H., Zhu, J.K., Bressan, R.A. and Hasegawa, P.M. (2001) ATHKT1 is a salt tolerance determinant that controls Na⁺ entry into plant roots. *Proc. Natl Acad. Sci. USA*, **98**, 14150–14155.
- Salisbury, F.B. and Ross, C.W. (1992) *Plant Physiology*, 4th edn. Belmont, California: Wadsworth Publications Co.
- Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H. and Galbraith, D.W. (1995) Green-fluorescent protein as a new vital marker in plant cells. *Plant J.* **8**, 777–784.
- Shi, H., Ishitani, M., Kim, C. and Zhu, J.-K. (2000) The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na⁺/H⁺ antiporter. *Proc. Natl Acad. Sci. USA*, **97**, 6896–6901.
- Shinozaki, K. and Yamaguchi-Shinozaki, K. (2000) Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. *Curr. Opin. Plant Biol.* **3**, 217–223.
- Utsugi, J., Inaba, K., Kuroda, T., Tsuda, M. and Tsuchiya, T.U. (1998) Cloning and sequencing of a novel Na⁺/H⁺ antiporter gene from *Pseudomonas aeruginosa*. *Biochim. Biophys. Acta*, **1398**, 330–334.
- Venema, K., Quintero, F.J., Pardo, J.M. and Donaire, J.P. (2002) The *Arabidopsis* Na⁺/H⁺ exchanger AtNHX1 catalyzes low affinity Na⁺ and K⁺ transport in reconstituted liposomes. *J. Biol. Chem.* **277**, 2413–2418.
- Yamaguchi, T., Fukuda-Tanaka, S., Inagaki, Y., Saito, N., Yonekura-Sakakibara, K., Tanaka, Y., Kusumi, T. and Iida, S.

- (2001) Genes encoding the vacuolar Na⁽⁺⁾/H⁽⁺⁾ exchanger and flower coloration. *Plant Cell Physiol.* **42**, 451–461.
- Zhang, H.X. and Blumwald, E.** (2001) Transgenic salt-tolerant tomato plants accumulate salt in foliage but not in fruit. *Nature Biotechnol.* **19**, 765–768.
- Zhang, H.X., Hodson, J.N., Williams, J.P. and Blumwald, E.** (2001) Engineering salt-tolerant Brassica plants: characterization of yield and seed oil quality in transgenic plants with increased sodium accumulation. *Proc. Natl Acad. Sci. USA*, **98**, 12832–12836.
- Zhu, J.-K.** (2000) Genetic analysis of plant salt tolerance using *Arabidopsis*. *Plant Physiol.* **124**, 941–948.
- Zhu, J.-K.** (2001a) Cell signaling under salt, water and cold stresses. *Curr. Opin. Plant Biol.* **4**, 401–406.
- Zhu, J.-K.** (2001b) Plant salt tolerance. *Trends Plant Sci.* **6**, 66–71.