Plant and Soil **00:** 245–256, 2003.
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Characterization of a family of vacuolar Na^+/H^+ antiporters in *Arabidopsis thaliana*

Gilad S. Aharon[1], Maris P. Apse[2], Shili Duan[1], Xuejun Hua[1] & Eduardo Blumwald[2,3]
[1]*Department of Botany, University of Toronto, Toronto, Ontario M5S 3B2, Canada.* [2]*Department of Pomology, University of California, One Shields Ave., Davis CA 95616, USA.* [3]*Corresponding author**

Received 4 April 2002. Accepted in revised form 7 March 2003

Key words: AtNHX, Na^+/H^+ antiporters, salinity, vacuoles

Abstract

A family of *AtNHX1*-like genes of *Arabidopsis thaliana*, coding for vacuolar Na^+/H^+ antiporters, was cloned and functionally characterized by their heterologous expression in yeast mutants lacking an endosomal vacuolar antiporter. The expression of all of the AtNHX members of the family provided a recovery of the salt sensitive yeast mutant, supporting their role in Na^+/H^+ exchange. RT-PCR, used to determine the relative abundance of the AtNHX transcripts, showed that while *AtNHX1* and *AtNHX2* transcripts were abundant and widely distributed in all tissues, *AtNHX3* and *AtNHX4* transcripts were almost exclusively detected in flower and root tissues, respectively. *AtNHX5* transcripts were observed at very low levels in all tissues. The potential for the use of these genes for the engineering of salt tolerance in crop plants is discussed.

Introduction

Agricultural productivity is severely affected by soil salinity, and the damaging effects of salt accumulation in agricultural soils have influenced ancient and modern civilizations. The detrimental effects of salt on plants are a consequence of both a water deficit that results from the relatively high solute concentrations in the soil, and a Na^+-specific stress resulting from altered K^+/Na^+ ratios and Na^+ ion concentrations that are inimical to plants. The alteration of ion ratios in the plant is due to the influx of Na^+ through pathways that function in the acquisition of K^+(Maathuis and Amtmann, 1999). Sodium entry is due to the similarity between the hydrated ionic radii of sodium and potassium, which makes difficult the discrimination between the two ions by transport proteins (Blumwald et al., 2000). This discrimination problem is also the basis for Na^+ toxicity, where key biochemical processes in the plant cell are inhibited by the competition by sodium for potassium binding sites. It has been shown that the sensitivity to salt of cytosolic enzymes is similar in both glycophytes (salt-sensitive plants) and halophytes (salt-tolerant plants) (Flowers et al., 1977; Glenn at al., 1999). This would suggest that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt and that salt tolerant plants should be able to keep Na^+ away from the cytosol. Plants can use two strategies for the maintenance of a low Na^+ concentration: sodium exclusion and sodium compartmentation. Sodium transport out of the cell can be accomplished by the operation of plasma membrane-bound Na^+/H^+ antiporters. Biochemical evidence for the operation of plasma membrane Na^+/H^+ antiporters (Blumwald et al., 2000) and the characterization of SOS1, a putative plasma membrane Na^+/H^+ antiporter from *Arabidopsis thaliana* (Shi et al., 2002), have been reported. Transport mechanisms can also actively move ions across the tonoplast into the vacuole, removing the potentially harmful ions from the cytosol. These ions, in turn, act as an osmoticum within the vacuole, which then maintain water flow into the cell. The presence of large, acidic-inside, vacuoles in plant cells allows the efficient compartmentation of sodium into the vacuole, through the operation of vacuolar

* FAX No: 530-752-8502. E-mail: eblumwald@ucdavis.edu

Na^+/H^+ antiporters (Apse et al., 1999; Blumwald and Poole, 1985). These antiporters use the protonmotive force generated by the vacuolar H^+-translocating enzymes, H^+-adenosine triphosphatase (ATPase) and H^+-inorganic pyrophosphatase (PP_iase) to couple the downhill movement of H^+ (down its electrochemical potential) with the uphill movement of Na^+ (against its electrochemical potential) (Blumwald, 1987).

Here we show the cloning and functional characterization of a family of *AtNHX*-like genes in *Arabidopsis thaliana.* The expression of these genes in different tissues is investigated and the potential utility of these Na^+/H^+ antiporters in the engineering of salt-tolerant plants is discussed.

Materials and methods

Cloning of AtNHX genes

(1)AtNHX1: The cloning of AtNHX1 cDNA is described in Apse et al. (1999).

(2)AtNHX2: PCR primers used for the amplification of the AtNHX2 sequence were based on a BAC DNA sequence (T9J14.2) with a predicted amino acid sequence that showed homology to AtNHX1: X2F, 5′-TTCGCCTCTTTAACCTCTAAAATG-3′, X2R, 5′-TGTAGGCAAGAGCCATAGATACAG-3′. A single band of approximately 1.2 kb was amplified from a size fractionated flower cDNA library (CD4-6, ABRC) and was used as a template for screening the flower cDNA library with ^{32}P-labeled probes. The largest clone obtained from this screen was incomplete at the N-terminus coding portion of the gene. The 5′ end of the cDNA was first determined using 5′-rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA amplification kit (Clontech), 3 μg of RNA isolated from flower tissues of *A.thaliana* (Col) and the following primer (according to manufacturer's instructions): X2-5′RACE1, 5′-TACAGAGTCGGTTGCAGCAAATATGGCG-3′. A single 800-bp fragment was amplified, cloned into Invitrogen pCR2.1 TOPO vector using the Invitrogen TOPO TA Cloning Kit according to the manufacturer's instructions. Using the most extreme 5′-sequence from the 5′-RACE and the most extreme 3′-sequence from the original screen, two primers were designed for amplification of the full-length cDNA: X2-3′END, 5′-CACCAATACTAGTCACCATAAGAGGGAAGAG-CA-3′; X2-5′END, 5′-CTGCCTCTCTCTCAACGC-AACTCAATCCA-3′.

Using these primers and the RACE-ready cDNA prepared from RNA isolated from flower tissues of *A. thaliana* (Col), a 2.1-kb product was amplified according to manufacturer's instructions. This was submitted for complete sequencing and compared with sequences obtained from the original library clone and RACE product.

(3)AtNHX3: The full-length AtNHX3 cDNA was cloned from an *Arabidopsis thaliana* (ecotype Columbia) seedling cDNA library (Kieber et al., 1993). PCR primers were designed for the amplification of the AtNHX3 sequence based on a BAC DNA sequence (MTE17) with a predicted amino acid sequence that showed homology to AtNHX1: X3F: 5′-CCTCAGGTGATACCAATCTCA-3′, X3R: 5′-GATCCAATGTAACACCGGAG-3′. A 1.2 kb product was amplified from the seedling library, and was used as a probe template for screening plaques from the library. Plaques hybridizing to the probe in the first round were subjected to a second round using the same probe. Pure plaques from the secondary screen were tested by PCR with the X3F/X3R primer combination. One plaque from the secondary screen supported the amplification of a 1.2 kb product as above. Sequencing of the clone revealed a full-length 1.9 kb cDNA.

(4)AtNHX4: PCR primers were designed for the amplification of the AtNHX4 sequence based on a BAC DNA sequence (F24P17.16) with a predicted amino acid sequence that showed homology to AtNHX1. A full-length cDNA was isolated by screening of the λ-PRL-2 *Arabidopsis* cDNA library (ABRC) essentially as described fro AtNHX3.

(5)AtNHX5: AtNHX5 was originally identified by tBlastn homology of BAC DNA sequence (F20D21) to both AtNHX1 and AtNHX3. A full-length cDNA was amplified using N- and C-terminus-specific primers that were determined from sequences obtained from 5′- and 3′-RACE as described for AtNHX2.

Yeast transformation

Yeast colonies were resuspended in 50 μL of sterile ddH2O. One μg of Carrier DNA was added along with 1 μg of plasmid DNA. 0.4 mL of PEG mix, 40% PEG in 100 m*M* LiAC/TE, was added and mixed by vortexing. The mixture was incubated at 30 °C for 30 min and then heat-shocked for 15 min at 42 °C. Aliquots of the mixture were plated on appropriate media plates.

Targeted disruption of the yeast Nhx1 gene

All Saccharomyces cerevisiae strains used were strains of W303 (ura3-1, can1-100, leu2-3112, trp1-1, his3-11). The Δ*nhx1::TRP1* genotype was generated as follows: An EcoRI/PstI fragment internal to ScNHX1 was replaced with an EcoRI/PstI fragment containing the marker gene TRP1. The linear fragment was used to transform a W303 yeast strain. Yeast was plated on Trp^- drop-out plates for selection of successful transformants. Δ*nhx1::TRP1* null mutants were confirmed by Southern analysis on yeast genomic DNA probed with the TRP1 gene.

Complementation studies

The complete coding sequences of AtNHX1-5 were cloned into p413MET25, a yeast vector in which the expression of the gene of interest is repressed by methionine, and whose selectable marker is HIS3 (Mumberg et al., 1994). Sodium tolerance tests were performed in arginine-phosphate (APG) medium consisting of the following: 2% glucose; 8 m*M* Phosphoric acid pH 6.5 (adjusted with Arginine); 0.2 m*M* KCl; 0.1 m*M* $CaCl_2$;0.1 m*M* $MgSO_4$; 1 m*M* $FeCl_3$ 200 ng/mL; 20 ng/mL Biotin; 500 ng/mL Calcium Panthothene; 500 ng/mL Nicotinic acid; 2 μg/mL Thiamine-ClH; 2 μg/mL Piridoxine-ClH; 20 ng/mL Folic acid; 200 ng/mL Riboflavine; 200 ng/mL p-aminobenzoic; 500 ng/mL Boric acid; 50 ng/mL $CuSO_4$; 100 ng/mL KI; 400 ng/mL $MnSO_4$; 200 ng/mL Sodium Molibdate and 400 ng/mL $ZnSO_4$. Amino acids were supplemented as needed in the following concentrations: adenine (30 μg/mL); histidine (30 μg/mL); leucine (100 μg/mL); tryptophan (80 μg/mL). NaCl was supplemented as required. For growth in liquid media, cells were grown for three days to saturation in APG (-HIS, -TRP;-MET,) media. Five μL were used to inoculate 3 ml of APG (-HIS, -TRP;-MET) supplemented with different concentrations of NaCl. Cell density was measured as absorbance at 600 nm after growth for 72 h at 30 °C.

RT-PCR

RT-PCR was carried out using the Ready-to-Go PCR and RT-PCR beads (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's conditions. Reactions were carried out on a 50 μL volume containing 45 μL DEPC-H_2O, 1 μl Oligo dT (0.5 μg/μL) and 0.5 μL of each of the primers described below and 2 μg RNA. Unique regions of each cDNA were selected for amplification of probe templates so that we could be certain that signals, when detected, were specific for the particular gene of interest. The following primer pairs were used:
AtNHX1, 5′-UTR, 348 bp;
X1ECO 5′-GAATTCGCCTCTCTGTTTCGTTCC-TC-3′;
X1PST 5′-CTGCAGACCACAGAAGCGTGATCA-GA-3′;
AtNHX2, 5′-UTR, 353 bp;
X2ECO 5′-GAATTCCTCAACGCAACTCAATCC-AC-3′;
X2PST 5′-CTGCAGGGCGAACATTGTCATCTT-TC-3′;
AtNHX3, 3′-UTR, 291 bp;
X3ECO 5′-GAATTCTGGTCTTTGGTTTCCTCA-CA-3′;
X3PST 5′-CTGCAGCTGGTTGGTTTTCTCGA-CG-3′;
AtNHX4, 3′-UTR, 474 bp;
X4ECO 5′-GAATTCCATTGAGAATAGTGTTCCG-CAA-3′;
X4PST 5′-CTGCAGGATTCGTGTCCCTTTGTTT-TG-3′;
AtNHX5, ORF central portion, 502 bp;
X5ECO 5′-GAATTCTCGCTTCAGTTGTTACTGG-TG-3′;
X5PST 5′-CTGCAGCGCTTCATAACAATTCCTGT.

In situ *hybridization and GUS-promoter fusion*

The primer pairs listed above were used to amplify PCR products for cloning into the pSPT18 and pSPT19 cloning vectors (Roche, Indianapolis, IN). DIG-labeled sense and antisense probes were synthesized, and labeling efficiency was evaluated, according to manufacturer's instructions (DIG RNA Labeling Kit, Roche, Indianapolis, IN). *In situ* hybridization was performed as described by Long et al. (1996), except for the following modifications. *Arabidopsis thaliana* ecotype Columbia plant tissues were fixed in 4% paraformaldehyde and dehydrated through an ethanol and ter-butyl alcohol series before embedding in paraffin. Seven-μm-thick sections were adhered to ProbeOn Plus slides (Fisher, Pittsburgh, PA), deparaffinized with Histoclear, rehydrated through an ethanol series, treated for 30 min with 1 μg/mL proteinase K, refixed with paraformaldehyde, treated with 0.1 M acetic anhydride, and dehydrated through an ethanol series. Slide pairs were incubated with equal concentrations of sense and antisense probes

at 55 °C overnight. Post-hybridization treatment, including washes and RNAse treatment, was also as described by Long et al. (1996). Alkaline-phosphatase conjugated anti-DIG antibodies were used to detect DIG-labeled probes. NBT/BCIP (Invitrogen, Carlsbad, CA) was incubated with the slides to generate a color reaction with the alkaline phosphatase bound to the antibody. Slides were dehydrated and mounted with Permount (Sigma, St. Louis, MO).

The promoter region for AtNHX3 was amplified from *Arabidopsis thaliana* (Col) genomic DNA using the following primer pair (restriction enzyme sites incorporated into the primers are underlined):

X3GUSF, 5′-AGCC*GTCGAC*GAACCAAACAGA-AGATTAAAAAAATACCG-3′

X3GUSR, 5′-AGG *CCCGGG*CCTAATCCGATA-CTCATTTTTCAC-3′

The PCR product was ligated into the SalI and SmaI sites of the pBI101 binary vector. Plants were transformed with *Agrobacterium* strain LB4404 harboring the vector construct above using the floral dipping method (Clough and Bent, 1998). Transformants were selected on agar media containing 0.5X Murashige and Skoog (MS) salts, supplemented with 25 mg/L^{-1} kanamycin. Plant tissues were dissected and submerged in staining solution containing 1 mg/mL X-gluc (5-bromo-4-chloro-3-indoyl-β-D-glucor onic acid). Tissues were incubated in staining solution at 37 °C overnight. To remove chlorophyll and excess stain, tissues were incubated at 37 °C with two changes of 70% ethanol overnight.

Plant material

Arabidopsis thaliana (ecotype Col) seeds were surface sterilized and incubated at 4 °C for 3 days to allow for imbibition. The growth medium is a modification of that described by Spalding et al. (1999) and contained 2.5 m*M* $Ca(NO_3)_2$, 2 m*M* $MgSO_4$, 80 μM $Ca(H_2PO_4)_2$, 25 μM $CaCl_2$, 25 μM H_3BO_3, 2 μM $MnSO_4$, 0.5 μM Na_2MoO_4, 0.5 μM $CuSO_4$, 0.01 μM $CoCl_2$, 2 μM $ZnSO_4$, 0.1 m*M* Na_2EDTA, 0.1 m*M* $FeSO_4$, 100 mg/L^{-1} myo-inositol, 0.5 mg/L^{-1} nicotinic acid, 0.5 mg/L^{-1} pyridoxine-HCl, 0.5 mg/L^{-1} thiamine-HCl, 2 mg/L^{-1} glycine, 0.7% agar (plant cell culture grade). pH was adjusted to 5.7 with 1 N KOH and KCl was adjusted to 1 m*M* with 1 M KOH. For growth of seedlings prior to transplantation to soil, 0.5% sucrose was added to the medium to speed growth. For mature tissues, 2 week-old seedlings were transplanted to soil and grown at 22 °C on a 12 h day/night cycle. Plants were watered with a nutrient medium as described above (excluding agar). For RNA isolation, leaves were harvested from 4-week-old rosettes. Flower and inflorescence stems were harvested from 6-week-old plants. Roots were harvested from seedlings grown on vertical plates (where the agar concentration was 1%). Tissues for *in situ* hybridization were harvested from plate and soil grown plants at different stages of development.

Results and discussion

Characterization of a family of vacuolar Na^+/H^+ antiporters in Arabidopsis thaliana

The completion of the *Arabidopsis thaliana* genome has allowed for the identification of plant genes with significant similarity to the *Saccharomyces cerevisiae NHX1* (**Na**/**H** e**X**changer 1) gene product (Nass et al., 1997). The predicted *ATNHX1* gene product is a protein of 538 amino acids and shows a high degree of similarity to Na^+/H^+ antiporters from *C. elegans*, human (mitochondrial, NHE6) and to the yeast ScNhx1, except within the N- and C- terminal regions (Gaxiola et al., 1999, Apse et al., 1999). Apse et al. (1999) provided evidence demonstrating that *AtNHX1* codes for a vacuolar Na^+/H^+ antiport. In order to assess the Na^+/H^+ exchange function, Na^+-dependent H^+ movement was measured in intact vacuoles isolated from both wild-type plants and plants overexpressing *AtNHX1*. While Na^+/H^+ exchange rates were very low in vacuoles from wild-type plants, much higher rates were observed in vacuoles from transgenic plants (Apse et al., 1999). The higher vacuolar Na^+/H^+ antiporter activity in the transgenic plants correlated with an increase in the amount of AtNHX1 protein. Moreover, transgenic plants overexpressing *AtNHX1* were able to grow in the presence of 200 mm NaCl, supporting the role of the vacuolar Na^+/H^+ antiport in salt tolerance (Apse et al., 1999). More recently Na^+/H^+ antiport activity was measured in vacuole of yeast Δnhx1 mutants transformed with *AtNHX1* (Darley et al., 2000). Further evidence for the function of AtNHX1 has been provided by the reconstitution of AtNHX1 purified from yeast expressing the protein (Venema et al., 2002). In our laboratory we have cloned a family of at least five vacuolar Na^+/H^+ antiporters (Figure 1). These proteins are homologous to the members of the Monovalent Cation:Proton Antiporter-1 (CPA1) Family *sensu* Paulsen (1988), and we have annotated them

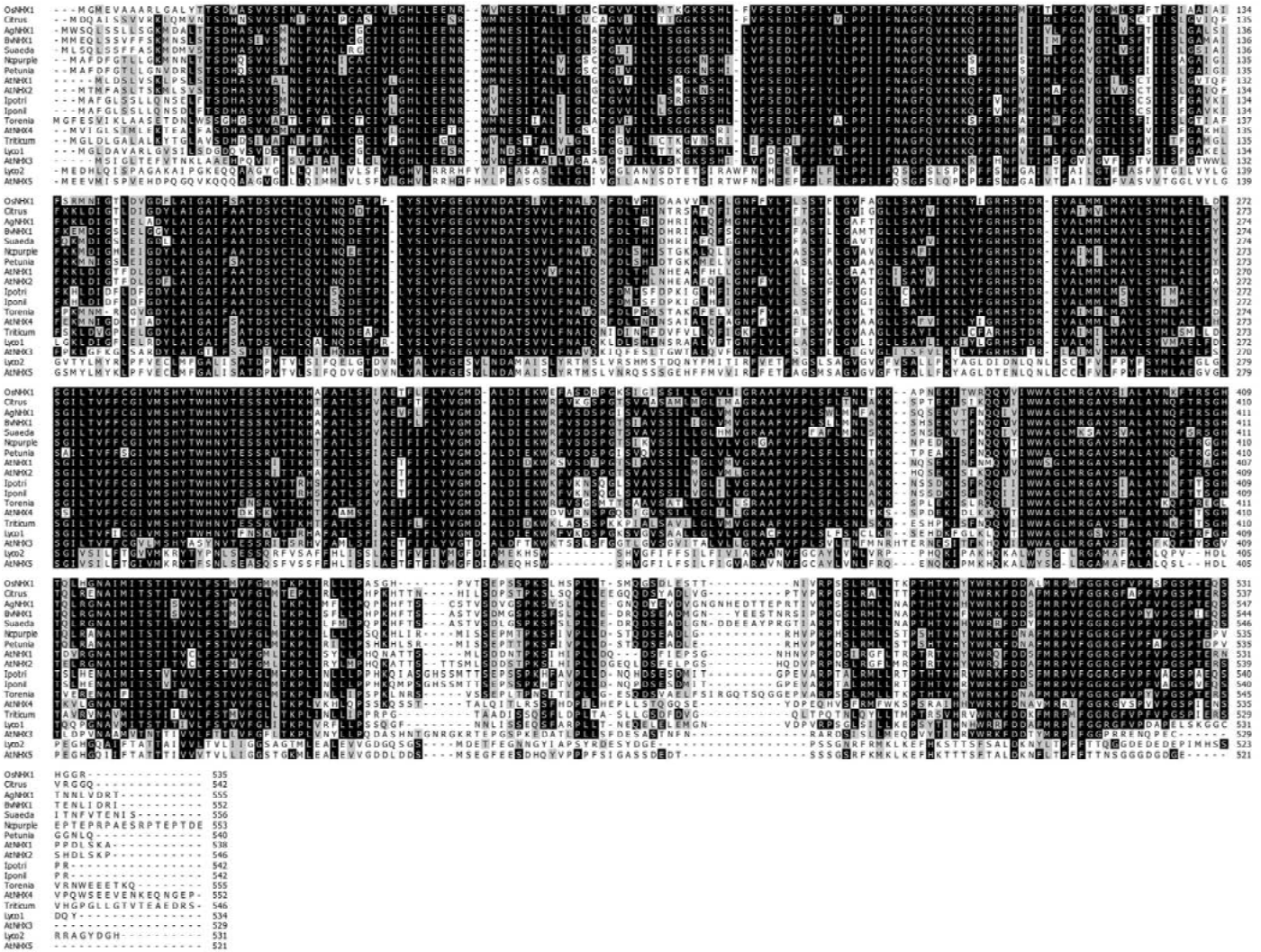

Figure 1. Alignment of full-length NHX-like proteins. Titles in the alignment correspond to accession numbers and species as follows: AgNHX1, BAB11940, *Atriplex gmelini*; AtNHX1, AAD16946, *Arabidopsis thaliana*; AtNHX2, AAG51408, *A. thaliana*; AtNHX3, BAB08564, *A. thaliana*; AtNHX4, AAF08577, *A. thaliana*; AtNHX5, *A. thaliana;* AAD25617; Citrus, AAK27314, *Citrus x paradisi*; Iponil, BAB16380, *Ipomoea nil*; Ipotri, BAB60901, *Ipomoea tricolor*; Lyco1, CAC84522, *Lycopersicon esculentum*; Ncpurple, BAB56106, *Nierembergia caerulea*; OsNHX1, BAA83337, *Oryza sativa*; Petunia, BAB56105, *Petunia x hybrida*; Suaeda, AAK53432, *Suaeda maritima* subsp. Salsa; Torenia, BAB56107, *Torenia hybrida*; Triticum, AAK76737, *Triticum aestivum*. Residues in black are identical; residues in grey are conservative substitutions.

Table 1. GeneBank plant ESTs similar to AtNHX1

Accession	Species	Tissue
AI938253	*Glycine max*	Leaves, 2–3 week old seedlings
AV408114	*Lotus japonicus*	Young plants
AW562918	*Zea mays* (Same as BM660978 and BM736947	Whole premieotic anthers to pollen shed
AW685820	*Medicago truncatula*	Rhizobium meliloti-inoculated roots
AW694621	*Medicago truncatula*	Internodal stem segments
BE420587	*Hordeum vulgare*	Leaf
BE433982	*Lycopersicon esculentum*	Pericarp breaker fruit stage
BE440835	*Glycine max*	Hypocotyl and Plumule, germinating seeds (3 day)
BE607262	*Pinus taeda*	Xylem Compression wood Inclined
BE643915	*Pinus taeda*	Xylem Compression wood Inclined
BE803390	*Glycine max*	Floral meristematic mRNA
BF275409	*Gossypium arboreum*	Fibers isolated from bolls harvested 7–10 dpa
BG649564	*Sorghum bicolor*	Embryos germinated for 24 h
BI138647	*Populus balsamifera* subsp. *Trichocarpa*	Flower
BI317267	*Glycine max*	Roots of 7 day old 'Bragg' supernodulating mutant NTS382 seedlings
BI471952	*Glycine max*	Leaf tissue
BI933988	*Lycopersicon esculentum*	Tomato flower, anthesis
BI934675	*Lycopersicon esculentum*	Tomato flower, anthesis
BJ198426	*Physcomitrella patens* subsp. *Patens*	Mix of chloronemata, caulonemata and protonemata
BM109451	*Solanum tuberosum*	Potato roots
BM173059	*Avicennia marina*	Mangrove leaf
BM660526	*Zea mays*	BMS (Black Mexican Sweet) suspension culture
BM660527	*Zea mays*	BMS (Black Mexican Sweet) suspension culture
BM660977	*Zea mays*	BMS (Black Mexican Sweet) suspension culture
BM660978	*Zea mays* (same as BM736947 and AW562918)	BMS (Black Mexican Sweet) suspension culture
BM736947	*Zea mays* (same as BM660978 and AW562918)	BMS (Black Mexican Sweet) suspension culture

according to the nomenclature of Maser et al. (2001). AtNHX1 and AtNHX2 share the greatest similarity, with 87% identical residues, while AtNHX5, which is the shortest member of the group, shares approximately 26% identical residues with the other four. The family of plant AtNHX-like Na^+/H^+ antiporters has grown to 18 fully sequenced members to date. These are aligned in Figure 1. This alignment reveals the extensive similarity between these proteins, with notable similarity between the beet, Atriplex and *Suaeda* proteins (>86% identity). The NHX-like proteins that are involved in regulating floral coloration also cluster together; *Ipomoea* nil and *tricolor* NHXs share 98% identity and *Petunia* NHX is 90% identical to *Nieremburgia* NHX. These similarities do not, however, differ from the phylogenetic relatedness of these species, and therefore, nothing about the particular function of these NHX proteins can be extrapolated from the alignment. The most important point about this alignment and the table of ESTs (Table 1) is that NHX-like genes are present across many and diverse taxonomic groups, from salt-tolerant to salt-sensitive

species, and from bryophytes to monocots and dicots. These data also support the hypothesis that, as in *Arabidopsis*, NHX-like genes are likely to be present as multiple isoforms in many species. There is conclusive evidence for this in species where sufficient numbers of ESTs have been sequenced (corn, tomato, alfalfa, and soybean); as EST sequencing projects develop in other plants, more NHX-like gene families should be uncovered.

The EST data (Table 1) also suggest that AtNHX1-like proteins are involved in different particular functions, by virtue of the diverse tissue types from which they were cloned. This is consistent with our observations in *Arabidopsis*, where AtNHX1-5 show distinct patterns of mRNA abundance in different tissues (Figure 2). We are currently unable to ascribe discrete functions for each antiport of the family, but T-DNA knockout lines and RNAi experiments will facilitate this course of investigation; a role for AtNHX1 in cellular ion homeostasis and leaf development has been suggested by the phenotype of an AtNHX1 knockout line in our lab (Apse et al., submitted).

AtNHX genes are differentially expressed in the organs of Arabidopsis

The expression of *Arabidopsis NHX*-like genes was analyzed to determine whether individual members of the family were expressed in particular organ(s). RT-PCR was used to determine relative transcript abundance in flowers, leaves, roots, and stem tissues for each of the five *AtNHX* genes. *AtNHX1* shows the most abundant and widely distributed transcripts, as the amplification product was detected in all tissues with ethidium bromide staining (Figure 2A). This is consistent with our previous findings using RNA gel blot analysis and *in situ* hybridization (Apse et al., submitted). *AtNHX2* shows a similar abundance in flowers and roots, but is marginally less abundant in leaves and almost undetectable in inflorescence stems (Figure 2B). The amplification products of *AtNHX3-5* were undetectable by ethidium bromide staining in agarose gels, and these required radioactive probing of DNA blots. *AtNHX3* and *AtNHX4* transcripts were almost exclusively detected in flower and root tissues, respectively (Figure 2C, D). *In situ* hybridizations for detection of *AtNHX2* and *AtNHX4* transcripts are consistent with the results of the RT-PCR. While *AtNHX4* signal was observed only in roots (see Figure 3I–K), Figure 3 (A–D) shows specific floral expression of *AtNHX2*. Although detectable in tapetal cells, *AtNHX2* is most strongly expressed in the septum of the developing silique; perhaps there is a role for this protein in pollen transmission. Signals for antisense probes of *AtNHX2* were not detected in other tissues (data not shown). Data from both *in situ* hybridizations and promoter-GUS fusion transgenic plants correlate well with the RT-PCR pattern of expression for *AtNHX3*. *In situ* hybridization did not detect signals for *AtNHX3* in leaves or stems (not shown), but flower specific expression was detected in sepals, very strongly in the receptacle parenchyma (Figure 3E–H), and particularly in anthers (Figure 3M). GUS activity was absent from most tissues except in anthers, where the signal appeared to be pollen-associated (Figure 3N). *AtNHX5* signal was not detected by *in situ* hybridization in all tissues tested (not shown), which is consistent with the low level of expression as determined by RT-PCR (Figure 2E). *AtNHX5* transcripts were observed at very low levels in all tissues, but were marginally more abundant in stem tissues. Although it is possible that these genes have overlapping functions in organs where their expression coincides, there is evidence from other species that supports organ-specific functions for the *NHX*-like genes. Yamaguchi et al. (2001) showed that in flower petals, the AtNHX1 homologue in morning glory (*Ipomoea nil*) mediates a developmentally regulated vacuolar alkalinization in the transition of purple buds to blue flowers. This alkalinization changes the absorption spectrum of the anthocyanins stored in the vacuole. As with AtNHX1, these other NHX proteins may function in development specific roles in floral tissues. The relatively strong presence of *AtNHX4* in roots raises the question of redundancy of NHX-like gene expression in roots, as *AtNHX1* also shows strong root expression (Shi and Zhu, 2002). The *AtNHX1* promoter was also found to drive GUS expression in lateral root primordia and root-hair-forming cells (Blumwald, unpublished). With the expression of AtNHX1 in petals, hypocotyls, young leaves, siliques, and stamens, it might be suggested that AtNHX1 has a role in rapidly expanding cells and tissues.

Heterologous expression of AtNHXs in yeast mutants

Heterologous complementation of *S. cerevisiae* mutants with plant genes provides a useful system for the functional assessment of plant proteins. Given the similarity in amino acid composition and function between the endosomal yeast and the plant vacuolar Na^+/H^+ antiporters, yeast mutants lacking Nhx1

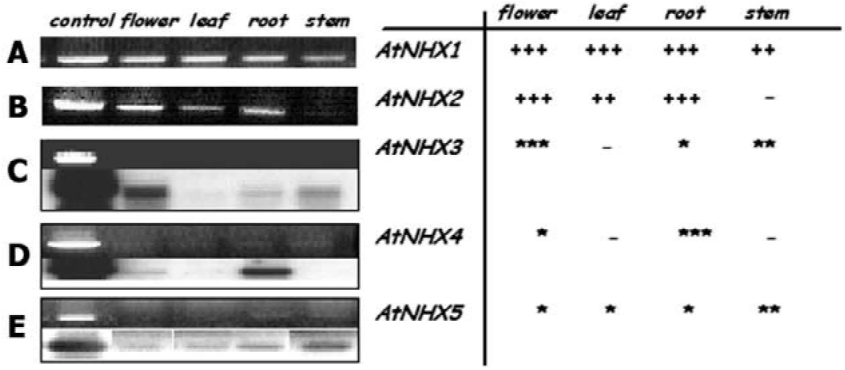

Figure 2. Relative transcript abundance of *AtNHX* genes in flowers, leaves, roots, and stem tissues. RT-PCR was performed as described in 'Materials and methods'. The RT-PCR products were detected by ethidium bromide staining after separation in agarose gels. **(A)** AtNHX1, **(B)** AtNHX2, **(C)** AtNHX3, **(D)** AtNHX4, **(E)** AtNHX5. When the amplification products were undetectable by ethidium bromide, the RT-PCR products were blotted onto nylon membranes and probed with ^{32}P-labelled probes specific for each gene (**C, D** and **E,** lower panels). (**+**) indicates the relative intensity of each signal in EtBr stained agarose gels, (*) indicates the relative intensity of signals in autoradiograms, (–) indicates that little or no signal was detected.

provide a unique tool for the assessment of the functional role of the AtNHX family (Quintero et al., 2000; Yokoi et al., 2002). The *AtNHX1* ORF was placed under the control of a conditional promoter for the expression of heterologous proteins in yeast. Studies were carried out to determine whether the *Arabidopsis NHX1* gene product could functionally complement the Δ*nhx1* yeast mutant by suppressing its observed phenotypes. Expression of AtNHX1 was studied in APG (-HIS, -TRP, -MET) media at pH 4.5–6.5 with varying NaCl concentrations between 0 and 250 m*M*. Partial complementation was observed at all pH ranges (Figure 4A–C) indicating a functional conservation between the yeast and *Arabidopsis* NHX gene products. Since Na^+ sensitivity of the Δ*nhx1* yeast strain was seen to increase with decreasing pH, complementation by *AtNHX1* expression was better observed at lower pH. At pH 5.5, complementation was seen to be more effective than at pH 4.5 in APG media. In an attempt to characterize the rest of the family members (*AtNHX2–5*), similar complementation studies were carried out at pH 5.5 for each one of the genes under the control of the *MET25* promoter (Figure 4E–H). Clearly, the expression of *AtNHX1-5* increased the ability of the Δ*nhx*1 mutant to grow in the presence of high NaCl concentrations. While the growth of the mutant yeast strain complemented with the different NHX genes was significantly different than that transformed with the control plasmids, there appears to be no significant difference between the NHX genes in the degree of this complementation. Because the yeast Nhx1 protein was shown to be involved in ion sequestration (Nass and Rao, 1997) and in vacuolar biogenesis (Bowers et al., 2000), the mechanism of the complementation of the mutant by the *AtNHXs* is not clear. However, even the vacuolar biogenesis function of the yeast Nhx1 protein is hypothesized to depend on its Na^+/H^+ exchange activity (Bowers et al., 2000). Therefore, it is reasonable to conclude that the expression of *AtNHX1–5* in the yeast Δ*nhx1* strain provides a recovery of the salt sensitive phenotype because it functions as a Na^+/H^+ antiporter. Yokoi et al. (2002) have shown complementation of a yeast *nhx1* mutant for AtNHX1, 2, and 5 during the review of this manuscript, which confirms the results we present here, but we add the functional complementation for AtNHX3 and 4 as well.

Role of AtNHX1 *in salt tolerance*

The sensitivity to salt of cytosolic enzymes is similar in both glycophytes and halophytes, indicating that the maintenance of a high cytosolic K^+/Na^+ concentration ratio is a key requirement for plant growth in high salt (Greenway and Munns, 1980). The compartmentation of Na^+ ions into vacuoles provides an efficient mechanism to avert the toxic effects of Na^+ in the cytosol. The transport of Na^+into the vacuoles is mediated by a Na^+/H^+ antiporter that is driven by the electrochemical gradient of protons generated by the vacuolar H^+-translocating enzymes, the H^+-ATPase and the H^+-PP_iase (Blumwald, 1987).

In *Arabidopsis*, the overexpression of *AtNHX1* resulted in transgenic plants that were able to grow in high salt concentrations (Apse et al., 1999). Analysis

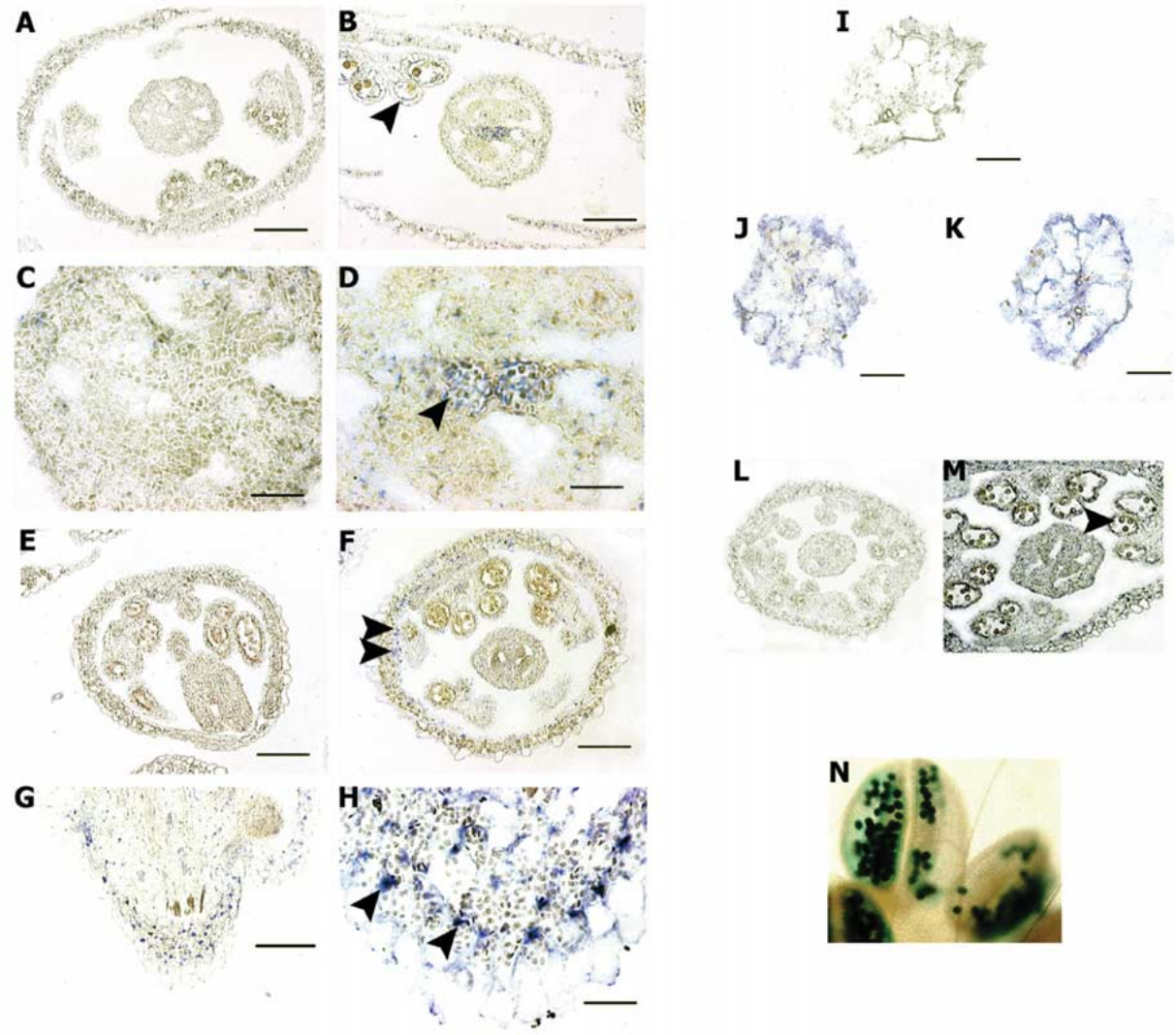

Figure 3. Localization of *AtNHX* transcripts in *Arabidopsis* tissues. *AtNHX2* transcripts were detected with antisense (**B**,**D**) but not control sense (**A**,**C**) probes in tapetal cells (arrow in **B**) and the septum of the carpel (arrow in **D**). AtNHX3 transcripts were detected with antisense (**F**,**G**,**H**,**M**) but not control sense (**E**,**G**,**L**) probes in sepals of unopened flowers (arrows in **F**), receptacle parenchyma (**G**, and arrows in **H**), and in anthers and pollen (arrow in **M**). X-gluc staining of plants containing *AtNHX3* promoter driving GUS expression stained very strongly for pollen and somewhat less intensely in anthers (**N**). *AtNHX4* transcripts were detected with antisense (**J**,**K**) but not sense (**I**) probes in roots. Bars = 25 μm except in (**A**,**B**,**E**,**F**,**G**) where bars = 100 μm.

of vacuolar and cytosolic ion contents is required to definitively address the mechanism of this acquired tolerance in the transgenic plants. However, the increased Na^+ content of transgenic shoots (relative to wild-type), together with the improved growth of the transgenic plants under Na^+ stress conditions, indicates the importance of vacuolar Na^+ compartmentation. Additional evidence supporting the role of vacuolar transport in salt tolerance has been provided. Transgenic *A. thaliana* plants overexpressing *AVP1,* coding for the vacuolar H^+-pyrophosphatase, displayed enhanced salt tolerance that was correlated with the increased ion content of the plants (Gaxiola et al., 2001). These results suggest that the enhanced vacuolar H^+-pumping in the transgenic plants provided additional driving force for vacuolar sodium accumulation via the vacuolar Na^+/H^+ antiporter. The role of sodium compartmentation in plant salt tolerance has been further demonstrated in transgenic tomato plants overexpressing *AtNHX1* (Zhang and Blumwald,

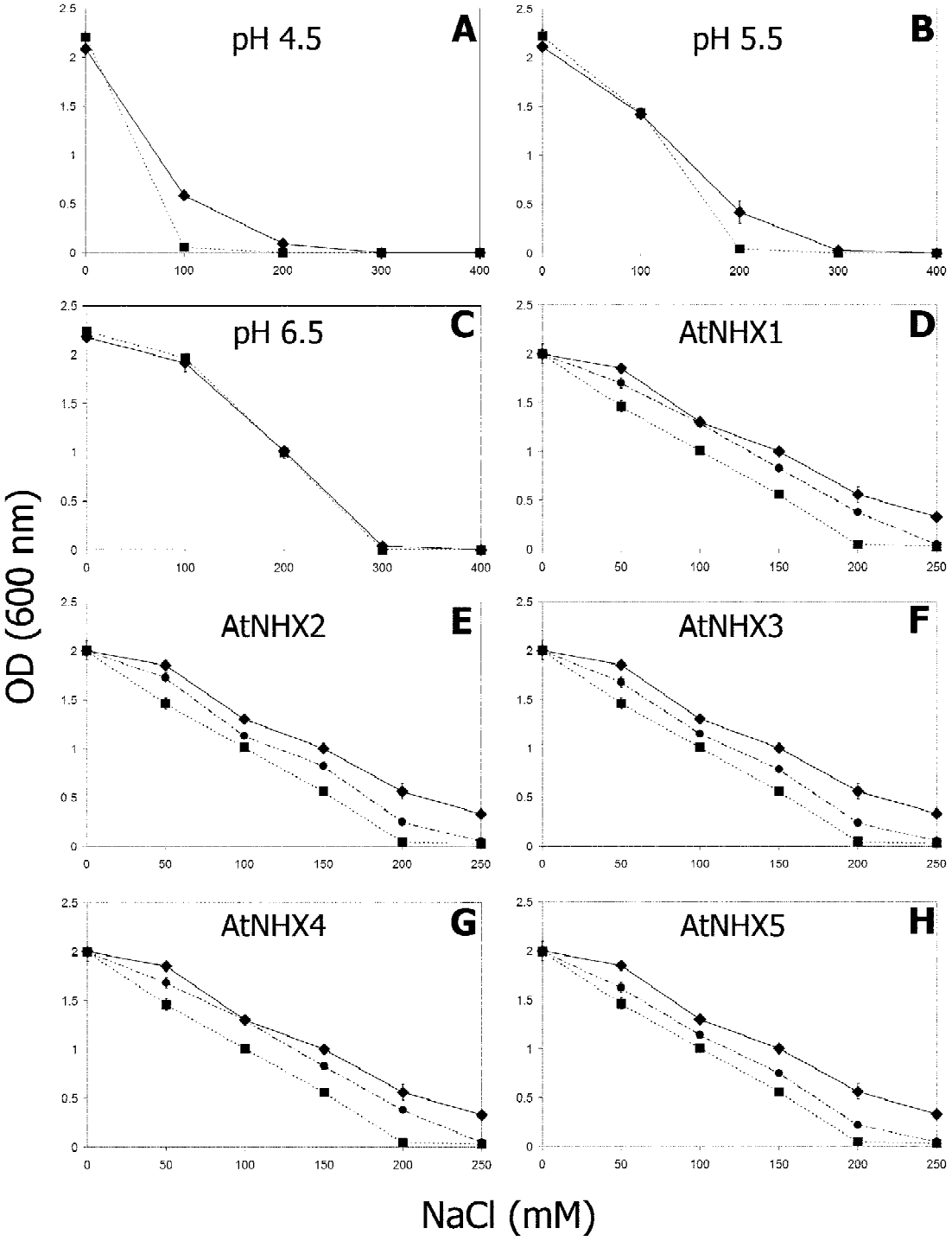

Figure 4. Functional complementation of *S. cerevisiae* mutants with *AtNHX* genes. Salt tolerance of wild type (♦) and *S. cerevisiae* Δ*nhx1* mutant (B) at different pH. (**A**) pH 4.5; (**B**) pH 5.5; (**C**) pH 6.5. Growth conditions as described in 'Materials and methods'. Salt tolerance of wild type (♦), Δ*nhx1* mutant (B), and Δ*nhx1* mutant transformed with the different AtNHX genes (CIRCLE) at pH 5.5. (**D**) AtNHX1; (**E**) AtNHX2; (**F**) AtNHX3; (**G**) AtNHX4; (**H**) AtNHX5. Growth conditions and transformation as described in 'Materials and methods'. Values are the Mean ± SD ($n = 4$).

2001). The transgenic tomato plants grown in the presence of 200 m*M* NaCl were able to grow, flower, and set fruit. Although the leaves accumulated high sodium concentrations, the tomato fruits displayed very low amounts of sodium (Zhang and Blumwald, 2001). Similar results were obtained with transgenic *Brassica napus* (Canola) overexpressing *AtNHX1* (Zhang et al., 2001).

An AtNHX1 homologue, BvNHX1, was recently identified in *Beta vulgaris* (Xia et al., 2002). Both transcript and protein abundance increased on exposure of cell suspension cultures and plants to NaCl treatment. These increases correlated with an enhanced vacuolar Na^+/H^+ exchange activity. The increase in the V_{max} of the Na^+/H^+ exchange with no significant change in the K_m (affinity) of the transporter for Na^+ was consistent with an increase in the number of Na^+/H^+ antiporters in NaCl-treated plants and cells. The response of BvNHX1 expression and activity to NaCl treatment contrasts with that of the *Arabidopsis* homologue, which shows only a relatively small induction by severe NaCl shock (Shi and Zhu, 2002). This highlights a critical difference between the adaptive responses of salt-tolerant and salt-sensitive plants. Whether these differences are due to different transcriptional and/or post-tranlational regulation is under investigation.

Acknowledgments

This work was supported by grants from NSERC and the National Science Foundation to E.B.

References

Apse M P, Aharon G S, Snedden W A and Blumwald E 1999 Overexpression of a vacuolar Na^+/H^+ antiport confers salt tolerance in *Arabidopsis*. Science 285, 1256–1258.

Blumwald E and Poole R J 1985 Na^+/H^+ antiport in isolated tonoplast vesicles from storage tissue of *Beta vulgaris*. Plant Physiol. 78, 163–167.

Blumwald E 1987 Tonoplast vesicles for the study of ion transport in plant vacuoles. Physiol. Plant. 69, 731–734.

Blumwald E, Aharon G S and Apse M P 2000 Na^+ transport in plant cells. Biochim. Biophys. Acta 1465, 140–151.

Bowers K, Levi B P, Patel F I and Stevens T H 2000 The sodium/proton exchanger Nhx1p is required for endosomal protein trafficking in the yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell 11, 4277–4294.

Clough S J and Bent A F 1998 Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. 16, 735–43.

Darley C P, van Wuytswinkel O C M, van der Woude K, Mager W H and de Boer A H 2000 *Arabidopsis thaliana* and *Saccharomyces cerevisiae* NHX1 genes encode amiloride sensitive electroneutral Na^+/H^+ exchangers. Biochem. J. 351, 241–249.

Flowers T, Troke P F and Yeo A R 1977 The mechanisms of salt tolerance in halophytes. Ann. Rev. Plant Physiol. 28, 89–121.

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.

Gaxiola R A, Li J, Undurraga S, Dang L M, Allen G J, Alper S L and Fink G R 2001 Drought- and salt-tolerant plants result from overexpression of the AVP1 H^+-pump. Proc. Natl. Acad. Sci. USA 98, 11444–11449.

Glenn E, Brown J J and Blumwald E 1999 Salt-tolerant mechanisms and crop potential of halophytes. Crit. Rev. Plant Sci. 18, 227–255.

Greenway H and Munns R 1980 Mechanisms of salt tolerance in nonhalohytes. Ann. Rev. Plant Physiol. 31, 149–190.

Kieber J J, Rothenberg M, Roman G, Feldmann K A and Ecker J R 1993 CTR1, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. Cell 72, 427–41.

Long J A, Moan, E I, Medford J I and Barton M K 1996 A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis.* Nature 379, 66–69.

Maathuis F J M and Amtmann A 1999 K^+ nutrition and Na^+ toxicity: The basis of cellular K^+/Na^+ ratios. Ann. Bot. 84, 123–133.

Maser P, Thomine S, Schroeder J I, Ward J M, Hirschi K, Sze H, Talke I N, Amtmann A, Maathuis F J M, Sanders D, Harper J F, Tchieu J, Gribskov M, Persans M W, Salt D E, Kim S A and Guerinot M L 2001 Phylogenetic relationships within cation transporter families of *Arabidopsis*. Plant Physiol. 126, 1646–1667.

Mumberg D, Muller R and Funk M 1994 Regulatable promoters of Saccharomyces cerevisiae: Comparison of transcriptional activity and their use for heterologous expression. Nucl. Acids Res. 22, 5767–5768.

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. Insights into mechanisms of sodium tolerance. J. Biol. Chem. 272, 26145–26152.

Nass R and Rao R 1998 Novel localization of a Na+/H+ exchanger in a late endosomal compartment of yeast. Implications for vacuole biogenesis J Biol. Chem. 273, 21054–21060.

Paulsen I T, Sliwinski M K and Saier M H 1998 Microbial genome analyses: global comparisons of transport capabilities based on phylogenies, bioenergetics and substrate specificities. J. Mol. Biol. 277, 573–592.

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.

Shi H, Quintero F J, Pardo J M and Zhu J K 2002 The putative plasma membrane Na^+/H^+ antiporter SOS1 controls long-distance Na^+ transport in plants. Plant Cell 14, 465–477.

Shi H and Zhu J K 2002 Regulation of the vacuolar Na^+/H^+ antiporter gene *AtNHX1* expression by salt stress and ABA. Plant Mol. Biol. 50, 543–550.

Spalding E P, Hirsch R E, Lewis D R, Qi Z, Sussman M R and Lewis B D 1999 Potassium uptake supporting plant growth in the absence of AKT1 channel activity-Inhibition by ammonium and stimulation by sodium. J. Gen. Physiol. 113, 909–918.

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, Fukada-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, 451–61.

Yokoi S, Quintero F J, Cubero B, Ruiz M T, Bressan R A, Hasegawa P M and Pardo J M 2002 Differential expression and function of *Arabidopsis thaliana* NHX Na^+/H^+ antiporters in the salt stress response. Plant J. 30, 529–539.

Zhang H X, Hodson J, Williams J P and Blumwald E 2001 Engineering salt-tolerant *Brassica* Plants: Characterization of yield and seed oil quality in transgenic plants with increased vacuolar sodium accumulation. Proc. Natl. Acad. Sci. USA 98, 12832–12836.

Zhang H X and Blumwald E 2001 Transgenic salt tolerant tomato plants accumulate salt in the foliage but not in the fruits. Nat. Biotech. 19, 765–768.