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Functional conservation between yeast and plant endosomal Na^+/H^+ antiporters[1]

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Abstract Vacuolar compartmentation of Na^+ is an essential mechanism for salinity tolerance since it lowers cytosolic Na^+ levels while contributing to osmotic adjustment for cell turgor and expansion. The AtNHX1 protein of *Arabidopsis thaliana* substituted functionally for ScNHX1, the endosomal Na^+/H^+ antiporter of yeast. Ion tolerance conferred by AtNHX1 and ScNHX1 correlated with ion uptake into an intracellular pool that was energetically dependent on the vacuolar (H^+)ATPase. AtNHX1 localized to vacuolar membrane fractions of yeast. Hence, both transporters share an evolutionarily conserved function in Na^+ compartmentation. *AtNHX1* mRNA levels were upregulated by ABA and NaCl treatment in leaf but not in root tissue.

Key words: Sodium/proton antiporter; Sodium compartmentation; Halotolerance; *Arabidopsis thaliana*; *Saccharomyces cerevisiae*

1. Introduction

When plants are exposed to excessive salinity, ions from the external environment accumulate in the apoplast and enter the cytosol causing imbalance of the cellular milieu. Transport processes that regulate ion fluxes, particularly those involved in the control of Na^+ uptake, efflux from the cell, and vacuolar compartmentation, are of critical importance for plant adaptation to high salinity [1,2]. Na^+ is removed from the cytosol by the activity of plasma membrane- and tonoplast-localized Na^+/H^+ antiporters [2,3]. Na^+ translocation against its electrochemical gradient is coupled to reverse H^+ flux and proton pumps located in these membranes provide the driving force required for Na^+ transport. Accordingly, NaCl treatment increases the activity of H^+ pumps and Na^+/H^+ antiporters in several plant species [2,3]. For instance, the halophyte *Mesembryanthemum crystallinum* (ice plant) accumulates large quantities of NaCl within vacuoles, exceeding 1 M in epidermal bladder cells. The activity of a Na^+/H^+ exchanger existing in leaves of *M. crystallinum* was enhanced further by salt treatment, concurrently with increased V-ATPase activity, presumably to energize secondary Na^+ transport [4]. Recently, the *Arabidopsis* AtNHX1 protein has been identified as a tonoplast Na^+/H^+ antiporter [5]. AtNHX1 overexpression in transgenic plants upheld the presumed significance of Na^+ compartmentation in NaCl tolerance [5]. In this paper, we show that AtNHX1 is functionally analogous to the endosomal Na^+/H^+ antiporter ScNHX1 of *Saccharomyces cerevisiae*. Ion tolerance conferred by AtNHX1 correlated with enhanced ion uptake and compartmentation that were commensurate with the activity of ScNHX1 and energetically subordinate to the vacuolar (H^+)ATPase. Furthermore, AtNHX1 localized to vacuolar membrane fractions.

2. Materials and methods

2.1. Strains and media

All *S. cerevisiae* strains used were derivatives of W303-1B (*MATα leu2-13,112 ura3-1 trp1-1 his3-11,15 ade2-1 can1-100*). Their relevant genotypes were: VATPc (*vma3::LEU2*) [6], ANT3 (*Δena1-4::HIS3 Δnha1::LEU2*) (gift from A. Rodríguez-Navarro, Universidad Politecnica de Madrid, Spain), AXT3 (*Δena1-4::HIS3 Δnha1::LEU2 Δnhx1::TRP1*) and WX1 (*Δnhx1::TRP1*). The null allele *nhx1::TRP1* was created by replacement of the *Eco*RI/*Pst*I fragment internal to *ScNHX1* with an *Eco*RI/*Pst*I fragment containing the marker gene *TRP1*. Attempts to disrupt *ScNHX1* in a *vma3* mutant were unsuccessful, presumably due to synthetic lethality. ρ^0 derivatives (lacking functional mitochondria) were isolated after two rounds of growth to saturation in liquid SD medium with 25 μg/ml ethidium bromide and testing for inability to grow on non-fermentable carbon sources. YPD, YPGly and SD synthetic media were prepared as described [7]. Na^+ and Li^+ tolerance tests were performed in arginine–phosphate (AP) medium [8], except when indicated otherwise. The AP medium is essentially free of alkali cations and was supplemented with 1 mM KCl, and NaCl or LiCl as required.

2.2. Isolation of AtNHX1 and expression in yeast

A full-length cDNA of *AtNHX1* was isolated by PCR from an *Arabidopsis* cDNA library [9] using the primers 5′-CACTCGAGCAATGTTGGATTCTCTAGTGTC and 5′-ATGCGGCCGCTCAAGCCTTACTAAGAT. Nucleotides were included to construct *Xho*I and *Not*I restriction sites on the 5′ and 3′ ends of the gene, respectively. The PCR product was fully sequenced in both strands. The *Arabidopsis* cDNA library was screened with the *AtNHX1* PCR product as probe to isolate additional full-length cDNAs. For expression in yeast, *AtNHX1* was subcloned into pDR195 [10] downstream of the *PMA1* promoter producing the plasmid pAtNHX1-1.

2.3. Determination of intracellular ion content

Because ScNHX1 and AtNHX1 antiporters catalyze Li^+ exchange, Li^+ was used as tracer for Na^+ transport in some experiments. To measure intracellular Na^+ and Li^+ contents, yeast cells were grown in AP medium with 1 mM KCl to $A_{550} \sim 0.3$, harvested, and resuspended in the same medium supplemented with NaCl or LiCl as indicated in each experiment. The *vma3* mutant is unable to grow in AP medium [11]. Therefore, in experiments in which the *vma3* mutants was included, cells were grown in SD medium buffered to pH 5.5 with 50 mM MES/Tris [6]. Cell samples were filtered through 0.8 μm Millipore membranes, washed with 20 mM $MgCl_2$, and extracted with acid. Ion contents of the samples were determined by atomic emission spectrophotometry. For ion efflux determination, preloaded cells were

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[1] The *AtNHX1* cDNA sequence has been deposited in the GenBank data base with accession number AF056190.

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quickly washed with buffer (10 mM MES adjusted to pH 5.5 with $Ca(OH)_2$, 10 mM KCl, 0.1 $MgCl_2$, 0.5 M sorbitol, and 2% glucose), resuspended in the same buffer, and samples were collected by filtration at time intervals to determine intracellular ion content.

2.4. DNA and RNA hybridizations

Arabidopsis thaliana L. var. Wassilewskija were grown for 4 weeks in liquid MS medium (Sigma M-5524) in sterile conditions. For salt or ABA treatment the MS medium was supplemented with NaCl 100 mM and ABA 50 μM, respectively. Total RNA was isolated from roots and leaves, electrophoretically separated on denaturing formaldehyde–agarose gels, and blotted onto nylon membranes. Nylon filters were hybridized with ^{32}P-labeled DNA probes following standard protocols [12]. Final washes were with 0.2×SSC at 65°C. RNA loading was assessed by re-probing the blots with a radish 18S rRNA gene probe. Hybridization signals from RNA blots were quantified by densitometric scanning of autoradiograms and relative abundance of *AtNHX1* transcripts was normalized to RNA loading. Nylon membrane lifts of bacterial colonies harboring the *Arabidopsis* cDNA library [9] were hybridized at 42°C in 5×SSC, 30% formamide. Final washes were with 0.2×SSC at 45°C.

2.5. Antibodies and immunoblotting

A translational fusion of the carboxy-terminal portion of AtNHX1 and the β-galactosidase was constructed using the *Bcl*I restriction site at amino acid residue I^{417} in AtNHX1 and the *Bam*HI site in the vector pEX2 [13]. Rabbit polyclonal antibodies were produced against the recombinant protein and were affinity-purified adsorbing the fusion protein to octadecyl silica [14]. Total membranes from cell lysates were fractionated by centrifugation in a 10-step sucrose density gradient (18–54% w/w sucrose, in 4% increments) [15]. Fractions were assayed for activity of marker enzymes of tonoplast (α-mannosidase), mitochondria (azide-sensitive ATPase) and plasma membrane (vanadate-sensitive ATPase) [16,17].

3. Results

3.1. Isolation of AtNHX1 cDNA

The sequencing of chromosome V of *Arabidopsis* revealed the presence of a putative gene, referred to as *AtNHX1* (accession number AF007271), encoding a predicted protein with sequence and topological similarities to animal Na^+/H^+ exchangers. Subsequently, AtNHX1 has been shown to impart NaCl tolerance when expressed in yeast and transgenic *Arabidopsis* [5,18]. To characterize further the AtNHX1 protein, the coding region of *AtNHX1* was amplified by PCR from a cDNA library using specific primers that annealed to start and stop codons. Complete sequencing of amplified cDNA indicated that the PCR product matched the genomic sequence at putative exons except for one silent nucleotide change. Three additional full-length cDNAs were isolated (out of 5×10^5 bacterial colonies screened) by hybridization screening under mild stringency of the *Arabidopsis* cDNA library with the amplified product of *AtNHX1* as probe. Nucleotide sequences demonstrated that these cDNAs also derived from the *AtNHX1* locus. Southern blot analysis under stringent conditions produced hybridization bands consistent with the restriction map of *AtNHX1*, suggesting that *AtNHX1* is present in the *Arabidopsis* genome as a single copy gene (data not shown). Nevertheless, sequences corresponding to other putative *NHX*-like genes have appeared in the Arabidopsis data base (data not shown) suggesting the presence of a small gene family encoding proteins related to NHE-type exchangers in *Arabidopsis*.

3.2. Complementation of yeast nhx1 mutant by AtNHX1

AtNHX1 encodes a predicted 59.5 kDa hydrophobic polypeptide that shares an overall 35–38% similarity with animal

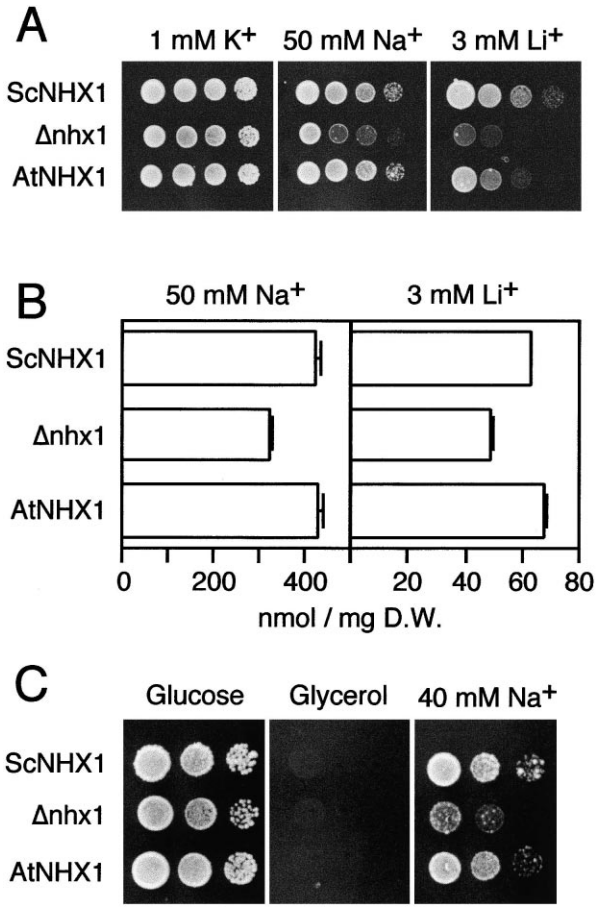

Fig. 1. AtNHX1 is functionally analogous to ScNHX1. A: Cells of strains ANT3 (*ScNHX1*), AXT3 (Δ*nhx1*), and AXT3 transformed with plasmid pAtNHX1-1 (*AtNHX1*) were grown overnight in selective AP medium. Aliquots (10 μl) and 10-fold serial dilutions were spotted onto AP plates supplemented with 50 mM NaCl or 3 mM LiCl as indicated on each panel. Plates were photographed after 4 days at 28°C. B: Cells of strains shown in A were grown in AP medium containing 50 mM NaCl or 3 mM LiCl until the cultures reached mid-log phase. Cell were then filtered and their internal contents of Na^+ or Li^+ determined. Shown are the average and S.E.M. of ion contents of three independent cultures of each strain. Units are nmol of ion per mg dry weight of cell samples. C: ρ^0 derivatives of the same strains shown in A were spotted onto AP medium containing either glucose or glycerol as carbon source, and in glucose medium supplemented with 40 mM NaCl.

NHE-type Na^+/H^+ exchangers and fungal NHX1 proteins of *S. cerevisiae* and *Schizosaccharomyces pombe*. The ScNHX1 proteins has a perivacuolar location and functions in intracellular compartmentation of Na^+ in *S. cerevisiae* [18–20]. On the other hand, the Sod2-like NHA1 exchanger localizes to the plasma membrane of *S. cerevisiae* where it can partially substitute for the Na^+-ATPases ENA1-4 in mediating Na^+ extrusion from the cell [21,22]. The NaCl tolerance of yeast is primarily dependent on the activity of ENA1-4 Na^+-ATPases but deletion of *NHA1* or *NHX1* genes further increases the NaCl sensitivity of an *ena1-4* mutant [19,21]. Hence, we tested the ability of AtNHX1 to suppress the Na^+ sensitivity of yeast mutants defective in endogenous Na^+/H^+ antiporters (in an *ena1-4* background to avoid masking the activity of antiporters). As shown in Fig. 1A, expression of AtNHX1 increased the tolerance to Na^+ of *nhx1* mutant cells to an extent similar to the endogenous ScNHX1, but failed to res-

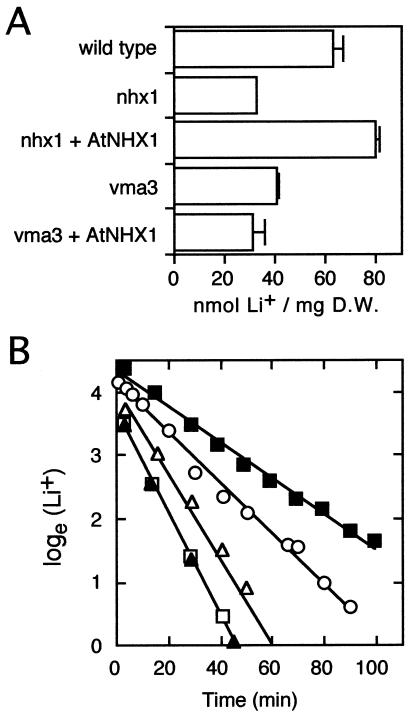

Fig. 2. Kinetics of ion efflux from preloaded cells. Wild type cells (circles), cells of strain WX1 (*nhx1*) (squares), strain VATPc (*vma3*) (triangles), and both mutant strains transformed with pAtNHX1-1 (filled symbols) were loaded for 1 h in SD medium with 300 mM LiCl. Subsequently, cells were transferred to Li^+-free buffer to allow extrusion of intracellular Li^+ and samples were collected at time intervals. A: Ion content of loaded cells. Values shown are the average and the S.E.M. of three or four independent cultures of each strain. B: Plot of $\log_e$ of intracellular Li^+ contents versus time fitted to linear first-order kinetics of efflux. Values are the average ion content of the replicate samples shown in A.

cue the tolerance of cells lacking the plasma membrane antiporter NHA1 (data not shown). We found that the *nhx1* mutation produced sensitivity to Li^+, a toxic analogue of Na^+, that was also partially suppressed by AtNHX1 (Fig. 1A), demonstrating that ScNHX1 and AtNHX1 transport both cations. To assess the tolerance mechanism conferred by AtNHX1, the intracellular ion content was measured in *nhx1* mutant and in cells expressing the endogenous or the plant antiporter, and after prolonged incubation in 50 mM NaCl or 3 mM LiCl, conditions in which these strains displayed differential sensitivity (see Fig. 1A). As shown in Fig. 1B, the internal Na^+ and Li^+ content was higher in transformants expressing AtNHX1 than in the *nhx1* mutant, and reached accumulation levels that were similar to that of control *NHX1* cells. Therefore, tolerance conferred by AtNHX1 and ScNHX1 correlated with higher intracellular ion content, as expected from a tolerance mechanism based on ion sequestration.

ScNHX1 (but not the plant AtNHX1) contains a putative targeting signal that could localize ScNHX1 to the mitochondrial inner membrane [23]. To test whether the contribution of ScNHX1 and AtNHX1 to NaCl tolerance required functional mitochondria, we performed Na^+ tolerance tests in ρ^0 derivatives of *NHX1* and *nhx1* strains with disabled mitochondrial respiration and energization. As depicted in Fig. 1C, ρ^0 cells were unable to use the non-fermentable carbon source glycerol, thus confirming loss of mitochondrial function. Disruption of *ScNHX1* in ρ^0 cells also caused sensitivity to Na^+ that was still suppressed by AtNHX1 (Fig. 1C), indicating that the role of both transporters in Na^+ tolerance was independent of mitochondria. Apart from increasing sensitivity to Na^+ and Li^+, disruption of *ScNHX1* in ρ^+ cells had no effect on the growth of yeast cells on fermentable (glucose) and non-fermentable (glycerol) carbon sources (data not shown).

3.3. Ion compartmentation in yeast cells expressing AtNHX1

An estimate of the magnitude of ion accumulation in endosomal compartments was obtained by determining the rate at which intracellular Li^+ was exchanged with the medium. To assess ion transport energetically dependent on the H^+ gradient established by the V-ATPase, a *vma3* mutant defective in proteolipid c was used. The *vma3* mutation disables vacuolar and endosomal acidification and membrane energization, thus impeding active ion uptake into these compartments [6]. Yeast cells were incubated in medium containing a high LiCl concentration (300 mM) to override the Li^+ efflux capacity of ENA1-4 and NHA1 proteins and to promote ion compartmentation. After loading, wild type cells (*NHX1 VMA3*) showed a higher ion content than isogenic mutants *nhx1* and *vma3* (Fig. 2A). Importantly, *nhx1* and *vma3* mutants had nearly identical intracellular ion contents, implying that ScNHX1 and the V-ATPase contribute equally to ion uptake and that these proteins are functionally coupled. As shown in Fig. 2B, intracellular ions were disposed at a greater rate in *nhx1* and *vma3* mutants (apparent kinetic constants of 0.069 min^{-1} and 0.080 min^{-1}, respectively, when fitted to linear first-order kinetics) than in wild type cells (0.039 min^{-1}), presumably because ions were not compartmentalized in these mutants but available to transporters in the plasma membrane for extrusion. AtNHX1 restored wild type ion uptake in a *nhx1* mutant and a low rate of Li^+ efflux (0.028 min^{-1}), indicating that ions were being compartmentalized. In accordance with endosomal ion accumulation, expression of AtNHX1 did not reverse the low ion loading and fast efflux

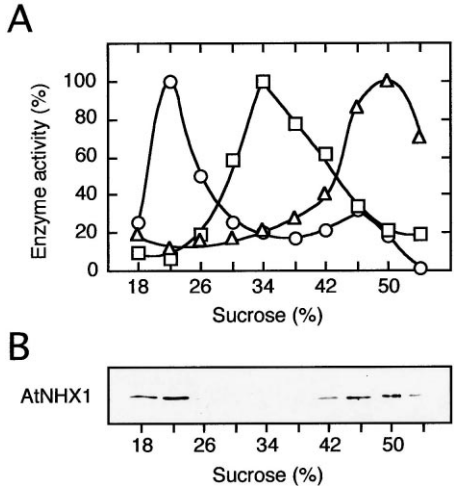

Fig. 3. Subcellular fractionation of AtNHX1. Cell membranes were fractionated on a discontinuous, 10-step sucrose gradient at 4% w/w increments. A: Activity of the marker enzymes α-mannosidase (circles), mitochondrial ATPase (squares), and plasma membrane ATPase (triangles). Values shown are normalized to peak activity fractions. B: Immunodetection of AtNHX1 in fractions shown in A using 90 μg of protein per lane.

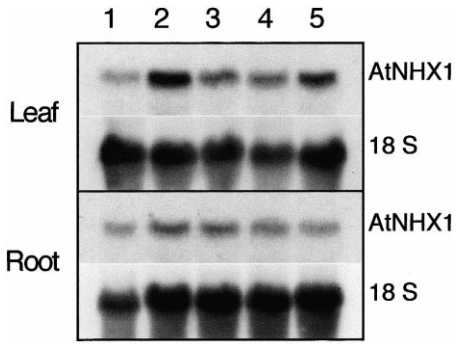

Fig. 4. RNA blot analysis of *AtNHX1* expression. Total RNA was purified from roots or leaves of *Arabidopsis* plants grown in liquid MS medium (lane 1), and subsequently treated with 50 μM ABA for 1 h (lane 2), or 100 mM NaCl for 1, 6, and 24 h (lanes 3, 4 and 5, respectively). 20 μg of RNA from each sample was hybridized with ^{32}P-radiolabeled probes derived from *AtNHX1* or radish 18S rDNA. The apparent size of bands hybridizing to *AtNHX1* was 1.9 kb.

rate of a *vma3* mutant (0.081 min^{-1}), demonstrating that AtNHX1 activity was energetically subordinate to the H^+ gradient generated by the V-ATPase. Accordingly, expression of AtNHX1 failed to improve the low Na^+ and Li^+ tolerance of *vma3* cells [11] (data not shown).

AtNHX1 was immunodetected to assess cellular localization in yeast. As shown in Fig. 3, AtNHX1 co-fractionated with the vacuolar marker α-mannosidase and away from the mitochondrial marker, the azide-sensitive ATPase. A signal was also observed in fractions of density similar to plasmalemma that may arise from transient residence in the endoplasmic reticulum of abundantly expressed AtNHX1.

3.4. Expression pattern of AtNHE1 in Arabidopsis

NaCl treatment is known to enhance tonoplast Na^+/H^+ exchange activity and Na^+ compartmentation in the vacuole of plants [2,3]. Northern blot analysis was carried out with RNA isolated from roots and leaves of 4-week-old *Arabidopsis* plants, plants exposed to NaCl for different periods of time, and plants treated with the stress hormone ABA for 1 h. Blots were hybridized with *AtNHX1* cDNA and re-hybridized to an 18S RNA probe to normalize RNA loading. *AtNHX1* transcript was detected in roots and leaves of control plants. A substantial and fast induction by ABA treatment was observed in the leaf (Fig. 4). *AtNHX1* transcript abundance increased seven-fold within 1 h of ABA addition. NaCl elicited a similar but slower response in the leaf, reaching a maximal four-fold induction at 24 h. Longer NaCl treatments did not result in a larger accumulation of the *AtNHX1* transcript (results not shown). In contrast, neither NaCl nor ABA treatment elicited noticeable changes in *AtNHX1* transcript abundance in the root. These results indicate that *AtNHX1* is responsive to NaCl stress and the stress-related hormone ABA in an organ-specific manner.

4. Discussion

The study of Na^+/H^+ antiporters is of paramount importance to the understanding of Na^+ homeostasis, pH regulation, and salinity tolerance in plants [2,3]. Recently, Gaxiola et al. [18] reported that the NHE-like protein AtNHX1 from *Arabidopsis* suppressed the NaCl and hygromycin B sensitivity of a yeast *nhx1* mutant and suggested that AtNHX1 is the plant ortholog of ScNHX1. Our results extend these findings and demonstrate that AtNHX1 functions in yeast through a mechanism involving ion sequestration. AtNHX1 failed to show cooperativity with the endogenous efflux proteins NHA1 and ENA1-4. Instead, AtNHX1 promoted ion uptake and compartmentation that was commensurate with ScNHX1 activity. ScNHX1 has been shown to co-fractionate with the VPH1 subunit of the vacuolar H^+ ATPase and the Cl^- channel GEF1 in the yeast prevacuolar compartment to achieve NaCl accumulation [18,20]. Accordingly, we show here that capacity to compartmentalize monovalent cations was equally impeded by mutations in either the ScNHX1 exchanger or the VMA3 subunit of the V-ATPase. Expression of AtNHX1 restored the capacity for ion accumulation and a low rate of ion efflux in *nhx1* cells, but not in a *vma3* mutant. Assuming that the rate of ion extrusion across the plasma membrane is proportional to the cytosolic ion content [11,24], from the data presented in Fig. 2 it can be estimated that the cytosolic Li^+ pool in AtNHX1-expressing cells was only 14.9 nmol Li^+ mg^{-1} at the shortest time measured, as opposed to an average of 36.7 nmol Li^+ mg^{-1} in *nhx1* and *vma3* mutants. Thus, AtNHX1 activity afforded compartmentation of ∼80% (ca. 64.5 nmol Li^+ mg^{-1}) of total intracellular Li^+. Apse et al. [5] have shown that AtNHX1 localizes to plant vacuoles and mediates Na^+ uptake into tonoplast vesicles. We show here that AtNHX1 localizes to vacuolar membrane fractions of yeast cells as well (Fig. 3) to implement ion compartmentation. Thus, AtNHX1 and ScNHX1 function have been evolutionarily preserved.

Vacuolar compartmentation of ions serves two functions. Confinement of toxic Na^+ in the vacuole provides a mechanism for the maintenance of sublethal ion levels in the cytosol and lends 'cheap' osmoticum for osmotic adjustment, cell turgor and expansion. The low water potential generated by salt accumulation within cell vacuoles helps to maintain water uptake. This is particularly true for higher plant cells where the vacuole constitutes ca. 95% of the volume of fully expanded cells. Thus, it is possible that capacity for Na^+ compartmentation is essential not only for survival to salt stress but is a basic component of cell growth also (reviewed in [2,3]). Accordingly, *AtNHX1* transcript was found both in leaf and root tissue. Moreover, the steady-state levels of *AtNHX1* mRNA in leaf increased rapidly (within 1 h) in response to the stress-related hormone ABA. Supplementation with NaCl elicited a similar but slower response, reaching a maximum at 24 h after the onset of the stress. Under our experimental conditions, the upregulation of *AtNHX1* transcript levels was restricted to leaves (Fig. 4). In glycophytes growing in saline conditions, Cl^- and Na^+ ions accumulate preferentially in fully expanded leaves, where they are compartmentalized into vacuoles (reviewed in [2]). Uptake of ions for osmotic adjustment must reach the leaves at a rate that does not exceed the capacity of leaf cells to accumulate them in the vacuole. Thus, salt exclusion in the root and salt sequestration in the leaf cell vacuoles are presumed to be critical co-ordinated determinants for salt tolerance. The upregulation of *AtNHX1* in leaves of *Arabidopsis* plants treated with ABA or NaCl is consistent with this model.

The identification of AtNHX1 as a bona fide plant Na^+/H^+ antiporter will enable molecular approaches to studying Na^+ homeostasis, pH regulation and salinity tolerance of plants.

Moreover, the feasibility of functional expression in a convenient heterologous system will also permit detailed biochemical studies of these important transporters.

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