Arabidopsis thaliana and *Saccharomyces cerevisiae* *NHX1* genes encode amiloride sensitive electroneutral Na^+/H^+ exchangers

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Sodium at high millimolar levels in the cytoplasm is toxic to plant and yeast cells. Sequestration of Na^+ ions into the vacuole is one mechanism to confer Na^+ -tolerance on these organisms. In the present study we provide direct evidence that the *Arabidopsis thaliana* *At-NHX1* gene and the yeast *NHX1* gene encode low-affinity electroneutral Na^+/H^+ exchangers. We took advantage of the ability of heterologously expressed *At-NHX1* to functionally complement the yeast *nhx1*-null mutant. Experiments on vacuolar vesicles isolated from yeast expressing *At-NHX1* or *NHX1* provided direct evidence for pH-gradient-energized Na^+ accumulation into the vacuole. A major difference between *NHX1* and *At-NHX1* is the presence of a cleavable N-terminal signal peptide (SP) in the former gene. Fusion of the SP to *At-*

NHX1 resulted in an increase in the magnitude of Na^+/H^+ exchange, indicating a role for the SP in protein targeting or regulation. Another distinguishing feature between the plant and yeast antiporters is their sensitivity to the diuretic compound amiloride. Whereas *At-NHX1* was completely inhibited by amiloride, *NHX1* activity was reduced by only 20–40%. These results show that yeast as a heterologous expression system provides a convenient model to analyse structural and regulatory features of plant Na^+/H^+ antiporters.

Key words: amiloride, antiport, salt tolerance, sodium proton exchanger, yeast.

INTRODUCTION

In both yeast and plant cells NaCl stress is a complex phenomenon involving both ionic toxicity and osmotic stress [1,2]. Yeast cells exhibit a network of integrated defence mechanisms that enable this unicellular organism to survive molar fluctuations in extracellular sodium. Higher plants also exhibit wide-ranging defence mechanisms at the whole plant and intracellular levels in order to withstand constant or transient fluctuations in NaCl. Whereas, specialized modifications, such as salt glands, are reserved for the true halophyte, more subtle intracellular mechanisms can permit the survival of most land plants under moderate salt-stress. Interestingly, it appears that at least some of the intracellular mechanisms are similar to those found in yeast. These similarities are exemplified by cognate modifications of ion transport processes across the plasma and vacuolar membranes in response to salt stress.

In *Saccharomyces cerevisiae*, exposure to excess NaCl induces the expression of the *ENA1/PMR2A* gene, encoding a plasma membrane Na^+ -ATPase, which pumps excess intracellular Na^+ ions out of the cell [3]. Although the Na^+ -ATPase represents the primary pathway for Na^+ exclusion in *S. cerevisiae*, Na^+/H^+ antiporters at the plasma membrane, encoded by the *NHA1* gene, also serve to reduce intracellular Na^+ [4]. The recent isolation of a novel antiporter gene, *NHX1* [5], points to a role for intracellular localized Na^+/H^+ antiporters mediating NaCl tolerance through sequestration within prevacuolar compartments. In addition to these efflux and sequestration mechanisms adaptation to salt-stress in *S. cerevisiae* is marked by reductions

in Na^+ influx through modulation of the K^+ uptake systems *TRK1* and *TRK2*, which shift from low-affinity K^+/Na^+ selectivity to high-affinity K^+ selectivity [6].

In higher plant species, physiological and biochemical data suggest that Na^+/H^+ antiport and/or alterations in channel selectivity play a more prominent role in adaptation to elevated cytosolic Na^+ than primary ion pump activity. Moreover, the dominance of a large central vacuole within all mature plant cells and its capacity to sequester toxic cations, bestows on this organelle a prominent role in salinity adaptations. A priori, compartmentation of Na^+ into the vacuolar lumen against its concentration gradient must be driven, either directly or indirectly, by energy-dependent mechanisms. Uphill transport of Na^+ into the vacuolar lumen is mediated by the operation of Na^+/H^+ antiport, energized by the co-ordinate action of the vacuolar ATPase (V-ATPase) and the vacuolar-pyrophosphatase [7–9]. Direct measurements of Na^+ -transport across isolated vacuolar membranes from halophytic and glycophytic plant species has shown an induction of Na^+/H^+ exchange activity upon salt stress. Intriguingly, these reported increases in antiporter activity may result from modifications of existing transporters in addition to increases in gene expression [7,10]. However, without the identification of the specific genes encoding vacuolar antiport proteins, more detailed characterization of their expression and function has not been possible.

Recently, genes encoding putative Na^+/H^+ antiporters from *Arabidopsis thaliana* (*At-NHX1*) and rice (*Oryza sativa*; *Os-NHX1*) have been reported [11–14]. These genes share sequence similarity with the *S. cerevisiae* antiporter *NHX1* but not with the

Abbreviations used: ACMA, 9-amino-6-chloro-2-methoxy-acridine; BTP, Bis-Tris propane; ΔpH , transmembrane pH gradient; DTT, dithiothreitol; EST, expressed sequence tag; NHE, Na^+/H^+ exchanger; ORF, open reading frame; SP, signal peptide.

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plasma membrane *NHA1*. *At-NHX1* can functionally complement some of the salt-sensitive phenotypes of *NHX1* null mutants ($\Delta nhx1$) under certain conditions [13] and overexpression in *Arabidopsis* promotes sustained growth at elevated NaCl [15].

The present study describes the functional expression of *At-NHX1* in *S. cerevisiae* and the Na^+/H^+ exchange characteristics of both *At-NHX1* and its yeast homologue *NHX1*. We provide direct evidence that both antiporters facilitate electroneutral Na^+/H^+ exchange and explore the function of specific motifs in subsequent Na^+/H^+ antiport regulation and localization.

MATERIALS AND METHODS

Materials

All chemicals were obtained from Sigma, unless otherwise indicated. *S. cerevisiae* strains R100 ($\Delta nhx1$) and K601 (wild type), and the plasmid pRIN73 were gifts from R. Rao (The Johns Hopkins University School of Medicine, Baltimore, MD, U.S.A.).

Plasmid vectors and cloning

The full length *At-NHX1* cDNA was isolated from two size-selected λ ZAP cDNA expression libraries of *A. thaliana* (CD-14, CD-15; [16]), obtained from the *Arabidopsis* Biological Resource Centre, University of Nottingham, Nottingham, U.K.). The libraries were probed with a ^{32}P -labelled *Arabidopsis* expressed sequence tag (EST) clone (T75860) following the protocol outlined in the Amersham N^+ Bond manual. Hybridizing plaques were checked by PCR using M13 forward and reverse primers revealing inserts of 2.1 kb. pBluescript KS⁻ plasmid was then excised from a single plaque, amplified and sequenced using exonuclease III to create a series of nested deletions. Sequence reactions were performed with the dideoxy chain termination method using the Ampli-Taq dye primer system (Applied Biosystems) and were analysed on an automated sequence analyser. Sequence data confirmed the insert as *At-NHX1*.

The construct pRIN73 was used to generate *pRS-AtNHX1/F*, *pRS-AtNHX1/T* and *pRS-AtNHX/SP*, with *At-NHX1* expression under the control of the yeast *NHX* promoter [5]. *NHX1* proximal promoter sequence was first obtained by PCR amplification using the primers 5'-CCCTCGAGGAATCTGGGTAT-TCAGCG-3' and 5'-CCCTGCAGATGGTATGTACTGACAGCG-3', and pRIN73 as the template. The 750-bp PCR product was then digested with *XhoI* and *PstI* and ligated into the corresponding sites in pRS305 [17] to generate *pRS-NHX1-promo*.

At-NHX1 was subcloned from pBluescript into *SmaI/XbaI* sites downstream of the *NHX1* promoter to generate the construct *pRS-AtNHX1/F*. *pRS-AtNHX1/T* was generated following transformation of JM110 cells (Stratagene) with the full-length construct. This allowed *XbaI* digestion of an adenine-methylated internal *XbaI* site directly upstream of the ATG start codon, releasing a fragment corresponding to the open reading frame (ORF) of *At-NHX1*. The chimaeric construct *pRS-AtNHX1/SP* was constructed by fusing the N-terminus of *NHX1* to the ORF of *At-NHX1*. *NHX1* sequence coding for the putative signal peptide (SP) was amplified by PCR using an *NHX1* genomic clone as a template and the primers 5'-CGCGGGTACCATTGCTATCCAAGGTATTGCTG-3' and 5'-CGCGGGATCCTGTAACAGGGTTTAAGTCTAC-3'. At the 5'-ends, *KpnI* and *BamHI* sites were added (underlined). The translation start codon is indicated in bold. The resulting PCR fragment was subcloned into the PCRII plasmid by T/A cloning (Invitrogen). Independently, another set of primers (5'-CGCGGGATCCTCT-

GTGGTTGCGTTGAATCTC-3' and 5'-CTGTAAAGCAAA-GCAAAGGTGTCTCG-3') were used to amplify the sequence coding for the N-terminus of *At-NHX1*. The use of these primers in a PCR reaction using the *At-NHX1/T* template allowed the amplification of a PCR fragment harbouring a *BamHI* site at its 5'-end (underlined). This second fragment was also cloned into the PCRII plasmid, cut by *BamHI* and *PstI* and subcloned in frame with the *NHX1* N-terminal sequence obtained previously. This fusion clone was then cut by *KpnI* and *BglII* and introduced into *pRS-At-NHX1/T*, replacing the sequence coding for the *At-NHX1* N-terminus.

Each new pRS construct was sequenced, and expression in yeast (see below) was verified by Northern-blot analysis using the labelled EST sequence as a probe. In complementation studies the empty plasmid pRS305 was also expressed in R100 and K601.

Yeast strains and growth conditions

The strains K601 (wild-type) and R100 ($\Delta nhx1::URA3$) used in these studies are isogenic to W303 (*ura3-1 can1-100 leu2-3 112trp1-1 his3-11, 15*). Generation of R100 is described by Nass et al. [5]. DNA transformations of K601 and R100 strains were performed as described by Klebe et al. [18]. Transformed, mutant and wild-type cells were grown to a D_{600} of 1.0–1.2 in SD medium [0.67% Yeast Nitrogen base (Difco, Sparks, MD, U.S.A.)/2% (w/v) glucose] with and without amino acid supplements as appropriate. The cells were harvested by centrifugation, washed with double-distilled water and then resuspended to a D_{600} of 0.8 (starter culture) in APG {10 mM arginine, 8 mM phosphoric acid, 2% (w/v) glucose, 2 mM MgSO_4 , 1 mM KCl, 0.2 mM CaCl_2 , trace vitamins and minerals, pH 4.0 with acetic acid; [5]}. For growth in liquid media, 500 μl of these cells were then used to inoculate flasks containing 0, 0.1, 0.2, 0.4, 0.6 and 0.8 M NaCl in 50 ml of APG or APGal, where glucose was replaced by galactose. After establishing the starting absorbance, the D_{600} of each culture was determined every 2 h post-inoculation for the first 10 h then every 4 h thereafter. For drop-tests 3 μl of serial (10^{-1}) dilutions of the starter culture were plated on to APG 1% agar with NaCl and supplements as described previously. For growth on hygromycin B drop-tests were also performed on YPD or YPGal [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose or galactose and 1% agar; Difco] with additions of 0.005, 0.01 or 0.05 mg/ml hygromycin B.

Vacuolar membrane isolation

Vesiculated purified yeast vacuoles were isolated following a modification of the method described by Roberts et al. [19]. Cell cultures were grown overnight to a D_{600} of approx. 0.8, after diluting 2 ml of stationary phase cells into 2 litres of APG plus appropriate supplements. The cells were collected by centrifugation, washed twice in double-distilled water, and resuspended in 100 ml of preincubation mix [100 mM Tris/HCl (pH 9.4) and 10 mM dithiothreitol (DTT)]. Following incubation with gentle shaking for 20 min at 30 °C, the cells were pelleted by centrifugation, resuspended in 50 ml of spheroplast mix [1% (w/v) yeast extract, 2% (w/v) peptone, 0.7 M sorbitol, 1% (w/v) glucose or 1% galactose, 100 mM Tris/HCl (pH 7.5) and 15 mg of Zymolase 100 T (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.)] and incubated at 30 °C with gentle shaking for 1 h. The resultant spheroplasts were then centrifuged through a cushion of 1.4 M sorbitol and resuspended in 25 ml of ice-cold lysis buffer A [10 mM Mes/Tris (pH 6.9), 0.1 mM MgCl_2 , 12% (w/v) Ficoll 800 (Pharmacia), 1 mM PMSF and 1 $\mu\text{g/ml}$ leupeptin].

The spheroplasts were then gently homogenized with a loose-fitting glass Potter–Elvehjem homogenizer and left on ice for 5 min to enable complete lysis. The crude lysate was centrifuged for 10 min at 1000 *g* using a Sigma 4K15 centrifuge pre-chilled to 4 °C. The resulting supernatant was divided equally into six SW41 (Beckman) centrifuge tubes overlaid with 4 ml of buffer A and centrifuged at 52000 *g* for 30 min at 4 °C. After centrifugation the white layer on top of the tubes was removed, resuspended in 24 ml of buffer A, aliquoted into six SW41 tubes and buffer B [10 mM Mes/Tris (pH 6.9), 0.5 mM MgCl₂ and 8 % (w/v) Ficoll 800] was layered on to the crude vacuole/buffer A mixture. The crude vacuole mixture was then subjected to a second round of centrifugation at 52000 *g* for 30 min. Subsequently, the white layer was removed and resuspended in 500 µl of buffer C [10 mM Mes/Tris (pH 6.9), 5 mM MgCl₂ and 25 mM KCl]. The protein concentration of the vacuolar vesicles was determined using an assay kit from Bio-Rad Laboratories using bovine γ-globulin as a standard. Membrane preparations were resuspended in buffer C to a final concentration of 1 mg/ml protein, snap frozen, and stored at –80 °C.

ATPase hydrolytic activities

ATPase hydrolytic activities were measured as the liberation of free P_i from ATP by the method of Ames [20]. Membrane protein (10–15 µg) was made up to a final volume of 100 µl with water, added to 200 µl aliquots of reaction mixture [3 mM Bis-Tris propane (BTP)-ATP, 3 mM MgSO₄, 50 mM KCl, 5 µM gramicidin D and 1 mM sodium molybdate, buffered to pH 8.0 with 30 mM BTP/Mes] and incubated for 20 min at 37 °C. The reaction was stopped by addition of 0.9 ml of Ames reagent [6 parts 0.4 % (w/v) ammonium molybdate in 0.5 M H₂SO₄ to 1 part 10 % (w/v) ascorbic acid] and allowed to stand for 30 min at room temperature. *D*₈₂₀ was measured in 1-ml cuvettes against a reagent blank. Reaction mixtures included inhibitors of specific ATPases: mitochondrial F-type ATPase (3 mM sodium azide), plasma membrane P-type-ATPase (0.25 mM sodium vanadate) or V-ATPase (100 nM bafilomycin A₁ or 50 mM KNO₃). Routinely, V-ATPase activity was calculated as the bafilomycin-sensitive activity for hydrolytic activity and the azide/vanadate insensitive portion of total ATPase activity for H⁺ pumping (see below).

Fluorescence measurements

Fluorescence quenching of 9-amino-6-chloro-2-methoxy-acridine (ACMA) [21] and Oxonol V [23] was used to monitor the formation and dissipation of pH and membrane potential essentially as described by Camarasa et al. [23]. The fluorescence intensities of these probes were recorded with an Aminco Bowman Series 2 Luminescence spectrophotometer. In experiments using ACMA, 50 µg of vacuolar membrane vesicles were added to a cuvette containing 2 ml of reaction buffer [10 mM BTP/Mes (pH 7.0), 25 mM KCl, 2 mM MgSO₄, 10 % (v/v) glycerol and 2 µM ACMA). Each vacuolar preparation was routinely assayed with and without azide and vanadate to assess any contaminating quench from mitochondrial or plasma membrane vesicles. The reaction was started by the addition of 1 mM BTP-ATP (pH 7.5). The pH response of ACMA was verified after the addition of nigericin (1 µM) or valinomycin (1 µM) to the reaction mixture. Additions of Na⁺ (as Na₂SO₄) were as indicated, and 10 mM (NH₄)₂SO₄ was added to equilibrate H⁺ across the membrane. Changes in membrane potential were monitored in 2 ml of reaction buffer with additions of Na₂SO₄ and Gramicidin D (5 µM) as indicated. The fluorescence buffer was stirred and maintained at 30 °C throughout the transport

assays. Emission signals were recorded at 480 nm (ACMA) and 645 nm (Oxonol V) after excitation at 412 nm (ACMA) and 585 nm (Oxonol V). After the transmembrane pH gradient (ΔpH) had reached steady-state, the rate of passive H⁺ leakage and ATP-independent Na⁺/H⁺ exchange was assessed following the addition of 3 mM Tris/EDTA to chelate all Mg²⁺. ATP-independence of Na⁺/H⁺ exchange was also assessed through the addition of 5 mM glucose plus 10 units/ml hexokinase in order to deplete ATP.

Na⁺ uptake measurements

Vacuolar membrane vesicles (100 µg) were incubated in 2 ml of buffer containing 5 mM BTP/Mes (pH 8.0), 1 mM BTP-ATP, 50 mM KCl, 0.1 mM vanadate, 0.2 mM azide and 10 % (w/v) glycerol, at 25 °C as described by Chardonens et al. [22]. V-ATPase activity was initiated by the addition of 1 mM MgSO₄ and vesicles were incubated to reach steady-state ΔpH. Na⁺ uptake was initiated by the addition of Na₂SO₄ to a final concentration of 20 mM. At the indicated times, 100 µl of the reaction mix was removed and the membranes were collected on 0.22 µm cellulose filters (Millipore). Filters were washed three times in ice-cold wash buffer [5 mM BTP/Mes and 10 % (v/v) glycerol], placed in glass vials and macerated in 2 ml of 0.1 % trifluoroacetic acid. Aliquots of this suspension were taken for Na⁺ analysis using flame emission spectrophotometry (Perkin Elmer 1100B Spectrophotometer). Vesicle membranes were permeabilized with Triton X-100 (final concentration 0.05 %) to show that the Na⁺ measurements were not due to non-specific binding of Na⁺ to the membranes.

RESULTS

Molecular characterization of At-NHX1

The full-length cDNA of *At-NHX1* was cloned as described in the Materials and methods section. Sequencing the 2152-bp cDNA revealed an ORF of 1614 bp with a 5'-untranslated region of 384 bp and a termination codon (TGA) at position 1116. The presence of an in-frame termination codon N-terminal to the translational initiation codon, indicated that the isolated cDNA contained the complete coding region for the respective protein. Figure 1 shows the structural alignment of the transcribed sequence of *At-NHX1* to the genomic sequence, mapped to chromosome V (*Arabidopsis* Genome Sequencing Project; BAC clone TM021B04). The amino acid sequence showed significant similarity to yeast vacuolar *NHX1* and to the mammalian *NHE* family, but little similarity to the plasma membrane *NHA1*. Of particular interest was the conservation of the motif ⁸¹LFFIYL-LPPI⁹⁰ identified as the binding site of the diuretic drug amiloride in mammalian *NHE* [24]. PSORT (prediction of protein sorting signals and localization sites in amino acid sequences) analysis

Figure 1 Structural alignment of transcribed sequence of *At-NHX1*

At-NHX1 is aligned to the genomic sequence mapped to chromosome V (*Arabidopsis* Genome Sequencing Project, BAC clone TM021B04). Black boxes indicate exon sequences and the line indicates intron sequences.

Figure 2 Complementation of R100 salt-sensitive phenotype by *At-NHX1* constructs

(A) Constructs used for complementation: full-length *At-NHX1* (*At-NHX1/F*); truncated *At-NHX1* (*At-NHX1/T*); N-terminus *NHX1/At-NHX1* (*At-NHX1/SP*). All constructs were expressed in the indicated yeast strain under the control of *NHX1* (promoter pRIN73). Growth of each strain was measured in APG (pH 4.0) at the indicated NaCl concentration. pRS305 is the empty plasmid control. The D_{600} of each strain 72 h post-inoculation is shown. The data are presented as means $\pm$ S.E.M. ($n = 3$).

[25] of *At-NHX1* did not suggest a membrane-specific location for the predicted protein. In contrast, *NHX1* appeared to encode a protein with a putative N-terminal targeting sequence.

Salt sensitivity of $\Delta nhx1$ (R100)

Consistent with data published elsewhere [5], we found that disruption of *NHX1* only resulted in a significant decrease in Na^+ tolerance when cells were grown on APG at acid pH (pH 4.0) and low K^+ concentration (1 mM). The lack of R100 Na^+ -sensitive phenotype under other conditions presumably reflects the ability of the plasma membrane Na^+ efflux transporters (*PMR2/ENA1* and *NHA1*) to compensate for any shortfall in intracellular compartmentation. Transformation of R100 with *NHX1* (back-complementation) under the control of its own full-length promoter (i.e. in pRIN73) or 650-bp promoter fragment recovers wild-type phenotype and expression in wild-type (K601 strain) but does not ameliorate salt-tolerance (results not shown).

Complementation of R100 with *At-NHX1*

For functional studies, we expressed three *At-NHX1* constructs (Figure 2A) in the R100 strain: (1) the full-length cDNA isolated from the phage library, *At-NHX1/F*; (2) a truncated construct containing the predicted ORF of *At-NHX1*, *At-NHX1/T*; (3) a

Figure 3 Suppression of the hygromycin-sensitive phenotype of R100 ($\Delta nhx1$) by *At-NHX1* constructs

Growth of wild-type, mutant and recombinant yeast strains in YPGal plus different concentrations of hygromycin: 0 (A), 5 (B) and (C) 50 $\mu\text{g/ml}$. Yeast strains: 1, K601; 2, $\Delta nhx1$; 3, $\Delta nhx1$ + empty plasmid; 4, $\Delta nhx1$ + *At-NHX1/T*; 5, $\Delta nhx1$ + *At-NHX1/SP*. The strains were plated as 3 μl , 10-fold dilutions with starting D_{600} of 0.8 and grown for 48 h at 30 $^{\circ}\text{C}$.

hybrid construct fusing the N-terminal sequence of *NHX1* to the ORF of *At-NHX1*, *At-NHX1/SP*.

Figure 2(B) demonstrates the ability of all three *At-NHX1* constructs to complement the salt-sensitive phenotype of R100 grown in synthetic minimal medium (APG). Expression of *At-NHX1* in K601 did not significantly increase salt tolerance. All yeast strains grew slowly in Na^+ -free APG, reaching stationary phase (D_{600} approx. 1.2) 72 h post-inoculation. Addition of NaCl resulted in a decrease in the growth rate of K601 and R100, with typical D_{600} values after 48 h in 0.2 M NaCl of 60 and 20% respectively. Addition of 0.6 M NaCl effectively arrested the growth of R100, while *At-NHX1*-transformed R100 retained wild-type growth rates. Drop-tests on solid APG showed that these increases in growth rate reflected a genuine recovery of wild-type phenotype and did not result from changes in cell morphology or size. It was not possible to resolve any statistically significant difference between the efficacy of complementation by the different *At-NHX1* constructs or between *At-NHX1* and *NHX1*.

In order to ascertain any differences between the constructs we took advantage of the acute sensitivity of R100 strains to hygromycin [13]. Hygromycin is a large, toxic alkali cation that accumulates intracellularly in response to the electrochemical proton gradient [26]. Figure 3 illustrates the hygromycin sensitivity of R100; clearly, net hygromycin uptake into the cell was increased and/or sequestration into the vacuole was disturbed in the absence of functional *NHX1*. Both *At-NHX1* constructs were capable of suppressing the hygromycin phenotype at low (5 $\mu\text{g/ml}$) hygromycin concentrations. However, at 50 $\mu\text{g/ml}$ hygromycin, only the *At-NHX1/SP* construct was able to maintain growth (Figure 3C). These results suggest that the N-terminal SP plays a role in targeting the protein to its target membrane or in regulation of the protein. There were no apparent differences between *At-NHX1/F* and *At-NHX1/T* (results not shown).

Figure 4 Sodium driven release of fluorescence quench in vacuolar vesicles isolated from yeast expressing *At-NHX1* constructs or *NHX1*

(A) ACMA fluorescence quench resulting from the activation of the vacuolar ATPase following addition of 1 mM BTP-ATP in membranes derived from K601 (1). Quench is reduced following the inclusion of 1 μ M nigericin in the reaction buffer and increased with the addition of 1 μ M valinomycin. (B) Effect of Na⁺ on the dissipation rate of the H⁺ gradient. 20 mM Na₂SO₄ was added at (2) and resulted in reversal of quench, indicative of Na⁺/H⁺ exchange in K601, R100-*At-NHX1/SP* and R100-*At-NHX1/T*, but not in R100. Addition of 10 mM (NH₄)₂SO₄ at (3) equilibrated H⁺ across the membrane, resulting in relaxation of quench. (C) Addition of 3 mM Tris/EDTA at (2) did not effect subsequent Na⁺/H⁺ exchange following addition of 20 mM Na⁺ at (3). 1 mM BTP-ATP and 10 mM (NH₄)₂SO₄ were added at (1) and (4) respectively.

Although these data clearly show that *At-NHX1* can functionally complement the $\Delta nhx1$ phenotype, they do not address the mechanisms by which *At-NHX1* (or *NHX1*) increase Na⁺ tolerance. Therefore we adopted a fluorimetric approach to measure the capacity for Δ pH-dependent Na⁺ uptake by vacuolar vesicles isolated from R100, K601 and *At-NHX1*-transformed strains.

Characterization of vacuolar membranes

Transport-competent vacuolar vesicles of high and consistent purity were purified from R100, K601 and *At-NHX1*-transformed strains. Marker enzyme assays using inhibitors of F-Type, P-type and V-type H⁺-ATPases were carried out to assess the purity of the vacuolar membrane preparation. In all preparations used in the present study the majority of the activity (> 80 %) was inhibited by bafilomycin A₁, a specific inhibitor of the V-ATPase (results not shown). Addition of 0.01 % Triton X-100 to the vacuolar preparation slightly increased the ATPase activity, suggesting a small population of inside-out vesicles.

Generation of Δ pH and dissipation by Na⁺

A fluorescence quench assay, using ACMA, was used to monitor the dissipation of Δ pH (inside acid) across the vacuolar mem-

Table 1 Maximum H⁺ quench and Na⁺-dependent dissipation from K601 and recombinant strains (expressed as maximum change in fluorescence per mg of membrane protein)

Measurements are representative of the mean $\pm$ S.E.M. of four (K601 and R100-*At-NHX1/SP*) or three (R100-*NHX1*, R100-*At-NHX1/T* and R100/*pRS-305*/empty plasmid) separate membrane preparations. Measurements of ATP-driven H⁺-pumping and Na⁺/H⁺ exchange in K601 and *At-NHX1/SP* following growth for 12 h in 0.2 M NaCl is indicated as +NaCl. N.D., not determined.

	ATP-dependent H ⁺ quench (% maximum quench/mg of protein)		Na ⁺ -dependent H ⁺ dissipation (% maximum recovery/mg of protein)	
	– NaCl	+ NaCl	– NaCl	+ NaCl
K601(wild-type)	41.3 $\pm$ 1.87	52.3 $\pm$ 2.11	20.3 $\pm$ 2.87	23.3 $\pm$ 1.58
R100/ <i>pRS-At NHX1/T</i>	45.7 $\pm$ 1.35	N. D.	26.5 $\pm$ 0.64	N. D.
R100/ <i>pRS-AtNHX1/SP</i>	43.2 $\pm$ 1.65	48.5 $\pm$ 2.30	27.5 $\pm$ 1.11	31.2 $\pm$ 2.00
R100/ <i>pRS-NHX1</i>	37.7 $\pm$ 2.75	N. D.	22.1 $\pm$ 2.29	N. D.
R100/ <i>pRS-305</i>	36.3 $\pm$ 0.80	41.5 $\pm$ 1.25	no dissipation	no dissipation

branes [21]. Figure 4(A) shows that the addition of ATP to vesicles derived from K601 resulted in a fluorescence quench of approx. 38 %, with maximum quench (Q_{max}) after 40 s. Changes in the rate of ACMA quench in the presence of nigericin (K⁺/H⁺ exchanger, dissipating a build-up of Δ pH) or valinomycin (K⁺ ionophore, stimulating ATP-driven H⁺ pump activity through dissipation of the membrane potential) support the application of this dye as an indicator of Δ pH.

Once steady-state levels of fluorescence quench were observed, 20 mM Na₂SO₄ was added to the reaction buffer. This resulted in the recovery of quench, indicative of Na⁺-dependent H⁺ efflux (Figure 4B). Inactivation of the H⁺ pump, either by chelating Mg²⁺ with Tris/EDTA (Figure 4C) or by depleting ATP through the addition of hexokinase/glucose (results not shown), still permitted Na⁺-dependent H⁺ efflux. These data strongly support the activity of an ATP-independent Na⁺/H⁺ exchange mechanism, i.e. antiport. Inhibitor studies showed that the H⁺ pump activity was due to the V-ATPase, as inclusion of nanomolar concentrations of bafilomycin A₁ arrested subsequent ATP-driven quench. Conversely, addition of azide or vanadate did not reduce the ATP-driven quench. All transport data were consistent with the isolation of tightly-sealed, right-side-out vacuolar membrane vesicles. Figure 4(B) illustrates typical rates and extents of V-ATPase driven quench and Na⁺-dependent H⁺ exchange in vesicles derived from wild-type, mutant and *At-NHX1* recombinant strains. Strikingly, there was no recovery of quench upon addition of Na⁺ to vesicles derived from R100. In contrast, all recombinant strains and R100 back-complemented with *NHX1* showed Na⁺-dependent H⁺ efflux, thus providing direct evidence that both genes encode vacuolar-localized Na⁺/H⁺ exchangers.

The extractable Na⁺-dependent H⁺ efflux was consistently greater in vesicles derived from *At-NHX1/SP*-expressing strains than from K601 (Student's *t* test value = 2.913; *P* < 0.1; 3 degrees of freedom) (Table 1). Analysis of the kinetics of Na⁺/H⁺ exchange in K601 and *At-NHX1/SP* demonstrated both a higher affinity for Na⁺ (apparent K_m of 16 and 11 mM respectively) and a 1.3-fold higher V_{max} for the plant antiporter (Figure 5). These data not only show that the *At-NHX1* constructs express functional Na⁺/H⁺ antiporters in *S. cerevisiae* but also suggest that the plant antiporter can catalyse Na⁺/H⁺ exchange equal to, and even above, the yeast antiporter. Fusion of the N-terminal sequence from *NHX1* to *At-NHX1* appeared to increase the

Figure 5 H^+ efflux (coupled to Na^+ influx) as a measure of substrate (Na^+) concentration in wild-type (K601) and *At-NHX1/SP*

Kinetics of initial rates of Na^+ -dependent H^+ fluxes (inset: double reciprocal plot). K_m values of 11 mM (*At-NHX1/SP*) and 16 mM (K601) were determined. The data points represent the average of three separate measurements. ■, K601 and □, *At-NHX1/SP*.

Figure 6 Na^+ uptake into vacuolar vesicles depends on the presence of *At-NHX1*

Na^+ uptake by vacuolar vesicles was measured by means of flame emission spectrophotometry. Vesicles were incubated in 20 mM Na_2SO_4 . Triton X-100 (0.05%) was added to permeabilize the membranes.

magnitude of Na^+/H^+ exchange, perhaps because of improved targeting of the expressed protein or of an as yet undefined regulatory role for the *NHX1* N-terminal domain.

The use of the endogenous *NHX1* promoter to drive expression of *At-NHX1* constructs allowed us to address the question of whether NaCl in the growth medium induced H^+ -dependent Na^+ exchange. Growth of K601 or *At-NHX1/SP*-transformed yeast in 0.2 M NaCl increased the magnitude and rate of ATP-driven H^+ quench and enhanced Na^+ -dependent H^+ exchange (Table 1). Northern-blot analysis of recombinant yeast grown in 0.2 M NaCl did not reveal any significant increase in *At-NHX1* transcripts (results not shown). Thus it would appear that increases in apparent ΔpH generated by the V-ATPase result in the observed increases in Na^+ -dependent H^+ exchange following salt treatments, as demonstrated in other systems [27,28].

Figure 7 Na^+/H^+ exchangers encoded by *At-NHX1* are electroneutral and amiloride sensitive

(A) Oxonol V fluorescence as a measure of electrical potential difference during Na^+/H^+ exchange across the vacuolar membrane isolated from *At-NHX1/T*. Addition of 1 mM ATP (1), 20 mM Na_2SO_4 (2) and 5 μM gramicidin D (3) are indicated. (B) ACMA fluorescence after preincubation of membrane vesicles from K601 (= wild type carrying the *NHX1* gene) and *At-NHX1/T* with 120 μM amiloride. Addition of 1 mM ATP (1) was followed by additions of 20 mM Na_2SO_4 (2) and 10 mM $(\text{NH}_4)_2\text{SO}_4$ (3).

In order to demonstrate that Na^+ ions were indeed taken up by the vesicles used in the fluorescent quench experiments, Na^+ uptake in the vesicles was measured by means of flame emission spectrophotometry (Figure 6). Consistent with fluorimetric data, vesicles isolated from R100 did not accumulate Na^+ , whereas wild-type and *At-NHX1* strains readily took up Na^+ and reached equilibrium within 4 min. Addition of the detergent Triton X-100 released accumulated Na^+ , thus showing that Na^+ uptake was not due to non-specific binding.

ΔpH energized Na^+ exchange is electroneutral and amiloride-sensitive

The generation of an inside-positive membrane potential in response to ATP-driven H^+ transport, was monitored by following the quench of the anionic fluorescent probe Oxonol V (Figure 7A). Addition of Na^+ to vesicles derived from *R100-At-NHX1/T* neither dissipated nor increased the Oxonol V quench, indicating the lack of net movement of charge across the membrane, suggesting electroneutral exchange of Na^+ for H^+ across the membrane. These findings are consistent with data derived from mammalian Na^+/H^+ exchangers (NHEs) [29] and those indicated by Na^+/H^+ exchange measured across the vacuolar membrane of other plant species [7,8].

A distinguishing feature of mammalian NHEs is their inhibition by micromolar concentrations of amiloride [24]. The identification of a putative binding site for amiloride in the predicted amino acid sequence of both *NHX1* and *At-NHX1* suggests that the proteins they encode may also be inhibited by this drug. In complementation studies, addition of amiloride to the yeast culture did not inhibit the ability of either *NHX1* or *At-*

NHX to recover the NaCl-tolerant phenotype. However, pre-incubation of membrane preparations with 120 μ M amiloride prior to the transport assay revealed a complete inhibition of *At-NHX1/T*-mediated Na⁺/H⁺ exchange and 20–40 % inhibition of that by *NHX1* (Figure 7B). These differential sensitivities of *At-NHX1* and *NHX1* may reflect the higher degree of amiloride binding site sequence conservation in the plant antiporter (see the Discussion section).

DISCUSSION

Sequestration of sodium into the vacuole is known to play an important role in plant tolerance to salinity [1]. A wealth of biochemical and physiological data points to Na⁺/H⁺ antiport as a general mechanism for Na⁺ uptake in the vacuole [7–9,30]. Despite these data the molecular and genetic identity of specific sodium transporters has until recently remained elusive. The present paper describes the cloning and transport characteristics of a vacuolar antiporter from *Arabidopsis*, *At-NHX1*, and makes comparisons in structure and function with its counterpart in yeast, *NHX1*.

Complementation studies

The full-length *At-NHX1* cDNA isolated from library screening revealed a 384-bp 5'-leader (transcribed-untranslated) sequence upstream of the first ATG. This 5'-sequence showed similarity to the 5'-untranslated region of 296 bp upstream of the first ATG in the rice homologue *Os-NHX1* [14]. Wakabayashi et al. [33] indicated that in mammalian Na⁺/H⁺ antiporters the 5'-untranslated region of the *NHE1* message inhibits translation to its corresponding protein. To assess the significance of the 5'-untranslated sequence upstream of the first ATG in full-length *At-NHX1* (*At-NHX1/F*) we produced a truncated construct, *At-NHX/T*, in which only the sequence corresponding to the predicted ORF of *At-NHX1* was expressed. Mammalian NHEs also possess an N-terminal region, which encodes a cleavable SP [33]. PSORT analysis of *NHX1* also revealed a sequence within the N-terminus, which may be involved in membrane targeting. Similar analysis of *At-NHX1/F* showed no putative N-terminal SP. To explore the significance of the N-terminal sequence in *NHX1* we engineered a chimaeric construct in which the N-terminus of *NHX1* was fused to *At-NHX1/T*, thus giving *At-NHX1/SP*.

All three plant constructs could recover wild-type salt-tolerant phenotype when expressed in R100 (Figure 2B). We could not resolve any significant difference between the ability of *At-NHX1* or *NHX1* constructs to complement R100 phenotype, possibly due to the weak salt-sensitive phenotype of R100 strains. This weak, pH-dependent, phenotype of R100 although not helpful for complementation studies does however give insight into the role of Na⁺-sequestration as a mechanism for salt tolerance in yeast. The dependence on acid pH is probably a reflection of suboptimal levels of the Na⁺-ATPase (*ENA1-4*) activity at low pH [32]. In other words, at low external pH the cells have a greater dependence on alternative Na⁺ efflux (*NHA1*) or Na⁺ sequestration (*NHX1*) mechanisms. Any disruption in either of these transporters then leads to the observed changes in salt sensitivity. We found that *NHX1* alone could not recover salt tolerance in *ENA1-4* and/or *NHA1* null mutants even at low pH and low K⁺ (results not shown). This suggests that *NHX1*, and therefore vacuolar sequestration of Na⁺, represents a relatively minor component of sodium elimination mechanisms in *S. cerevisiae*.

The acute sensitivity of R100 to hygromycin provided a high-resolution screen to assess the relative effectiveness of *NHX1* and

At-NHX1 constructs in suppressing the hygromycin phenotype (Figure 3). Hygromycin is driven into the cell or vacuolar lumen in response to a negative membrane potential or Δ pH [26,34]. Since Na⁺/H⁺ exchange is electroneutral (Figure 7A) and assuming that *At-NHX1* does not directly catalyse the transport of hygromycin, uptake into the vacuole in wild-type cells must be affected by changes in Δ pH across the vacuolar membrane as a result of Na⁺-dependent H⁺ exchange. Thus sensitivity of *NHX1* mutants to hygromycin probably results from impaired sequestration of this toxic cation into the vacuolar lumen. At low hygromycin concentrations (5 μ g/ml) all *At-NHX1* constructs were able to rescue hygromycin tolerance (Figure 3). However, at a 10-fold higher concentration only wild-type and *At-NHX1/SP* were able to sustain growth. These findings suggest that the N-terminal sequence of *NHX1* plays a role in facilitating Na⁺/H⁺ exchange by *At-NHX1*. This could be achieved through targeting the protein to the correct functional location or by direct modification of Na⁺/H⁺ exchange. Preliminary studies using hygromycin concentrations toxic to wild-type cells still permitted limited growth of *At-NHX1/SP*, suggesting that the plant gene encodes a more 'efficient' Na⁺/H⁺ antiport mechanism than the endogenous yeast gene. *At-NHX1/F* and *At-NHX/T* both recovered wild-type growth to the same extent at 5 μ g/ml hygromycin. This suggests that the 5'-untranslated region of the cDNA does not affect the translation and subsequent activity of *At-NHX1*.

Transport studies

Unequivocal evidence that both *NHX1* and *At-NHX1* encode vacuolar-localized Na⁺/H⁺ antiporters was achieved through the measurement of Δ pH-dependent Na⁺/H⁺ exchange and Na⁺ uptake (Figures 4–6). Our studies showed an ATP-independent, Δ pH-dependent Na⁺/H⁺ exchange across the membranes of all strains carrying *At-NHX1* or *NHX1*. Interestingly, expression of the construct *At-NHX/SP* resulted in higher rates and extents of Na⁺/H⁺ exchange than other strains used in the present study, consistent with the hygromycin tolerance of *At-NHX1/SP*-expressing strains (see above). In K601 and *At-NHX1/SP* strains, Na⁺-dependent H⁺ efflux displayed saturation kinetics with respect to extravesicular Na⁺ concentrations (Figure 5). *At-NHX1/SP* displayed a higher $V_{\max}$ and a lower apparent K_m than wild-type yeast. These results tentatively suggest that in *At-NHX1/SP*-expressing cells the turn-over rate and/or the number of transporters is higher and that the encoded antiporter possesses a greater affinity for Na⁺ than *NHX1*. The K_m value of 11 mM is in good agreement with that reported for plant vacuoles [7,28,30].

Na⁺/H⁺ exchange is electroneutral

In the present study two lines of data suggest that Na⁺/H⁺ exchange mediated by *At-NHX1* and *NHX1* is electroneutral. Firstly, similar rates of Na⁺-dependent H⁺ efflux were observed in the presence of valinomycin and K⁺, i.e. when the membrane potential was clamped at 0 mV. Secondly, we observed no charge-dependent change in the Oxonol V signal following the addition of Na⁺ (Figure 7A). These data are consistent with the notion that *At-NHX1* and *NHX1* are structurally and functionally similar to mammalian *NHE* genes, which also catalyse electroneutral Na⁺/H⁺ exchange.

Effect of NaCl on *NHX1* expression and activity

Since we expressed *At-NHX1* behind the endogenous yeast promoter, we were able to monitor Na⁺-induced gene expression in transformed yeast strains. Since Northern-blot analysis of *At-*

Table 2 Conservation of amiloride binding domains in mammalian, plant and yeast Na⁺/H⁺ antiporters

Underlined amino acids are different from NHE1 amino acids.

cDNA	Amiloride binding site	Amiloride sensitivity
<i>NHE1</i>	¹⁶⁴ VFFFLFLLPPI ¹⁷³	IC ₅₀ 3 µM*
<i>NHE1</i> Leu ¹⁶⁷ → Phe	VFFFFLLPPI	IC ₅₀ 15 µM*
<i>NHE1</i> Leu ¹⁶⁷ → Phe	VFFFYLLPPI	IC ₅₀ 15 µM*
Phe ¹⁶⁸ → Tyr		
<i>NHE2</i>	VFFLYLLPPI	IC ₅₀ 3 µM*
<i>At-NHX1</i> †	⁸¹ LFYLLPPI ⁹⁰	100% inhibition by 120 µM amiloride
<i>NHX1</i>	<u>Y</u> FFNVLLPPI	20–40% inhibition by 120 µM amiloride

* Counillon et al. [24].

† Identical sequences are found in *Os-NHX1* [14] and two recently sequenced *At-NHX1* homologues (accession numbers AAF08577 and AAD56988).

NHX1 transcripts showed no significant increase in expression of *At-NHX1* or *NHX1* following growth in 0.2 M NaCl, increases in Na⁺-dependent H⁺ exchange might be due to the larger ΔpH across the membrane. However 3-fold increases in the *NHX1* protein level in yeast have been reported after exposure to 400 mM NaCl [35] and the possibility that an increase in turnover rate and/or amount of transport protein influences Na⁺/H⁺ exchange cannot be discounted. In addition mammalian NHE activity can be modulated by an intrinsic H⁺ modifier site, which gives rise to steep activation of exchange at a lowered intracellular pH. It is possible that the lowered vacuolar pH resulting from increased V-ATPase activity may similarly activate exchange in *At-NHX1* and *NHX1*. Mammalian NHEs can also be regulated by phosphorylation, and the predicted amino acid sequence of *NHX1* and *At-NHX1* reveals several potential phosphorylation targets in the N- and particularly C-termini of both proteins. Since salt stress is known to elicit protein kinase cascades in both yeast and plants [2,36], there is considerable merit in studying whether (de)phosphorylation modulates Na⁺/H⁺ exchange *in vivo*. Further studies will be undertaken to correlate changes in transport kinetics to a study of C-terminal deletion mutants to assess any regulatory function.

Mammalian NHEs possess a highly conserved sequence located in the centre of a putative transmembrane domain in *At-NHX1*, which is the target of amiloride binding and inhibition. In mammals amiloride competitively inhibits Na⁺/H⁺ exchange with a K_i of 1–100 µM depending on cell type [31]. This sequence is conserved in both *At-NHX1* and *NHX1* (Table 2). The presence of this conserved domain in *At-NHX1* suggests that Na⁺/H⁺ exchange may be similarly inhibited. We have shown in transport assays across vacuolar membranes in yeast expressing *At-NHX1* that amiloride was able to inhibit Na⁺/H⁺ exchange (Figure 7B). These data corroborate previous biochemical transport data which have shown that amiloride inhibits Na⁺/H⁺ exchange across vacuolar membranes from red beet [7] and sunflower [9]. Interestingly, Na⁺/H⁺ exchange mediated by yeast *NHX1* was much less sensitive to amiloride (Figure 7B). This amiloride resistance of *NHX1* may be due to the presence of an aspartic acid residue and a valine residue at positions 4 and 5 respectively of the conserved domain (Table 2). Counillon et al. [24] showed that in these positions the presence of a leucine residue and a phenylalanine/tyrosine residue yields proteins with high affinity for amiloride. *At-NHX1*, *Os-NHX1* and two new potential isoforms of *At-NHX1* all contain an isoleucine residue and a tyrosine residue at positions 4 and 5 of the amiloride domain,

similarly to NHE-2, showing high affinity amiloride binding (Table 2). These new *At-NHX* isoforms show 90% overall identity to *At-NHX1* but significant C- and N-terminal sequence diversity.

What is the *in planta* function of *Arabidopsis At-NHX1*?

Conflicting data regarding the induction of *At-NHX1* expression in *Arabidopsis* have been published. Gaxiola et al. [13] reported a 4-fold increase in *At-NHX1* transcripts after suddenly exposing seedlings to 250 mM NaCl for a period of 6 h. Interestingly, the same level of induction was reached by addition of KCl, which points to an osmotic rather than an ion-specific effect. In contrast, Apse et al. [15] did not detect any change in the level of *At-NHX1* transcripts or in the amount of protein if plants were gradually exposed to 200 mM NaCl. In view of the severe growth inhibition of wild-type *Arabidopsis*, the presence of the endogenous *At-NHX1* gene does not appear to ameliorate Na⁺ tolerance. Taking into account a recent hypothesis that *NHX1* plays a role in adaptation to acute hypertonic shock [37], *At-NHX1* may have a function in short-term adaptation to rapid changes in external osmolarity. Clearly, plants forced to overexpress *At-NHX1* in large amounts can sustain growth in soil supplied with 200 mM NaCl [15]. However, these experiments give no insight into the function of the endogenous *At-NHX1* gene, since in wild-type plants the gene is not 'used' to ameliorate long-term growth under saline conditions. In this respect, a recent report that an Na⁺/H⁺ antiporter in the flower of Japanese morning glory controls the formation of the blue colour, points to a role for this antiporter in vacuolar pH regulation rather than Na⁺-detoxification [38]. So, in our opinion, the question as to what the function of *At-NHX1* is *in vivo* remains to be answered.

Overexpression studies [15] raise other intriguing questions. Namely, sequestration of Na⁺ in the vacuole requires at least two other adaptations in plant metabolism: (1) vacuolar H⁺-pumps must shift to a higher gear in order to sustain vacuolar Na⁺ concentrations of more than 100 mM; (2) the cytoplasm must be provided with compatible solutes to balance the more negative osmotic potential of the vacuolar sap. How these processes are triggered and why the large amount of energy necessary for these processes has no negative effect on growth [15] requires further experimentation.

Now that genes encoding Na⁺/H⁺ antiporters have been identified and cloned, rapid progress has already been made towards understanding the molecular nature of these transporters. The potential of vacuolar Na⁺-sequestration in plants in protecting against salt stress has been demonstrated by *Arabidopsis* plants overexpressing *At-NHX1* [15]. However, in order to engineer salt-tolerant crop plants it seems essential to create plants that do not constitutively express the antiporters, but rather respond to periods of increased salt levels in the soil, for example during periods of drought. Even though *At-NHX1* clearly facilitates Na⁺/H⁺ exchange, *in vivo* functions of *At-NHX1* remain to be shown. Antiporters from real halophytes, which accumulate large amounts of Na⁺ in their vacuoles, may provide better tools to engineer salt-tolerant crop plants. Moreover, it should be kept in mind that the activity of known Na⁺/H⁺ antiporters is regulated in a complex fashion, and without sufficient knowledge of regulatory mechanisms of plant antiporters the behaviour of engineered plants will remain unpredictable.

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