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 hnx1-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 NHAI 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 (Ar-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
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 (*Δnhx1*) and K601 (wild type), and the plasmid pRIN73 were gifts from R. Rao (The John 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 A3AP 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 32P-labelled *Arabidopsis* expressed sequence tag (EST) clone (T75860) following the protocol outlined in the Amersham Nucleic Acid Analysis 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-AtNHX1/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'-CCCTCAGGAAATCTGGGTATTCAGGC-3' and 5'-CCCTCAGGAGTTATGTAATGCAAGCG-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 *Smal*/*XbaI* sites downstream of the *NHX1* promoter to generate the construct pRS-AtNHX1/F, pRS-AtNHX1/T. pRS-AtNHX1/SP was generated following transformation of JM110 cells (Stratagene) with the full-length *At-NHX1* genomic clone as a template and the primers 5'-CGCGGGATCCATGGG-3' and 5'-CGCGGGATCCCT-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'-CGCGGGATCCCTCTC-. GTGTTGCGTGATATCTC-3' and 5'-CTGTAAGCACAAGCAGTTGCTCG-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 (*Δnhx1::URA3*) used in these studies are isogenic to W303 (ura3-1 can1-100 leu2-3 1122trpl-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*~opt~ of 1.0–2.2 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*~opt~ of 0.8 (starter culture) in APG [10 mM arginine, 8 mM phosphoric acid, 2 % (w/v) glucose, 2 mM MgSO₄, 1 mM KCl, 0.2 mM CaCl₂, 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 and 0.6 and 0.8 M NaCl in 50 ml of APG or APGal, where glucose was replaced by galactose. After establishing the starting absorbence, the *D*~opt~ frame with the *NHX1* promoter to generate the construct *NHX1* promoter [5]. *NHX1* proximal promoter sequence was first obtained by PCR amplification using the primers 5'-CCCTCAGGAAATCTGGGTATTCAGGC-3' and 5'-CCCTCAGGAGTTATGTAATGCAAGCG-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.

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#### Vacular 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*~opt~ 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 to a *D*~opt~ of 0.8 (starter culture) in APG [10 mM arginine, 8 mM phosphoric acid, 2 % (w/v) glucose, 2 mM MgSO₄, 1 mM KCl, 0.2 mM CaCl₂, 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 and 0.6 and 0.8 M NaCl in 50 ml of APG or APGal, where glucose was replaced by galactose. After establishing the starting absorbence, the *D*~opt~ 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^−x^) 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.
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 10000 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 centrifuged at 52000 g. SW41 (Beckman) centrifuge tubes overlaid with 4 ml of buffer A 4°C the reaction mixture. Additions of Na+ and Gramicidin D (5 μM) were stirred and maintained at 30 °C for 20 min at 37 °C. The reaction was stopped by addition of 0.9 ml of Malachite green 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. D540 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 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 Pi 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%], resuspended in 24 ml of buffer A, aliquoted into six SW41 tubes and centrifuged 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.

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 NHAI. Of particular interest was the conservation of the motif "LLFFIYL-LLPIT*" identified as the binding site of the diuretic drugamiloride 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.
Salt sensitivity of \( \Delta \text{nhx1} \) (R100)

Consistent with data published elsewhere [5], we found that disruption of \( \text{NHX1} \) 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 (\( \text{PMR2/ENA1} \) and \( \text{NHA1} \)) to compensate for any shortfall in intracellular compartmentation. Transformation of R100 with \( \text{NHX1} \) (back-complementation) under the control of its own full-length promoter (i.e. in \( \text{pRIN73} \)) 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 \( \text{At-NHX1} \)

For functional studies, we expressed three \( \text{At-NHX1} \) constructs (Figure 2A) in the R100 strain: (1) the full-length cDNA isolated from the plasmid library, \( \text{At-NHX1/F} \); (2) a truncated construct containing the predicted ORF of \( \text{At-NHX1} \), \( \text{At-NHX1/T} \); (3) a hybrid construct fusing the N-terminal sequence of \( \text{NHX1} \) to the ORF of \( \text{At-NHX1} \), \( \text{At-NHX1/SP} \).

Figure 2 demonstrates the ability of all three \( \text{At-NHX1} \) constructs to complement the salt-sensitive phenotype of R100 grown in synthetic minimal medium (APG). Expression of \( \text{At-NHX1} \) 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 \( \text{At-NHX1} \)-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 \( \text{At-NHX1} \) constructs or between \( \text{At-NHX1} \) and \( \text{NHX1} \).

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 \( \text{NHX1} \). Both \( \text{At-NHX1} \) constructs were capable of suppressing the hygromycin phenotype at low (5 \( \mu \)g/ml) hygromycin concentrations. However, at 50 \( \mu \)g/ml hygromycin, only the \( \text{At-NHX1/SP} \) 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 \( \text{At-NHX1/F} \) and \( \text{At-NHX1/T} \) (results not shown).
The dissipation of H⁺

A fluorescence quench assay, using ACMA, was used to monitor the generation of D

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 ΔpH and dissipation by Na⁺

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

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 μM nigericin in the reaction buffer and increased with the addition 1 μM 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-AHXX1/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 Δnhx1 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 Na⁺/H⁺ exchange following 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 ΔpH and dissipation by Na⁺

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

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 (Qₘₓ) after 40 s. Changes in the rate of ACMA quench in the presence of nigericin (K⁺/H⁺ exchanger, dissipating a build-up of ΔpH) 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 ΔpH.

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⁺/H⁺ exchange following 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 Kₘ of 16 and 11 mM respectively) and a 1.3-fold higher Vₘₓ 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

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)

<table>
<thead>
<tr>
<th>Strain</th>
<th>At-NHX1 quench (mg of protein)</th>
<th>Na⁺-dependent H⁺ quench (mg of protein)</th>
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<tbody>
<tr>
<td>K601</td>
<td>41.3 ± 1.8</td>
<td>52.3 ± 2.11</td>
</tr>
<tr>
<td>R100/pRS-At-NHX1/T</td>
<td>45.7 ± 1.35</td>
<td>N. D.</td>
</tr>
<tr>
<td>R100/pRS-At-NHX1/SP</td>
<td>43.2 ± 1.65</td>
<td>48.5 ± 2.30</td>
</tr>
<tr>
<td>R100/pRS-NHX1</td>
<td>37.7 ± 2.75</td>
<td>N. D.</td>
</tr>
<tr>
<td>R100/pRS-305</td>
<td>36.3 ± 0.80</td>
<td>41.5 ± 1.25</td>
</tr>
</tbody>
</table>

Measurements are representative of the mean ± S.E.M. of four (K601 and R100-At-NHX1/SP) or three (R100-At-NHX1, R100-At-NHX1/7 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.
Magnitude of Na\textsuperscript{+}/H\textsuperscript{+} 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\textsuperscript{+}-dependent Na\textsuperscript{+} exchange. Growth of K601 or At-NHX1/SP-transformed yeast in 0.2 M NaCl increased the magnitude and rate of ATP-driven H\textsuperscript{+} quench and enhanced Na\textsuperscript{+}-dependent H\textsuperscript{+} 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\textsuperscript{+}-dependent H\textsuperscript{+} exchange following salt treatments, as demonstrated in other systems [27,28].
NHX to recover the NaCl-tolerant phenotype. However, pre-incubation of membrane preparations with 120 μM amiloride prior to the transport assay revealed a complete inhibition of At-NHX1/T-mediated Na<sup>+</sup>/H<sup>+</sup> 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<sup>+</sup>/H<sup>+</sup> antiport as a general mechanism for Na<sup>+</sup> 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<sup>+</sup>/H<sup>+</sup> 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-terminal, 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<sup>+</sup>-sequestration as a mechanism for salt tolerance in yeast. The dependence on acid pH is probably a reflection of suboptimal levels of the Na<sup>+</sup>-ATPase (EN1-A4) activity at low pH [32]. In other words, at low external pH the cells have a greater dependence on alternative Na<sup>+</sup> efflux (NHA1) or Na<sup>+</sup> 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 EN1-A4 and/or NHA1 null mutants even at low pH and low K<sup>+</sup> (results not shown). This suggests that NHX1, and therefore vacuolar sequestration of Na<sup>+</sup>, 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 ΔpH [26,34]. Since Na<sup>+</sup>/H<sup>+</sup> 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 ΔpH across the vacuolar membrane as a result of Na<sup>+</sup>-dependent H<sup>+</sup> 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 μg/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<sup>+</sup>/H<sup>+</sup> exchange by At-NHX1. This could be achieved through targeting the protein to the correct functional location or by direct modification of Na<sup>+</sup>/H<sup>+</sup> 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<sup>+</sup>/H<sup>+</sup> antiport mechanism than the endogenous yeast gene. At-NHX1/F and At-NHX1/T both recovered wild-type growth to the same extent at 5 μg/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<sup>+</sup>/H<sup>+</sup> antiporters was achieved through the measurement of ΔpH-dependent Na<sup>+</sup>/H<sup>+</sup> exchange and Na<sup>+</sup> uptake (Figures 4–6). Our studies showed an ATP-independent, ΔpH-dependent Na<sup>+</sup>/H<sup>+</sup> exchange across the membranes of all strains carrying At-NHX1 or NHX1. Interestingly, expression of the construct At-NHX1/SP resulted in higher rates and extents of Na<sup>+</sup>/H<sup>+</sup> 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<sup>+</sup>-dependent H<sup>+</sup> efflux displayed saturation kinetics with respect to extravesicular Na<sup>+</sup> concentrations (Figure 5). At-NHX1/SP displayed a higher V<sub>max</sub> and a lower apparent K<sub>m</sub> 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<sup>+</sup> than NHX1. The K<sub>m</sub> value of 11 mM is in good agreement with that reported for plant vacuoles [7,28,30].

**Na<sup>+</sup>/H<sup>+</sup> exchange is electroneutral**

In the present study two lines of data suggest that Na<sup>+</sup>/H<sup>+</sup> exchange mediated by At-NHX1 and NHX1 is electroneutral. Firstly, similar rates of Na<sup>+</sup>-dependent H<sup>+</sup> efflux were observed in the presence of valinomycin and K<sup>+</sup>, 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<sup>+</sup> (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<sup>+</sup>/H<sup>+</sup> 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<sup>+</sup>-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

<table>
<thead>
<tr>
<th>cDNA</th>
<th>Amiloride binding site</th>
<th>Amiloride sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE1</td>
<td>\textsuperscript{164}VFF\textsuperscript{173}LFPPI</td>
<td>IC\textsubscript{50} 3 \textmu M*</td>
</tr>
<tr>
<td>NHE1 Leu\textsuperscript{167} \rightarrow Phe</td>
<td>V\textsuperscript{165}V\textsuperscript{166}VFF\textsuperscript{173}LFPPI</td>
<td>IC\textsubscript{50} 15 \textmu M*</td>
</tr>
<tr>
<td>NHE1 Leu\textsuperscript{167} \rightarrow Phe</td>
<td>V\textsuperscript{165}V\textsuperscript{166}VFF\textsuperscript{173}LFPPI</td>
<td>IC\textsubscript{50} 15 \textmu M*</td>
</tr>
<tr>
<td>Ph\textsuperscript{168} \rightarrow Tyr</td>
<td>V\textsuperscript{165}V\textsuperscript{166}VFF\textsuperscript{173}LFPPI</td>
<td>IC\textsubscript{50} 3 \textmu M*</td>
</tr>
<tr>
<td>NHE2</td>
<td>V\textsuperscript{248}V\textsuperscript{249}V\textsuperscript{250}LFPPI</td>
<td>IC\textsubscript{50} 3 \textmu M*</td>
</tr>
<tr>
<td>At-NHX1\textsuperscript{†}</td>
<td>V\textsuperscript{248}V\textsuperscript{249}V\textsuperscript{250}LFPPI</td>
<td>100% inhibition by 120 \textmu M amiloride</td>
</tr>
<tr>
<td>NhX1</td>
<td>V\textsuperscript{248}V\textsuperscript{249}V\textsuperscript{250}LFPPI</td>
<td>20–40% inhibition by 120 \textmu M amiloride</td>
</tr>
</tbody>
</table>

* 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\textsuperscript{+}-dependent H\textsuperscript{+} 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\textsuperscript{+}/H\textsuperscript{+} exchange cannot be discounted. In addition, mammalian NHE activity can be modulated by an intrinsic H\textsuperscript{+} 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\textsuperscript{+}/H\textsuperscript{+} 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\textsuperscript{+}/H\textsuperscript{+} exchange with a \textit{K}\textsubscript{s} of 1–100 \textmu M 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\textsuperscript{+}/H\textsuperscript{+} 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\textsuperscript{+}/H\textsuperscript{+} exchange (Figure 7B). These data corroborate previous biochemical transport data which have shown that amiloride inhibits Na\textsuperscript{+}/H\textsuperscript{+} exchange across vacuolar membranes from red beet [7] and sunflower [9]. Interestingly, Na\textsuperscript{+}/H\textsuperscript{+} 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 \textit{in planta} function of Arabidopsis At-NHX1?

Conflicting data regarding the induction of At-NHX1 expression in \textit{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, Ape 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 \textit{Arabidopsis}, the presence of the endogenous At-NHX1 gene does not appear to ameliorate Na\textsuperscript{+} 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\textsuperscript{+}/H\textsuperscript{+} 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\textsuperscript{+}-detoxification [38]. So, in our opinion, the question as to what the function of At-NHX1 \textit{is} in \textit{vivo} remains to be answered.

Overexpression studies [15] raise other intriguing questions. Namely, sequestration of Na\textsuperscript{+} in the vacuole requires at least two other adaptations in plant metabolism: (1) vacuolar H\textsuperscript{+}-pumps must shift to a higher gear in order to sustain vacuolar Na\textsuperscript{+} 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\textsuperscript{+}/H\textsuperscript{+} 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\textsuperscript{+}-sequestration in plants in protecting against salt stress has been demonstrated by \textit{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\textsuperscript{+}/H\textsuperscript{+} exchange, \textit{in planta} functions of At-NHX1 remain to be shown. Antiporters from real halophytes, which accumulate large amounts of Na\textsuperscript{+} 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\textsuperscript{+}/H\textsuperscript{+} 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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REFERENCES

