Mutants of the Arabidopsis thaliana Cation/H⁺ Antiporter AtNHX1 Conferring Increased Salt Tolerance in Yeast

THE ENDO一些/PREVACUOLAR COMPARTMENT IS A TARGET FOR SALT TOXICITY

Received for publication, August 11, 2008, and in revised form, March 19, 2009 Published, JBC Papers in Press, March 23, 2009, DOI 10.1074/jbc.M806203200

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Mutants of the plant cation/H⁺ antiporter AtNHX1 that confer greater halotolerance were generated by random mutagenesis and selected in yeast by phenotypic complementation. The amino acid substitutions that were selected were conservative and occurred in the second half of the membrane-associated tail domain. AtNHX1 complemented the lack of endogenous ScNHX1 in endosomal protein trafficking assays. Growth enhancement on hygromycin B and vanadate media agreed with a generally improved endosomal/pervacuolar function of the mutated proteins. In vivo measurements by 31P NMR revealed that wild-type and mutant AtNHX1 transporters did not affect cytosolic or vacuolar pH. Surprisingly, when yeast cells were challenged with lithium, a tracer for sodium, the main effect of the mutations in AtNHX1 was a reduction in the amount of compartmentalized lithium. When purified and reconstituted into proteoliposomes or assayed in intact vacuoles isolated from yeast cells, a representative mutant transporter (V318I) showed a greater cation discrimination favoring potassium transport over that of sodium or lithium. Together, our data suggest that the endosome/pervacuolar compartment is a target for salt toxicity. Poisoning by toxic cations in the endosome/pervacuolar compartment is detrimental for cell functions, but it can be alleviated by improving the discrimination of transported alkali cations by the resident cation/H⁺ antiporter.

The Arabidopsis thaliana vacuolar alkali cation transporter AtNHX1 has been shown to increase salt tolerance in transgenic plants of several species (1). In Saccharomyces cerevisiae, its ortholog (ScNHX1) is mainly localized in late endosomes, where it is thought to contribute to vacuole biogenesis by regulating pH and vesicle volume (2). ScNHX1 itself has a role in halotolerance. Deletion of ScNHX1 confers salt sensitivity and diminishes Na⁺ compartmentalization, albeit indirectly, since the unrelated VNX1 exchanger accounts for most of the cation/H⁺ antiporter activity in the tonoplast of yeast (3, 4). However, AtNHX1 complements a yeast mutant defective in ScNHX1 and restores cation compartmentalization (5).

Improving the salt tolerance of crop plants is an important goal in biotechnology. In addition to the mechanisms by which a cell can cope with increased concentrations of toxic cations, it is important to know the identity of salt-sensitive cellular targets. Only a few key processes have been identified. In yeast, HAL2, an inositol phosphatase that catalyzes the dephosphorylation of 3'-phosphoadenosine-5'-phosphate to AMP, has been found to be inhibited by Li⁺ and Na⁺. Inhibition of HAL2 during salt stress results in the accumulation of 3'-phosphoadenosine-5'-phosphate in the cell, which has the potential to produce a variety of toxic effects, such as the inhibition of sulfotransferases and RNA-processing enzymes (6). Another possible target is the KEX2/furin family of proteases of the Golgi/secretory pathway. The activity of KEX2 in vitro has been shown to respond differently, depending on the alkali cation and concentration present in the medium (7). Here, we show that the endosomal system is an additional target for Na⁺ toxicity.

The Golgi apparatus, trans-Golgi network, and endosome/pervacuolar compartment form a continuum where proteins and membranes are modified en route to their final destinations (8–10). The late endosome/pervacuolar compartment is considered a key point in intracellular vesicle and protein trafficking. In addition to being the previous stage for vacuolar sorted proteins and cargo, this is where both the exocytic and endocytic pathways converge (10, 11). Ion homeostasis in these organelles is increasingly regarded as an important feature for intracellular transport processes (12–15). In particular, K⁺ concentration may regulate the activity and specificity of enzymes modifying proteins posttranslationally, such as the above mentioned KEX2/furin protease family (7). Lumenal pH has been reported also to regulate selective protein aggregation in secretory vesicles (12). In this respect, it is noteworthy that yeast nhx1 mutants have been characterized as class E vps mutants with impaired vacuolar biogenesis and protein sorting (15).

AtNHX1 is thought to increase salt tolerance in plants through the intracellular compartmentation of Na⁺. However, using purified protein, it has been shown that this antiporter can exchange H⁺ for K⁺, Na⁺, or Li⁺, albeit the last one with

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lower affinity (16). The poor K⁺/Na⁺ selectivity raises the question of whether Na⁺ transport is the primary function of AtNHX1 in plant cells and if AtNHX1 is amenable to selection of better alleles for salt tolerance. Mutagenesis of cation transporters has proved to be a valuable tool to obtain alleles with modified transport activities (17, 18). At the same time, this provides information about the important amino acid residues that affect the mechanism of protein function. In this work, we sought to produce hypermorphic AtNHX1 alleles conferring greater salt tolerance, by either improved Na⁺/K⁺ discrimination or altered protein regulation. We show here that nhx1-deficient yeast cells that express mutated forms of AtNHX1 display improved halotolerance compared with cells that express the wild-type AtNHX1. The mutations responsible for these changes were scattered throughout the hydrophobic N terminus of the protein, and their effect was to introduce bulkier side chain amino acids. Surprisingly, the result of these mutations was not increased compartmentalization of toxic alkali cations. Instead, all of these mutants showed a decreased content of Li⁺ (a tracer for Na⁺), whereas full amounts of K⁺ were retained. Biochemical characterization of a selected mutant transporter showed greater cation discrimination favoring K⁺ transport. AtNHX1 is localized to the vacuole and late endosome/prevacuolar compartment. Together, these results suggest that the endomembrane system is a cellular target of Na⁺ intoxication.

MATERIALS AND METHODS

Strains and Culture—S. cerevisiae strains AXT3 (Δena1-4::HIS3, Δnha1::LEU2, Δnhx1::TRP1), WX1 (Δnhx1::TRP1), and OC2 (Δnhx1::HIS3, nux1::KanMX4) have been described previously (4, 5). For cation tolerance testing, saturated cultures were diluted to 2 × 10⁶ cell/ml, and 5-μl aliquots of 10-fold serial dilutions from saturated yeast cultures were spotted onto AP plates (8 mM phosphoric acid, 10 mM l-arginine, 2 mM MgSO₄, 0.2 mM CaCl₂, 2% glucose plus vitamins and trace elements) supplemented with KCl, NaCl, or LiCl (19). Li⁺ was used as a tracer for Na⁺. Resistance to hygromycin B and vanadate was assayed in YPD medium as described above. YPD and SD media lacking the appropriate amino acids were prepared according to Sherman (20). Yeast expression plasmids pDR195 (2μ, PMA1 promoter, URA3) and pKV61 (2μ, PMA1 promoter, LEU2) have been described elsewhere (16, 18).

In Vitro Random Mutagenesis—An Sphl-Sphl fragment from pFA6a-KanMX4 containing a Geneticin resistance cassette (21) was generated by PCR using primers containing Sphl linkers and introduced into the pDR195AtNHX1 plasmid to assess the degree of mutagenesis imposed (pDRKanAtNHX1). Plasmid mutagenesis was done according to previous description (17). In brief, pDRKanAtNHX1 plasmid (2 μg) was dissolved in 30 μl of 400 mM hydroxylamine, 50 mM KH₂PO₄, 0.9 mM EDTA, pH 6.5, cooled at 4 °C for 45 min, and then heated at 65 °C for 30 min. After precipitation, mutagenized plasmid was introduced into AXT3 cells using the lithium acetate method (22). Transformants showing a greater resistance than control cells containing the AtNHX1 wild-type allele were selected by replica-plating onto agar plates with AP medium supplemented with 70 mM NaCl and YPD medium containing 30 μg/ml hygromycin B to avoid false positives (23). The extent of mutagenesis was estimated to be greater than 1%, as assessed by loss of function of the plasmid-borne Geneticin resistance gene. Mutant plasmids were recovered, their AtNHX1 open reading frames were sequenced in both directions, and the phenotype was confirmed by retransformation with nonmutagenized pDR195 expression vectors carrying appropriate mutant open reading frames.

Introduction of His Tags and Western Blotting—Hexahistidine tags were introduced by PCR using the primers 5’-GCCAGGATCCCTCGAGGTTGATGGTGATGCGATCCACGGAG-3’ and 5’-GCCAGAATTCCGGGACACAGATGTACGCGG-3’ and appropriate templates. PCR products were cloned, sequenced, and excised as BsmI-BamHI fragments, which were swapped for the corresponding part in each mutant. Tagged versions were introduced into pKV61 yeast expression plasmid (16) as Xhol-SpeI fragments. Tagged versions were checked for complementation on NaCl and hygromycin B-containing plates.

WX1 cells bearing appropriate pKV1-derived plasmids were grown in SD medium to 4×10⁶ cell/ml, spun, washed, and broken in 100 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM dithiothreitol, 0.2 mM sorbitol, 1 mM phenylmethylsulfon fluoride, 1 mM benzamidine, and protease inhibitor mixture (Roche Applied Science) by vortexing with glass beads. Extracts were centrifuged at 500 × g for 5 min to pellet cell debris, and supernatants were spun at 30,000 × g for 30 min to obtain a microsomal fraction. Proteins (25 μg) were separated by SDS-PAGE according to Laemmli (24), transferred to polyvinylidene difluoride membranes, and blocked with 5% non-fat dry milk in Tris-buffered saline. Anti-H₄ antibody (Qiagen) was used at 0.1 μg/ml, and immunocomplexes were visualized with horseradish peroxidase-conjugated antibodies and an ECL kit (Amersham Biosciences).

Microscopy—Wild-type W303 (NHX1) and WX1 (Δnhx1) cells were co-transformed with His-tagged versions of AtNHX1 and plasmid pJLU34 bearing a chimeric Ste3-GFP reporter gene (15). Cells were grown to midlog phase in liquid AP medium (1 mM KCl) adjusted to pH 4.0 with acetic acid or pH 6.0. Cells were collected and stained with FM4–64 as described in Ref. 25. For co-localization experiments, an AtNHX1-GFP translational fusion was created by introducing NotI restriction sites at the C terminus of AtNHX1 and the N terminus of the GFP. The resulting in-frame fusion was cloned into the Xhol-BamHI sites of plasmid pDR195. The open reading frame encoding the fluorescent protein mCherry was cloned in the NotI site of vector pRIN73 (2), generating an in-frame fusion of mCherry to the C terminus of ScNHX1 (ScNHX1-ChFP) and appropriate vector fragments. Wild-type W303 cells were co-transformed with plasmids containing the tagged proteins AtNHX1-GFP and ScNHX1-ChFP. Cells were grown in liquid YNB medium with and without 300 mM NaCl to late log phase. NaCl was used to induce the expression of the ScNHX1 gene promoter in pRIN73 (2). Images were acquired by epifluorescence microscopy with a Zeiss Axioscope 3 The abbreviations used are: GFP, green fluorescent protein; Mes, 4-morpholineethanesulfonic acid; BTP, 1,3-bis[(hydroxymethyl)methylamino]-propane.
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equipped with a filter set, Chroma 41018 (exciter HQ470/40, emitter HQ500LP), or using a laser confocal microscope, Olympus Fluoview1000. Experiments were carried out a minimum of two times.

³¹P NMR—Cytosolic and vacuolar pH determinations were done as described in Ref. 26. Cells were grown to $A_{600} = 1$ on AP medium (1 mM KC1) medium, collected by centrifugation, washed twice in 100 mM Mes/Tris, pH 6.5, with 2% glucose, and resuspended to yield $A_{600} = 100$. ³¹P experiments were recorded at 298 K on a Bruker AVANCE 400 apparatus. Chemical shifts are expressed in ppm using the signal of the trisodium salt of methylene-diphosphonic acid as reference. Each experiment was recorded using 1024 scans of 16,204 points, and ¹H was externally weighted (line broadening of 5 Hz).

Plasma Membrane Permeabilization and Ion Content Determination—WX1 cells ($\Delta$nx1) transformed with AtNHX1 variants were grown in AP medium (1 mM KC1) to $A_{600} = 0.2$. Then 2 mM LiCl was added to the medium, and cells were collected when $A_{600} = 1$. Cell permeabilization was done according to Ref. 27 with the following modifications: cells were washed twice in permeabilization buffer (1 mM sorbitol, 20 mM MgCl₂, 50 mM HEPES/BTP, pH 7.0) and resuspended in the same buffer without magnesium to $A_{600}$ ~50. Nystatin was added to a final concentration of 0.4 mg/ml, and cells were incubated at 30 °C for 10 min. Permeabilization was stopped by adding 10 volumes of ice-cold permeabilization buffer supplemented with 20 mM MgCl₂. Cells were spun and washed once. For ion content determination, both supernatants were combined (cytosol), whereas pellets were used for determination of compartmentalized ions. Permeabilization was monitored by measuring glucose-6-phosphate dehydrogenase activities and quinacrine accumulation (27, 28). Ion contents were determined by atomic emission spectrophotometry (5).

Purification of Histidine-tagged AtNHX1 by Ni²⁺ Affinity Chromatography and Reconstitution into Liposomes—The procedure was essentially that described in Ref. 16. Briefly, yeast microsomes (4 ml, 5 mg protein/ml) were mixed with 20 ml of solubilization buffer (50 mM KH₂PO₄, pH 7.4, 500 mM NaCl, 10 mM imidazole, 20% glycerol, 0.5% n-dodecyl-β-D-maltoside, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg/ml chymostatin, 2 μg/ml pepstatin) and incubated for 30 min at 4 °C under gentle shaking. Unsolubilized material was removed by centrifugation for 30 min at 30,000 × g. The supernatant was mixed with 1 ml of pentadentate chelator resin charged with Ni²⁺ (Affilant) and incubated overnight at 4 °C with gentle stirring. The resin was then poured into a polypropylene column and washed according to the manufacturer’s instructions: three times with 4 ml of buffer A (50 mM Na₂HPO₄, 0.5 M NaCl, pH 6.5), three times with 4 ml of buffer B (same as above but without NaCl), and once more with 4 ml of buffer A (buffers supplemented with protease inhibitors as above and 0.15% n-dodecyl-β-D-maltoside). Thereafter, the protein was eluted with an imidazole step gradient of 8-ml fractions containing 0, 50, 100, 200, 300, and 1000 mM imidazole, pH 7.4, in 50 mM KH₂PO₄, pH 7.4, 500 mM NaCl, 20% glycerol, 0.075% n-dodecyl-β-D-maltoside and protease inhibitors. The purified protein eluted in the 100–200 mM imidazole fractions. The sample was frozen in liquid nitrogen and stored at −80 °C.

For reconstitution of purified AtNHX1 forms in proteoliposomes, protein (4 μg) was mixed with soybean phospholipids type II-S (Sigma) at a lipid/protein ratio of 585 in a total volume of 208 μl of reconstitution buffer containing 20 mM BTP-Mes, pH 7.5, 10% glycerol, 25 mM (NH₄)₂SO₄, and 2.5 mM pyrimine (converted to BTP salt using Dowex 50WX8 ion exchange resin). The sample was solubilized by the addition of 12 μl of 1 M octyl glucoside and loaded onto a 2.5-ml spin column filled with Sephadex G-50 (fine; Amersham Biosciences, Inc.) preloaded with 200 μl of 2.5 mM pyrimine in reconstitution buffer. After centrifugation for 5 min at 180 × g, the eluate was incubated for 30 min at room temperature with 100 mg of wet Biobeads (SM-2; Bio-Rad) and passed again over a G-50 spin column.

Measurement of Cation/H⁺ Exchange—For proteoliposomes, fluorescence of the sample was adjusted to adequate levels by diluting the sample with reconstitution buffer. Next, 50 μl of liposomes containing the histidine-tagged protein AtNHX1-RGSH₄ were diluted in reconstitution buffer without (NH₄)₂SO₄ in a 1-ml stirred reaction cuvette thermostated at 20 °C. The 20-fold NH₄⁺ dilution results in pH 6.6 inside the vesicles due to outward diffusion of NH₃. Thereafter, infinite inward cation gradients were imposed by the addition of chloride salts at the outside of the vesicles. Proton efflux coupled to cation influx was monitored from the increase of pyranine fluorescence recorded at 463-nm excitation wavelength and 510-nm emission wavelength. For tonoplast vesicles, intact vacuoles were isolated essentially as described (28). The formation of ΔpH was established by the activity of the tonoplast H⁺-ATPase in the following reaction mixture (1 ml): 5 μM quinacrine, 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, and 50 μg of vacuolar membrane protein. The assay was initiated with the addition of 3 mM ATP. When ΔpH reached steady state, equal amounts of various concentrations of sulfate salt stock solutions were added to the reaction mixture. Specific activity was calculated by dividing the initial rate (30 s after the addition of salts) of fluorescence recovery, expressed as a ratio of the preformed pH gradient, by the amount of plasma membrane protein in the reaction and time (ΔF% = ΔF/F₀/F_max × F_min × 100%). Quinacrine fluorescence was measured at excitation and emission wavelengths of 430 and 500 nm, respectively. Kinetic parameters were estimated by nonlinear regression (29).

RESULTS

Screening for AtNHX1 Mutants with Improved Halotolerance—We sought to generate AtNHX1 alleles with improved capacity for Na⁺ compartmentation as a way to enhance plant halotolerance and, at the same time, gain knowledge concerning the amino acid residues that influence ion transport. Random mutants of AtNHX1 were generated in vitro, as described under “Materials and Methods.” Mutagenized plasmids were introduced into the AXT3 strain devoid of the main Na⁺ transporters that contribute to salt tolerance in yeast (ENA1 to -4, NHA1, and NHX1). Deletion of ScNHX1 also renders yeast cells hypersensitive to hygromycin B. Mutant screening was
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performed on 70 mM NaCl in AP medium supplemented with 1 mM KCl and on YPD plates with 30 μg/ml hygromycin B. Since salt-tolerant extragenic suppressors arise at high frequency (30), mutants were also tested in hygromycin B to avoid false positives. AXT3 cells transformed with an empty vector could not grow on these media, whereas cells expressing wild-type AtNHX1 grew poorly. A total of 25 putative salt- and hygromycin B-resistant mutants with robust growth on both selection media were selected after a first screening. Mutants were rescreened, and their plasmids were recovered. The AtNHX1 coding sequence was excised and introduced into fresh pDR195 plasmids for confirmation of the phenotype. Those with a reconfirmed halotolerance phenotype had their AtNHX1 insert sequenced in both directions. Single base mutations were found in all cases except for mutant HAT#8, which showed a double G to A transition at positions 1141 and 1144, corresponding to valine residues at positions 381 and 382 in the protein. The identities of the deduced changes in the amino acid sequence and their position in the AtNHX1 polypeptide are shown in Fig. 1.

Halotolerance Phenotype of Random Mutants of AtNHX1—A finer comparison of the halotolerance phenotype was done by drop tests. All mutants showed improved growth capacity on NaCl- and LiCl-containing plates (Fig. 2). Phenotypes observed on lithium-containing plates were somewhat stronger than those on NaCl plates. No growth differences were found on 1 mM KCl plates, a concentration that exerts a mild stress and growth inhibition on these strains (Fig. 2). Since differences in protein levels of the AtNHX1 mutants could account for the phenotypes observed, His6-tagged versions were constructed to estimate protein abundance. His6-tagged proteins showed no deviation when compared with their untagged parental on NaCl and LiCl plates (data not shown). The amount of AtNHX1 polypeptide for each mutant was then examined by Western blot. Densitometric analysis showed that mutant V251I was expressed at a slightly greater level than wild-type AtNHX1, whereas V381I and V382M mutant levels were somewhat lower (~1.42 ± 0.05 and 0.54 ± 0.13-fold, respectively, n = 2). Mutants G144S and V318I showed similar levels of expression compared with wild-type (Fig. 2B). Despite several attempts, we could not detect mutant T428I on Western blots.

Intracellular pH Measurements—The yeast ScNHX1 has been proposed to be involved in intracellular pH homeostasis in acid-stressed cells (pHext < 3) (31). Therefore, we investigated the effect that the expression of wild-type and mutant alleles of AtNHX1 had on cytosolic and vacuolar pH in yeast. In vivo 31P NMR allows direct simultaneous measurement of both cytosolic and vacuolar pH with a minimum of handling. This is accomplished through analysis of resonance shifts corresponding to free phosphate and terminal phosphate from polyphosphates, respectively (26). Changes in cytosolic or vacuolar pH are visualized as shifts of the resonance peaks. Measurements of cytosolic pH revealed that heterologous expression of wild-type AtNHX1 had an influence on yeast cytosolic pH under conditions similar to those used in halotolerance tests (AP medium, pH 6.5) (25), compared with empty vector-transformed AXT3 cells (Fig. 3). Similarly, no changes were appreciable in the chemical shift of terminal phosphate from vacuolar polyphosphates (Fig. 3). Identical results were obtained for mutant V318I (Fig. 3). The presence of NaCl in the growth medium and/or buffer had no effect on the measured cytosolic pH, which remained at approximately pH 6.8 (data not shown). In addition to those shown in Fig. 3, these determinations were

FIGURE 1. Mutations on the AtNHX1 protein. The topological model was constructed according to the model given for human NHE1 and AtNHX1 in Ref. 36. The hatched square depicts the predicted ion binding site. Open squares correspond to amino acids altered by random mutagenesis with hydroxylamine. Mutational changes are indicated by arrows. Open circles correspond to other relevant amino acids referred to under “Discussion.”

FIGURE 2. Salt tolerance of yeast cells bearing mutated versions of AtNHX1. A, saturated cultures of AXT3 transformants were diluted to 2 × 10⁶ cell/ml, and 5 μl of 10-fold serial dilutions were spotted onto AP medium (1 mM KCl) supplemented as follows: control (no additions), LiCl (4 mM), NaCl (70 mM), KCl (1 M). B, expression of the different mutant versions of AtNHX1. Microsomal membrane proteins (25 μg) were separated electrophoretically and subjected to Western blot using anti-His antibody.
also performed with the other AtNHX1 mutants, but none showed any deviation in cytosolic or vacuolar pH (data not shown). On the other hand, the technique was capable of detecting the outcome of treatments known to affect cytosolic pH, such as the addition of a permeable organic acid (25, 26). Upon the addition of the acidulant, the cytosolic pH shifted to more acidic values, and the signal from the terminal phosphate of vacuolar polyphosphates disappeared (Fig. 3), presumably due to the lumenal pH reaching values below pH 4.5 (26). These results indicate that the alkali cation and hygromycin B resistance imparted by wild-type and mutant AtNHX1 proteins was not caused by overt changes in cytoplasmic or vacuolar pH due to altered cation/proton exchange.

Complementation of vps Phenotypes—The yeast protein NHX1/VPS44 is known to be important for endosomal trafficking and protein sorting of the late endosome/prevacuolar compartment (25, 31). To test whether Arabidopsis AtNHX1 suppressed the defects in intracellular protein trafficking of nhx1 cells, we monitored the subcellular localization of a Ste3-GFP chimera as a marker for endosome-to-vacuole traffic. Ste3p-GFP accumulates in the vacuole of wild-type cells, but in the class E compartment of the nhx1/vps44 mutant (18). At pH 4.0, ~65% of nhx1 cells showed impairment of Ste3-GFP sorting to the vacuole (Fig. 4). This could be reverted if cells were grown at pH 6.0, in accordance with previous reports (31). A majority (>90%) of cells transformed with wild-type AtNHX1 and mutant V318I showed correct sorting of Ste3-GFP to the vacuole at low pH. This indicates that the Arabidopsis exchanger suppressed this defect of nhx1 cells.

AtNHX1 localizes to the tonoplast of plants (1). In yeast, an AtNHX1-GFP fusion protein was predominantly targeted to the vacuole (Fig. 5A), but the labeling of additional punctate bodies and the phenotypic complementation of nhx1/vps44 mutation indicated that it was localized also to the same late endosome compartment as ScNHX1, presumably due to transient residence while en route to the vacuole. To test this, AtNHX1-GFP was co-expressed with the yeast protein ScNHX1 fused to the fluorescent protein mCherry (ScNHX1-ChFP). The coding region of ScNHX1 in the plasmid pRIN73 (2) was replaced with the ScNHX1-ChFP construct to attain expression from the ScNHX1 gene promoter. In the presence of 300 mM NaCl to induce the expression of ScNHX1-ChFP (2), the two reporter proteins showed strong co-localization in endosomal membranes (Fig. 5B, middle row). Salt stress produced the fragmentation of the large central vacuole (Fig. 5B, top row). The prevacuolar/late endosome compartment could be visualized as the preferential accumulation site of ScNHX1-
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AtNHX1-GFP and DIC

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**FIGURE 5. Co-localization of plant and yeast NHX1 proteins.** A, AXT3 cells were transformed with an AtNHX1-GFP construct and pictured under epifluorescence microscopy (DIC). GFP fluorescence was predominant in membranes of the vacuole and smaller endosomes. The punctate pattern corresponds to the late endosome/prevacuolar compartment. B, wild-type cells were co-transformed with plasmids harboring the reporter constructs AtNHX1-GFP or ScNHX1-ChFP, grown in AP medium with 300 mM NaCl to late exponential phase, and observed under confocal microscopy. **Top row,** cells expressing one (cell 1, AtNHX1-GFP; cell 2, ScNHX1-ChFP) or both (cell 3) reporter proteins demonstrating no fluorescence bleed-through from the green and red channels. **Middle row,** cells showing strong co-localization of AtNHX1-GFP and ScNHX1-ChFP in the endosomal membranes. **Bottom row,** cells pictured at low fluorescence intensity showing preferential accumulation of both reporter proteins in endosomal bodies peripheral to the central vacuole that correspond to the late endosome/prevacuolar compartment.

ChFP in cells with moderate levels of expression and/or excited with low laser intensity to enhance regions of maximal fluorescence (Fig. 5B, bottom row). In these conditions, AtNHX1-GFP significantly co-localized with ScNHX1-ChFP in punctate bodies (Fig. 5B, bottom row) that have been ascribed to the prevacuolar/late endosomes (2). Without salt, the expression of ScNHX1-GFP was tenuous, and its localization was restricted to the prevacuolar and late endosomes, as previously reported (2). Observation of single-plasmid transformants demonstrated no fluorescence bleed-through from GFP and ChFP markers (Fig. 5B, top panels).

Defects at the endosome/prevacuolar compartment translate into a series of growth characteristics common to a family of yeast mutants collectively known as *vps*. Hygromycin B sensitivity is a shared phenotype of many *vps* mutants, including *nhx1/vps44* (30). To ascertain if improvement of halotolerance by AtNHX1 mutant alleles was linked to the functions of the endosome/prevacuolar compartment that are ascribed to ScNHX1, a drop test series of experiments were done. As anticipated from the selection screening, growth on hygromycin B-containing plates was improved for yeast cells bearing AtNHX1 mutant alleles (Fig. 6). Since resistance to the polycationic drug hygromycin B could come about through effects on the endosome/prevacuolar compartment or the plasma membrane (32, 33), we chose vanadate, an oxyanion known to reflect defects in *vps* genes (34), in order to confirm these results. Again, all mutants of AtNHX1 showed improved growth on vanadate-containing plates (Fig. 6). Growth tests on liquid cultures over the pH range 5–8.5 or on plates buffered to pH 8.0 showed no differences in pH sensitivity for any AtNHX1 mutant relative to wild-type (data not shown), indicating that the vacuole was not significantly affected. Together, these results indicate that AtNHX1 mutants improved the proficiency of wild-type AtNHX1 when substituting for ScNHX1 in endosome/prevacuolar functions.

**Ion Contents in AtNHX1 Mutants—Vacuolar sequestration of toxic alkali cations is thought to account for the salt tolerance imparted by AtNHX1 in plants and yeast (1, 5).** Hence, the amounts of compartmentalized and cytosolic ions were investigated next in cells expressing wild-type and mutant AtNHX1.

Li⁺ was used as a tracer for Na⁺ in these experiments, since the latter must be added at too high concentration in the medium to make reliable intracellular ion content measurements (e.g. 70 mM NaCl versus just 2 mM LiCl). Also, AtNHX1 mutants imparted greater tolerance to LiCl compared with NaCl (Fig. 2). Surprisingly, cells grown on 2 mM LiCl-containing AP medium (1 mM KCl) showed no differences in cytosolic K⁺ or Li⁺ contents, with the exception of the strain transformed with an empty vector (Table 1). The slight drop in cytosolic ion contents in the AXT3 strain might be a reflection of the defective ion accumulation brought about by the *nhx1* mutation (5). The amounts of ions compartmentalized into organelles (fraction resistant to nystatin) dropped to ~70% for both Li⁺ and K⁺ in empty vector-transformed cells, as compared with wild-type AtNHX1-bearing cells. Unexpectedly, cells expressing mutants of AtNHX1 also showed decreased levels of compartmentalized...
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<th>Cellular ion contents of yeast cells grown on LiCl-supplemented medium</th>
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<td>Data are shown as percentage of wild type ± S.E. Cytosol: 100% Li = 27.70 ± 0.70 nmol/mg dry weight; 100% K = 43.69 ± 7.71 nmol/mg dry weight. Compartimentalized: 100% Li = 24.00 ± 1.41 nmol/mg dry weight, 100% K = 13.58 ± 1.37 nmol/mg dry weight.</td>
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|         | ![Diagram](http://www.jbc.org/)

*Li\(^{+}\)* compared with wild-type AtNHX1. Compartimentalized K\(^{+}\) contents in these conditions remained comparable (Table 1). The extent of these decreases in Li\(^{+}\) contents correlated well with the observed phenotypes on LiCl or NaCl agar plates (Fig. 2). These changes reflect an increase in the K\(^{+}\)/Li\(^{+}\) ratio of compartmentalized ions (Table 1).

**Cation Selectivity for the V318I AtNHX1 Mutant** —The above results suggested that AtNHX1 mutants had improved discrimination for K\(^{+}\) over Na\(^{+}\) and Li\(^{+}\). To confirm that, we purified and reconstituted in proteoliposomes the AtNHX1 mutant V318I as a representative. Thereby, any differences observed can be unequivocally ascribed to the mutations introduced rather than to expression levels or to the membrane environment. We compared mutant transport capacity with that of the wild-type transporter (Fig. 7). At 100 mM cation concentration, a consistent difference in K\(^{+}\) transport versus Na\(^{+}\) was observed for the V318I mutant (Fig. 7A), whereas the wild-type transporter showed none, as previously reported (6). A representative experiment is shown in Fig. 7B. These determinations were also done with a range of cation concentrations, and similar results were found (Fig. 7C). Another two different protein preparations rendered similar results (data not shown). Consistently, the velocity of cation transport over the full range of concentrations was lower for V318I than for the wild-type protein (Fig. 7B), irrespective of the cation species. Importantly, Na\(^{+}\) transport was less effective than K\(^{+}\) in the V318I mutant at any given cation concentration, whereas the wild-type protein showed no differences. The ratio of K\(^{+}\) transport over Na\(^{+}\) transport across the whole range of concentrations tested was constant at 0.99 ± 0.02 for the wild type but increased to 1.28 ± 0.04 for the V318I mutant. This difference was found statistically significant (unpaired t test, p < 0.05).

**FIGURE 7. Cation transport activity in proteoliposomes.** Wild-type AtNHX1 and mutant V318I proteins were purified and reconstituted into liposomes to measure their cation/H\(^{+}\) exchange activity. A, proton transport assays were done as zero-trans experiments at 100 mM sodium, lithium or potassium chloride and using pyranine fluorescence to monitor H\(^{+}\) movements. Shown are means and S.E. of two independent preparations of both wild-type and V318I mutant. B, representative transport tracers for V318I mutant. At the arrows, cation-chloride (assay start (a) or NH\(_{4}\)Cl (assay end (b)) was added. D, concentration dependence of cation/H\(^{+}\) exchange velocity. Transport assays were done as in A over a 12.5–100 mM cation concentration range. A representative experiment of three with similar results is shown. C and D, wild-type AtNHX1; closed symbols, V318I mutant; □ and ▲, NaCl; ○ and ●, LiCl. D, alkali cation transport activities in tonoplast membranes from yeast OCO2 (nhx1Δ vnx1Δ) transformed with an empty vector pDR195 or the same plasmid containing wild-type AtNHX1 or mutant V318I. Transport was measured as the change of the V-ATPase-generated ΔpH gradient by the addition of 37.5 mM sulfate salts. Closed bars, K\(^{+}\) transport; open bars, Na\(^{+}\) transport.

To corroborate the improved K\(^{+}\)/Na\(^{+}\) selectivity in a biological membrane, wild-type AtNHX1 and mutant V318I were expressed in Δvnx1 cells, which are almost devoid of background K\(^{+}\)/H\(^{+}\) and Na\(^{+}\)/H\(^{+}\) exchanges. Transport assays with intact vacuoles demonstrated that mutant V318I had a greatly diminished capacity for Na\(^{+}\) transport relative to K\(^{+}\) compared with wild-type AtNHX1 (Fig. 7D). Once the background K\(^{+}\)/H\(^{+}\) exchange was subtracted, the K\(^{+}\) to Na\(^{+}\) transport ratio at 75 mM each was 1.27 for wild-type AtNHX1 and 7.73 for the V318I mutant, a 6-fold difference. In intact vacuoles, the lower rates of both K\(^{+}\)/H\(^{+}\) and Na\(^{+}\)/H\(^{+}\) exchange of V318I mutant relative to wild type were also observed and could not be attributed to differential protein abundance (see Fig. 2B). Together, these results indicate that mutation V318I reduced net cation exchange while enhancing K\(^{+}\)/Na\(^{+}\) selectivity. The reason for the much higher selectivity ratio measured in vacuoles (Fig. 7D) compared with proteoliposomes (Fig. 7A) is unclear. It could be due to posttranslational modification(s) or protein–protein interactions of AtNHX1 in the yeast cell affecting the K\(^{+}\)/Na\(^{+}\)
selectivity of the exchanger, which would be missing in recon-
stituted proteoliposomes. The C-terminal hydrophilic region
of AtNHX1 plays an important role in regulating the antiporter
activity (35). The binding of AtCaM15 to this domain of
AtNHX1 was Ca$^{2+}$- and pH-dependent and modified the
Na$^+$-K$^+$ selectivity of the antiporter, decreasing its Na$^+$/H$^+$
exchange activity (35). Mutation V318I seems to mimic this
effect by an as yet unknown mechanism.

**DISCUSSION**

Topological and mutational analyses of AtNHX1 have
revealed both similarities and differences with mammalian
counterparts, mostly dealing with the arrangement of trans-
membrane segments or the orientation of the long C terminus
(36, 37). However, site-directed mutagenesis is limited by the
predictions that can be made by the importance of the residues
under study. Random mutagenesis, instead, can reveal unsus-
pected critical residues in a protein. Our results indicate that
the mutations selected were, in most cases, of a conservative
nature (e.g. valine to isoleucine). In all cases, they introduced a
bulky side chain (Fig. 1). This suggests that the effect of these
mutations may rely on steric changes in the AtNHX1 molecule.
None of the original residues mutated was ionizable and the
only polar amino acid, Thr$^{428}$, was substituted by isoleucine.
Therefore, none of the mutated residues are likely to be can-
dates for sensing pH or cation concentrations, nor for coordi-
nating cations with their side chains. On the other hand, the
kind of residues mutated, the substitutions incorporated, and
their effect on the discrimination between K$^+$ and Na$^+$ for
transport suggest that these sites comprise structurally
important points at the molecule that may be affecting
parameters such as pore size or distance between cation
coordinating residues.

The mutations selected by our screening mapped between
transmembrane segments 8 and 12 with the exception of G144S
(Fig. 1). This last one lies at the beginning of what is considered
the cation-binding site (positions 147–193), by homology to
other transporters of the same family. This mutation had a
modest effect on both cation profile (Table 1) and their associ-
ated phenotypes (Figs. 2 and 6). Mutation V251I mapped to a
region believed to be associated with the membrane but not
forming a transmembrane segment (Fig. 1). This stretch is
homologous to IL4 in human NHE1 (38). Furthermore, Val$^{251}$
is near an aspartate residue (Glu$^{250}$) conserved in human iso-
forms NHE1 and -3 and yeast ScNHX1. Mutation V318I is
embedded in a highly conserved region between transmem-
brane segments 9 and 10 and near Glu$^{311}$, which is equivalent to Glu$^{391}$
in human NHE1 and Glu$^{335}$ in ScNHX1. These residues have
been shown to be important for transport activity (39). A site-
directed mutagenesis study on ScNHX1 showed that hydro-
phobic residues in region H10 between transmembrane seg-
ments 9 and 10 could not be replaced by conservative amino
acid substitutions (40). Residue Tyr$^{217}$ in ScNHX1 (Tyr$^{217}$ in
AtNHX1) is conserved in all intracellular isoforms of NHE/
NXH proteins (40, 41) and is essential for function (40). Even
conservative mutations (Y361F) produced an inactive protein,
indicating a strict requirement for structural specificity within
this region that must be maintained for function. Mutation
V318I falls within the equivalent region of AtNHX1 and lies
next to the conserved residue Tyr$^{217}$ (Fig. 1). Paired mutations
V381I and V382M are close to a short conserved cluster among
35 NHE-like proteins of various origins (consensus: gGlxRGA),
as identified using PROSITE. This region has been suggested
to form a P-loop in rabbit NHE3 (42), but it is considered a true
transmembrane segment in human NHE1 (38). At similar posi-
tions, mammalian NHE3 isoforms present a double valine,
whereas yeast ScNHX1 has a double methionine, and a double
isoleucine is found in mammalian isoforms 1, 2, and 4. There-
fore, residues Val$^{381}$ and Val$^{382}$ were mutated to alternative
residues found in other family members. Sequence comparison
produced no remarkable features for Thr$^{428}$. These results sug-
gest that, like cation coordination (39), ion specificity is dictated
by sites dispersed throughout the molecule. In addition, the
binding of AtCaM15 to the C-terminal domain of AtNHX1
modified the Na$^+$-K$^+$ selectivity of the antiporter (35), presum-
ably through conformational changes that could conceivably be
brought about by mutations in other critical amino acid
residues.

AtNHX1 is a tonoplast protein in plants that is also localized
to the vacuole of yeast (Fig. 5A). Cation/H$^+$ antiporters have
been repeatedly shown to be involved in intracellular pH home-
ostasis. Thus, mammalian NHE1 to -5 are critical players at the
plasma membrane for maintaining intracellular pH (43–45); NhaA from *Escherichia coli*, NhaA from *Saccharomyces* and Sod2 from *Schizosaccharomyces pombe* also have been demonstrated
to be involved in cytosolic pH homeostasis (46–48). ScNHX1
protects against hyperacidification of both vacuolar and cyto-
plasmic compartments in acid stress growth tests (40). How-
ever, in our experiments, cytosolic and vacuolar pH values were
not significantly affected by the expression of wild-type
AtNHX1 under the growth conditions used to test halotoler-
ance. None of the AtNHX1 mutants departed from this pattern
(Fig. 3) (data not shown). The cytosolic and vacuolar pH were
measured directly by the $^{31}$P chemical shifts in the cytosolic
pool of inorganic phosphate and the terminal phosphate of
polyphosphates in the vacuole (25).

AtNHX1 and ScNHX1 proteins showed extensive co-local-
ization in the endosomal system of yeast (Fig. 5B). Complimen-
tation of *vps* phenotypes of the nlhX1/vps44 mutant involved the
presence of AtNHX1 in the late endosome/prevacuolar, per-
haps while en route to the vacuole. Mutant forms of AtNHX1
improved growth, compared with the wild-type allele, on
hygromycin B and vanadate media, two phenotypes ascribed to
defects in *vps* genes (34). Hygromycin B sensitivity has also been
associated with alteration of the electrochemical gradient at the
plasma membrane (32, 33, 49). However, AtNHX1 is an intra-
cellular and electroneutral cation/H$^+$ exchanger (50), and thus,
it should theoretically not affect $\Delta \Psi$. We did not find differ-
ences in the incorporation of the $\Delta \Psi$-sensitive Dio-C9 probe (data
not shown). Furthermore, vanadate is an oxyanion, and changes in $\Delta \Psi$ across the plasma membrane could not explain
the concomitant improvement of growth on toxic cations
(hygromycin B, lithium, and sodium) and anions (vanadate).

The trans-Golgi network and endosomal and prevacuolar
compartments comprise part of the pathway where proteins
and membranes are sorted and modified while in transit to their
AtNHX1 Mutants with Improved K⁺-Na⁺ Discrimination

![Diagram of ion transporters in cell membranes]

**FIGURE 8. Sodium and potassium transporters in cell membranes.** Na⁺ ions are extruded back to the extracellular medium by the array of P-type pumps ENA1-ENA4 and the Na⁺, K⁺/H⁺ exchanger NHA1 at the plasma membrane or compartmentalized into the vacuole by the Na⁺, K⁺/H⁺ exchangers NHX1 and VNX1. The yeast NHX1 protein is primarily localized to the late endosome/prevacuolar (LE) compartment, where it has been suggested to control luminal pH, a key feature for correct intracellular vesicle and protein trafficking. Some proteins leaving the Golgi follow a pathway to the vacuole that includes transport through the LE, whereas other proteins reach the vacuole via an alternative route, bypassing the LE (heavy arrows). NHX1 functions to mediate the exit of vesicles from the late endosome/prevacuolar compartment, and this process is inhibited in nhx1 mutant cells (dashed arrows). Antiporters NHA1, NHX1, and VNX1 catalyze the exchange of Na⁺ or K⁺ for H⁺. Our results indicate that to achieve salt tolerance, a greater K⁺/Na⁺ selectivity is needed by endosomal NHX antiporters in order to prevent Na⁺ toxicity in the lumen. GA, Golgi apparatus; LE, late endosome; V, vacuole; PM, plasma membrane.

final destinations (8, 10, 51–53). Their functions are, therefore, multiple, and several phenotypes have been described in association with defects in proteins present in these organelles (54). Intracellular NHE/NHX isoforms localized to endosomal compartments seem to play critical roles in luminal pH control and vesicle trafficking (55). In mammalian cells, a Na⁺/K⁺ exchanger (NHE7) has been described to localize at the trans-Golgi network (56), and both NHE3 and NHE6 are present and functional in recycling endosomes (57, 58). Human NHE6–NHE9 are distributed along Golgi and post-Golgi endocytic compartments, and their overexpression increased the luminal pH of these compartments from the mildly acidic pH to the cytosolic pH, suggesting that their in vivo function is to regulate the pH and monovalent cation concentration in these organelles (59). Moreover, monensin, a Na⁺/H⁺ exchanger, blocks endosome-to-Golgi transport (60). Finally, in yeast, the involvement of ScNHX1 in endosomal pH homeostasis and its effect on endosomal functions are well documented (15, 31, 40) (Fig. 8). The critical requirement for a fine-tuned ionic environment of the endosomal system can explain in part the improvements derived from AtNHX1 mutants, such as better growth in NaCl, LiCl, hygromycin B, and vanadate. Initially, this work aimed to isolate AtNHX1 alleles that enhanced Na⁺ sequestration. That we obtained halotolerance with reduced Li⁺ compartmentalization (a tracer for Na⁺; Table 1) seems paradoxical. One would have expected that, in order to avert Na⁺ toxicity in the cytosol, the sequestration of Na⁺ into the vacuole and prevacuolar compartment needed to be enhanced. However, our results show a very different mechanism: the enhancement of K⁺/Na⁺ selectivity (Fig. 8). K⁺ homeostasis is emerging as a key requirement in endosomes (61). Consequently, Na⁺ and Li⁺, if they were to displace K⁺, could impair endosomal functions. Maintaining the luminal K⁺ contents while increasing the compartmentalization of toxic cations in the vacuole should require manipulating other exchangers, such as the recently identified vacuolar exchanger VNX1 (4), or the production of a greater H⁺ gradient in order to make more protons available for the vacuolar exchangers. Accordingly, previous reports showed that increasing the H⁺ gradient in yeast vacuoles by the introduction of a membrane-bound H⁺-transporting pyrophosphatase from *A. thaliana* gave rise to halotolerance (23). Conversely, impaired endosomal functions should result in salt-sensitive cells. In this regard, deletion of the yeast *ScNHX1* gene has already been reported to produce salt sensitivity (3, 5) in addition to a class E vps phenotype (15). Furthermore, many other *vps* mutants show some degree of salt sensitivity (62). The data presented here point at luminal K⁺ homeostasis as a key part of endosomal function and, thus, may help to understand these observations. A similar situation may occur in plants, where the importance of K⁺ to the endosomal system has been highlighted (61). In this regard, it must be mentioned that other isoforms of plant exchangers (AtNHX5 and -6 of *Arabidopsis* and the tomato LeNHX2) are located in cell compartments other than the vacuole, and at least the last one has shown a greater affinity for K⁺ transport compared with Na⁺, whereas vacuolar counterparts present similar transport affinities for both cations (55, 63).

The present report complements the emerging view that K⁺ homeostasis is crucial to the yeast and plant endosomal systems. It also suggests the novel perspective that the presence of Na⁺ and Li⁺ in endosomes, far from being indifferent to the cell, is detrimental for cell growth even in the presence of normal K⁺ levels. Probably these toxic cations compete with K⁺ ions for their luminal functions. This hypothesis is in agreement with the likely specialization of plant NHX antiporters based on their subcellular localization (55). These results are important for fully understanding the basis of halotolerance in plants and yeast and the interplay of intracellular organelles and their ion transporters.

Acknowledgments—We thank Rajini Rao for plasmid pRIN73, Robert C. Piper for plasmid pJLU34, and Eduardo Blumwald for strain OC02.

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Mutants of the *Arabidopsis thaliana* Cation/H\(^+\) Antiporter AtNHX1 Conferring Increased Salt Tolerance in Yeast: THE ENDOSONE/PREVACUOLAR COMPARTMENT IS A TARGET FOR SALT TOXICITY

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doi: 10.1074/jbc.M806203200 originally published online March 23, 2009

Access the most updated version of this article at doi: 10.1074/jbc.M806203200

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