Identification and characterization of a NaCl-inducible vacuolar Na$^+$/H$^+$ antiporter in *Beta vulgaris*

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We have cloned, by RT-PCR and the use of degenerate oligo-nucleotide primers, a Na$^+$/H$^+$ antiporter from *Beta vulgaris* that is homologous to *NHX1* of *Arabidopsis thaliana* and is a member of the family of recently cloned plant *NHX*-genes. This antiporter, *BvNHX1*, partially complements the salt-sensitive phenotype of a *Δena1–4Δnhx1* yeast strain. Antibodies were raised against a central portion of the *BvNHX1* open reading frame that was predicted from the cloned cDNA. This antiporter was found to be highly enriched in tonoplast membranes isolated from plant tissues. *BvNHX1* transcript abundance increased after salt treatments, in both suspension-cell cultures and whole plants. BvNHX1 protein abundance in the tonoplast-enriched membranes was also elevated after salt treatments. The vacuolar Na$^+$/H$^+$ antiporter activity increased up to 3-fold when the cell were exposed to 100 m*M* NaCl. The increase in protein abundance in response to the salt treatment, together with the salt-induced vacuolar Na$^+$/H$^+$ antiporter activity in *B. vulgaris* suggests that BvNHX1 plays an important role in salinity tolerance.

Introduction

The study of the cellular basis of salinity tolerance in plants has, of late, enjoyed a renewal with the characterization and cloning of a number of genes involved in salinity stress. Among these is the family of plant Na$^+$/H$^+$ antiporters, which has grown to between six fully sequenced members in *Arabidopsis thaliana* and at least 40 potential other members, based on EST information available in the public databases (Maser et al. 2001).

Materials and methods

Cloning of BvNHX1

Degenerate primers were designed for the cloning of an antiporter from *Beta vulgaris* mRNA. These were determined on the basis of an alignment of amino acid sequences of yeast, rat, human, nematode and plant Na$^+$/H$^+$ antiporters (not shown). The homologous regions are highlighted in the alignment shown in . The two corresponding degenerate primers are: NHX1F: 5′-CC-(AT)CC(GC)AT(CT)AT(AC)TTCAATGCAGG(CGT)-TTTCA-3′ and NHX1R:5′-T(AC)ACAACACC(CT)-TC(AGT)CC(AG)AA(GT)AC(AC)AGACTGTA-3′.

RNA was extracted from beet (*B. vulgaris* L.) cell suspension cultures using the Trizol reagent method (Invitrogen, Carlsbad, CA, USA). One µg of total RNA was used in RT-PCR reactions as specified in the Ready-To-Go™ Kit (Amersham Pharmacia, Piscataway, NJ, USA), and 2-step protocols described by the manufacturer. cDNA synthesis was carried out at 42°C for 30 min using the P6 (N6) primer. The two degenerate primers were added for PCR amplification under the following temperature cycling conditions: 32 cycles of 95°C for 30 s, 52°C for 30 s, 72°C for 1 min; and an additional polymerization step at 72°C for 5 min. The amplified fragments were purified from agarose gels and ligated into the pCR2.1-TOPO vector (Invitrogen). These constructs were used to transform *E. coli* TOPO10 (Invitrogen). On the basis of the sequences of this RT-PCR fragment, two additional primers were designed to amplify the full-length cDNA using the Smart™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA): NHX1-3R: 5′-GGAGCCATTGGCACTTTGATAT-CGTTCACC-3′ and NHX1-5R: 5′-CGAGACTGTAG-AGAAGGGGAGTTTCATCCTGG-3′.

Approximately 1.5 µg total RNA was used for first-

strand cDNA synthesis (42°C for 1.5 h) using the primers provided in the Kit. For 5′-RACE, 5 µl of 5′-RACE-Ready cDNA was used as the template. The PCR reaction was conducted under the following temperature cycling conditions: 94°C for 2 min; 32 cycles of 94°C for 5 min, 65°C for 30 s, 72°C for 3 min; and an additional polymerization step at 72°C for 5 min. For 5′-RACE the primer combination was NHX1-5R and the UPM primer provided in the kit. For 3′-RACE, 5 µl of 3′-RACE-Ready cDNA was used as the template. The PCR reaction was conducted under the following temperature cycling conditions: 94°C for 2 min; 30 cycles of 94°C for 5 min, 68°C for 10 s, 72°C for 3 min; and an additional polymerization step at 72°C for 5 min. The primers for 3′-RACE were NHX1-3R and the UPM primer provided in the kit. The amplified fragments were purified by agarose gel electrophoresis and then ligated into the pCR2.1-TOPO vector (Invitrogen). These constructs were used to transform *E. coli* TOPO10 (Invitrogen). The cloned products of the original PCR and the 5′- and 3′-RACE reactions were sequenced; contiguous sequences were assembled to provide the full-length BvNHX1 cDNAFig. 1.

Yeast complementation studies

The ORF of BvNHX1 was amplified by PCR using primers with restriction sites suitable for subcloning into the pYPGE15 yeast expression vector (gift of Ramon Serrano, Valencia, Spain). NHX1YF: 5′-GCCG(CCCG-GG)ATGAGTTTCTGAGGGTCTGG-3′ and NHX1YR: 5′-CCGGC(GTCGAC)ATATTCTGTCTA-TCAAATTTTCGG-3′.

The amplified fragment was gel purified, digested with *Sma*I and *Sal*I, and ligated into the pYPGE15 vector that had been digested with the same enzymes. *E. coli* cells were transformed and clones containing the BvNHX1 insert were selected on ampicillin; those with the ORF ligated in a sense orientation were selected by sequencing. These constructs were used for complementation studies in yeast. The three *Saccharomyces cerevisiae* mutant strains used were deletion mutants that were isogenic to the wild type strain w303 (*ura3-1 can1-100 leu2-3, 112trp1-1his3-11, 15*). The *ena1-4* (*Δena1::HIS3*) and *ena1-4nha1* (*Δena1::HIS3Δnha1::TRP1*) strains were provided by Ramon Serrano (Valencia, Spain). The *ena1-4nh×1* strain was constructed by insertion of TRYP1 into the NHX1 locus (*Δnhx1::TRP1*). Yeast were transformed by the lithium acetate method (Gietz et al. 1992). All strains were transformed with the BvNHX1 ORF or an empty pYPGE15 plasmid vector. Transformants were selected on APG media supplemented with the appropriate amino acids for each strain. APG is a synthetic minimal medium containing 10 mM arginine, 8 mM phosphoric acid, 2% (w/v) glucose, 2 mM MgSO$_4$, 1 mM KCl, 0.2 mM CaCl$_2$, trace minerals, and vitamins (Rodriguez-Navarro and Ramos 1984). For complementation, saturated liquid media cultures of each strain were serially diluted (1:10, 1:100,

1:1000) and these dilutions were spotted onto solid APG medium at different pH values (4.5, 5.5, 6.5, 7.5, adjusted with arginine) and supplemented with different concentrations of NaCl (0 mM, 25 mM, 50 mM, 75 mM). All growth was at 30°C.

Preparation of antibodies

The region corresponding to the predicted amino acid residues 122–185 of the ORF of BvNHX1 was selected for recombinant protein production in fusion with GST. The coding sequence for this region was amplified by PCR using two primers containing restriction sites (in brackets): NHX1AF: 5′-CCCGC (GGATCC)A-CATTGATATCGTTCAC-3′ and NHX1AR: 5′-CGCCG (GAATTC)TTCACAACACCCTCACC-3′.

The gel purified PCR product was digested using *Bam*HI and *Eco*RI, and then subcloned into the pGEX-2TK vector (Pharmacia, Piscataway, NJ). The fusion protein was affinity-purified on glutathione-agarose columns (Pharmacia) followed by SDS-PAGE. The fusion protein was excised from the acrylamide gels, electroeluted, and then concentrated. Purified GST-BvNHX1 fusion protein was used to as an antigen to raise antibodies in rabbits. Antisera were collected after 6 weeks. Antibodies were purified by affinity blot purification (Harlow and Lane 1988).

Plant material and isolation of membrane proteins

Beet cell suspension cultures (Blumwald and Poole 1987) were maintained in MS medium and grown in the dark at 26°C with shaking. For the salt treatment, 7-day-old cultures were supplemented with 100 mM NaCl for 1, 2 and 7 days. For analysis of BvNHX1 transcripts, total RNA was isolated from these cultures using the Trizol reagent method (Invitrogen). Thirty µg of total RNA from each sample were blotted and probed with a 3′-portion of the BvNHX1 cDNA. Beet plants were obtained from a local grocery store and were maintained in 1/2 strength MS medium supplemented with either 0 mM NaCl or 200 mM NaCl at room temperature for the 2-day treatment. Membrane proteins were isolated from cell suspension cultures as described (Blumwald and Poole 1987) with some modifications. Tonoplast-, Golgi/ER-, and plasma membrane-enriched fractions were collected from the 0%/20%, 20%/30%, and 30%/40% sucrose (w/v) interfaces, respectively. Chymostatin, leupeptin, aprotinin, and were added to the homogenization and resuspension buffers to a final concentration of 10 µM each. For isolation of membranes from beet tissues, leaves and storage roots were homogenized with sand by mortar and pestle.

Protein blots

Membrane fractions were assayed for protein concentration using the CBBR-250 dye binding and blotting assay (Ghosh et al. 1998) with BSA as a standard. Equal

amounts of protein were separated by SDS-PAGE on 10% (w/v) gels and electroblotted onto nitrocellulose. The blotted proteins were blocked for 1 h at room temperature in PBS containing 0.1% (v/v) Tween-20 (PBST) and 5% (w/v) skim milk powder. Blots were incubated with primary antibodies (diluted 1/1000 for purified BvNHX1 antibodies and 1/2000 for anti-V-PP$_i$ase antibodies) in PBST containing 1% (w/v) skim milk powder (1% MPBST) at 4°C overnight. After washing three times in PBST, blots were incubated with secondary antibodies (HRP-conjugated goat antirabbit antibodies) at a dilution of 1/10000 in 1% MPBST. Blots were washed again in PBST three times followed by detection of secondary antibodies by chemiluminescence. Signal intensities were quantified using Scion Image software (Frederick, MD, USA).

Measurement of Na$^+$/H$^+$ exchange

The fluorescence quenching of acridine orange was used to monitor the H$^+$-dependent Na$^+$ transport in tonoplast vesicles as described previously (Blumwald and Poole 1985). In all experiments, 20 µg of tonoplast vesicles were added to 0.8 ml buffer containing 0.4 M mannitol, 1 mM DTT, 10 mM Tris/Mes (pH 8.0), 1.5 mM Tris-ATP and 5 µl acridine orange, and the reaction was initiated by the addition of 3 mM MgSO$_4$. Changes in fluorescence were measured with a Hitachi 4000 spectrofluorimeter at 25°C at excitation and emission wavelengths of 495 and 540 nm, respectively, and a slit width of 3 nm for both excitation and emission.

Results

Cloning of BvNHX1

The cloned cDNA of BvNHX1 is 2602 nucleotides long, with a 5′-untranslated region of 516 nucleotides, a predicted ORF of 1657 nucleotides, and a 3′-untranslated region of 429 nucleotides. The predicted ORF encodes a protein of 552 amino acids with a calculated molecular mass of 61.2 kDa. BvNHX1 is homologous to the members of the Monovalent Cation:Proton Antiporter-1 (CPA1) Family *sensu* Paulsen et al. 1998. More specifically, BvNHX1 clusters with other plant NHX-like genes within a newly defined group of intracellular Na$^+$/H$^+$ exchangers (Nass and Rao 1998). Nine members of this family are shown in the alignment in Fig. 1 and their similarity to one another is graphically represented in Fig. 2. The predicted protein sequences of these NHX-

like genes share extensive regions of similarity that are interrupted with a few less well conserved regions, most notably about 70–100 amino acids from the C-terminus of BvNHX1. BvNHX1 is most similar to the *Atriplex gmelini* NHX1 protein (Hamada et al. 2001), but the relatedness between these two proteins cannot be taken as an indication of conservation between antiporters of 'salt-tolerant' plants.

BvNHX1 complements salt-sensitive yeast mutant

Expression of BvNHX1 in yeast provides at least partial complementation of the salt-sensitive phenotype of the *ena1-4nhx1* mutant. In order to test complementation, we used a range of pH (4.5, 5.5, 6.5, and 7.5), each with a series of NaCl concentrations (0 mM, 25 mM, 50 mM, and 75 mM). Figure 3 shows the result for the conditions providing the best complementation (pH 5.5 and 25 mM NaCl). Because the growth of the ena1-4 mutant is reduced both at lower and higher pH and higher NaCl concentrations (data not shown), these conditions were not suitable to show a recovery of growth with the expression of BvNHX1. In the presence of sodium, the growth of the ena1-4nhx1 mutant (3rd row) is restored to the levels shown by the ena1-4 mutant (2nd row) with the expression of BvNHX1 (4th-7th rows). This implies that BvNHX1 is at least partially replacing the yeast nhx1 function.

BvNHX1 localization and salt-stress effect on protein and mRNA transcript abundance

Immunoblots of membrane fractions from sugarbeet cell suspension cultures were used to assess the subcellular localization of BvNHX1. Antibodies raised against a

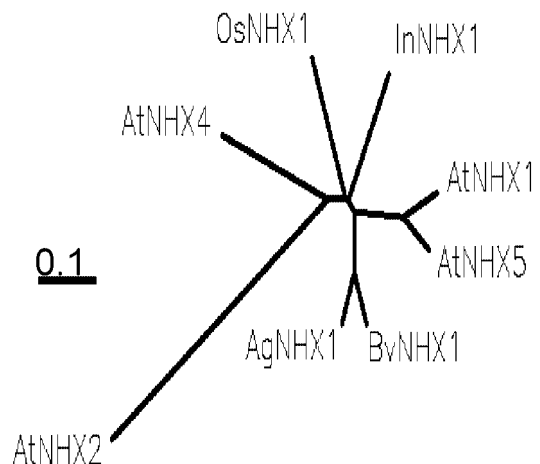Fig. 2. Phenogram of antiporters in alignment of Fig. 1. Distances were calculated using PHYLIP (Felsenstein 1993): PROTDIST was used to generate the distance matrix and FITCH was used to generate the tree. Bar corresponds to branch length of 0.1 where branch length estimates are in terms of the expected underlying numbers of changes per site (some of the changes may occur in the same site and overlie or even reverse each other).
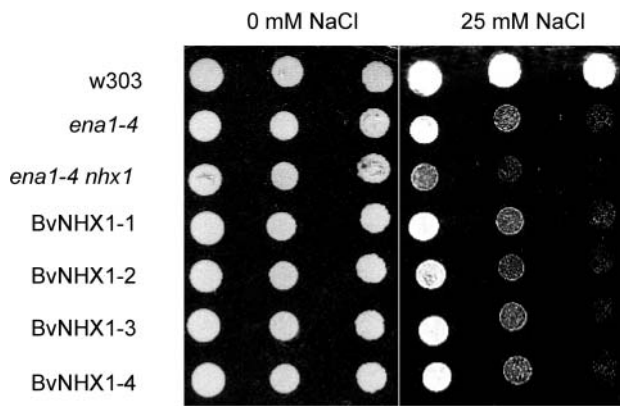
Fig. 3. Complementation of salt-sensitive phenotype by BvNHX1 of yeast mutant *ena1-4nhx1*. Three serial dilutions (1:10, 1:100, 1:1000) of saturated cultures were spotted onto APG solid media at pH 5.5 supplemented with NaCl as indicated (0 or 25 m*M* NaCl). Growth of colonies was assessed after 2 days at 30°C. The figure compares the results for wild type yeast (w303), *ena1-4*, *ena1-4nhx1*, and four independently derived clones *ena1-4nhx1BvNHX1* (BvNHX1-1, −2, −3, −4). Strains not transformed with BvNHX1-containing vector were transformed with the empty pYP-GE15 vector.

Fig. 4. Subcellular localization of BvNHX1 and the effect of salinity on BvNHX1 mRNA and protein abundance. (A) Membrane fractions were isolated from cell suspension cultures on sucrose step gradients as in methods. Equal amounts of protein (2 μg) were loaded onto a 10% SDS-polyacrylamide gel that was electroblotted onto a nitrocellulose membrane. Polyclonal antibodies, raised against the BvNHX1, were used for the immunoblot at a dilution of (1:1000), which was developed using chemiluminescence. The lane numbers correspond as follows: (1) tonoplast enriched fraction; (2) golgi/ER enriched fraction; (3) plasma membrane enriched fraction; and (4) mitochondrial fraction. (B) Tonoplast-enriched fractions were isolated from suspension cells that had been treated with 100 m*M* NaCl. Equal amounts of protein (0.5 μg) were separated and immunoblotted as above. The lane numbers correspond as follows: (0) untreated control cells; (1) 1-day treatment with NaCl; (2) 2-day treatment with NaCl; and (3) 7-day treatment with NaCl. The second panel shows the same electroblotted membrane incubated with antibodies raised against the vacuolar H^+-PP_iase (1:2000 dilution). (C) Tonoplast-enriched fractions were isolated from roots and leaves of red beet plants whose storage roots had been immersed in 1/2 MS medium supplemented with either 0 m*M* or 200 m*M* NaCl for 2 days. Equal amounts of protein (5 μg) were separated and immunoblotted as above. The blotted proteins in the top and bottom panels were incubated with antibodies raised against BvNHX1 and the V-PP_iase, respectively. Lane numbers correspond as follows: (1) leaves (0 m*M* NaCl); (2) leaves (200 m*M* NaCl); (3) roots (0 m*M* NaCl); (4) roots (200 m*M* NaCl). In all panels, the migration of proteins of known molecular masses indicated on the left in kilodaltons. (D) mRNA abundance of BvNHX1. Thirty μg of total RNA from control (lane 1) and salt-treated (lane 2) sugarbeet cell suspension cultures were blotted and probed with a 3′-portion of the BvNHX1 cDNA. Loading control is shown by EtBr staining of 35S rRNA bands.

portion of BvNHX1 detected a protein of approximately 53 kDa in the tonoplast-enriched membrane fraction (Fig. 4A). No noticeable cross-reactivity with these antibodies was observed in plasma membrane-, Golgi/ER-, and mitochondria-enriched fractions (Fig. 4A). The apparent molecular mass of BvNHX1 (53 kDa) is somewhat lower than that calculated for the predicted amino acid sequence of the BvNHX1 ORF and may reflect anomalous migration during SDS-PAGE (Ros et al. 1998) or specific cleavage (or degradation). In order to investigate the effect of salinity on the expression of BvNHX1, beet cell suspension cultures and plants were subjected to salt-treatments. Immunoblots of tonoplast-enriched fractions isolated from these tissues are shown in Fig 4B,C. In both cases, the BvNHX1 protein abundance increased after the salt treatment. BvNHX1 protein from suspension cells exposed to 100 m*M* NaCl for 1, 2 and 7 days showed 4- to 10-fold higher expression than control cells (Fig. 4B). BvNHX1 proteins from roots and tissues treated by 200 m*M* NaCl for 2 days were about 3- to 5-fold more abundant than that of control tissues (Fig. 4C). The increase in protein abundance is similar to the observed increase in BvNHX1 transcript abundance in total RNA isolated from control and salt-treated suspension cell cultures (Fig. 4D). These results suggest the induction of BvNHX1 expression by salt treatment.

Na^+/H^+ exchange activity correlates with BvNHX1 expression

The effect of NaCl on the vacuolar antiporter activity was tested in tonoplast vesicles from beet cell suspension cultures grown in the presence of 100 m*M* NaCl for a period of 1, 2 or 7 days. Initial rates of Na^+-dependent
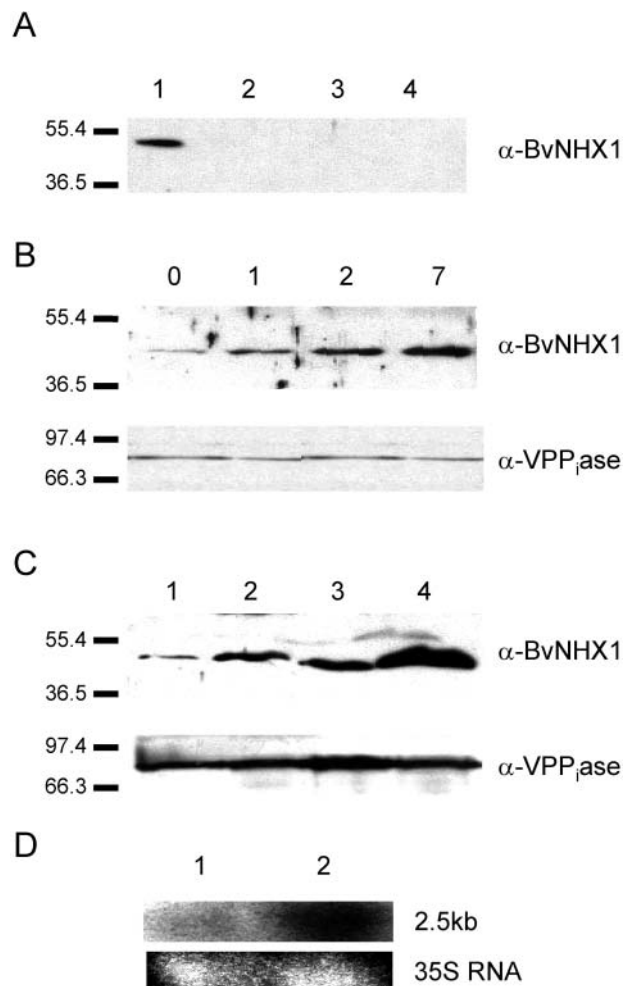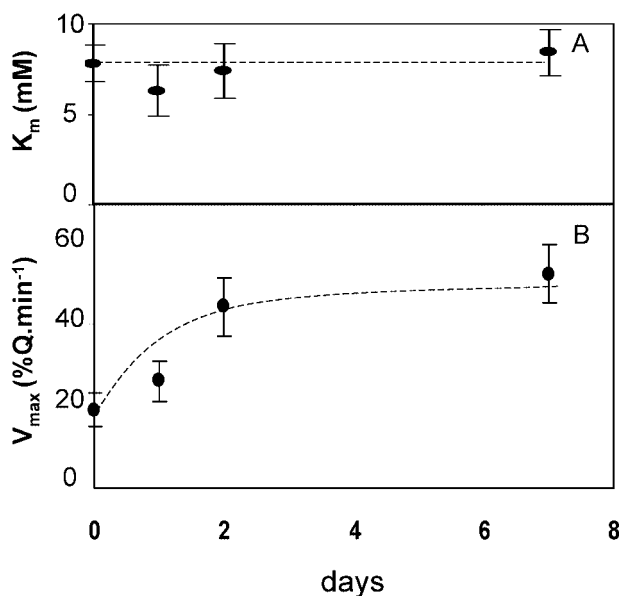
Fig. 5. Effect of NaCl in the growth medium on Na^+-dependent H^+ transported measured in tonoplast vesicles isolated from beet cell suspension. Cell suspensions were grown in the presence of 100 mM NaCl for a period of 1, 2 and 7 days. The apparent K_m and the V_{max} were calculated from plots of the initial rates of Na^+-dependent H^+ movements. Values are Mean $\pm$ SD (n = 3).

H^+ transport were measured as a percent of fluorescence quenching (Q) per minute using a range of Na^+ concentrations. The apparent K_m and V_{max} of the Na^+/H^+ exchange were determined (Fig. 5). No significant changes were seen in the apparent K_m for Na^+/H^+ exchange in tonoplast vesicles from cells grown in the absence or presence of 100 mM NaCl. On the other hand the V_{max} for the Na^+/H^+ exchange increased to almost 3-fold after 7 days of growth in the presence of salt.

Discussion

Based on the presence of a family of cloned NHX-like genes in *Arabidopsis* and the wide distribution of putative NHX-homologues in other plants, it is highly probable that NHX-like proteins occur as multiple isoforms in each plant across the plant kingdom. The yeast NHX1 gene product, Nhx1p, has been localized to the pre-vacuolar compartment (PVC) (Nass and Rao 1998) and has been shown to play a role both in osmotolerance following hypotonic shock (Nass and Rao 1999) and in endosomal protein trafficking (Bowers et al. 2000). The human endosomal sodium/proton antiporters, NHE6 and NHE7, localize to the mitochondria and *trans*-Golgi network, respectively (Numata et al. 1998, Numata and Orlowski 2001), and may have a role similar to that of the yeast Nhx1p. In plants, NHX proteins have been directly associated with the accumulation/sequestration of sodium in the vacuole (Apse et al. 1999, Zhang and Blumwald 2001, Zhang and Blumwald 2001, Zhang et al. 2001).

In *A. thaliana*, AtNHX1 expression is not significantly induced in response to a salt stress (Shi and Blumwald, unpublished). In contrast, NHXs-like transcripts are induced in response to salt in *M. crystallinum* (Chauhan et al. 2000), and as shown in this study, in *B. vulgaris*. The established hypothesis for the role of these antiporters in salinity tolerance has been that halophytes, which readily accumulate Na^+ in the vacuole, exhibit antiporter activity while glycophytes do not. This observation has carried the implication that such antiporters occur in halophytes and do not occur in glycophytes. Given the occurrence in public databases of ESTs similar to NHX1 in many plants, our observations in *Arabidopsis* and tomato (Yamaguchi and Blumwald, unpublished), it is clear that glycophytes not only possess such antiporters, but they may occur as multiple gene families. It is necessary then to modify the notion that correlates salt tolerance in plants with the existence of vacuolar Na^+/H^+ antiporters. Thus, the ability of a plant to sustain growth under high salinity conditions is not directly correlated with the presence of a gene coding for the antiporter — since it appears that these antiporters are present across taxa — but is more likely to be correlated with the antiporter activity (Apse et al. 1999, Chauhan et al. 2000).

The BvNHX1 gene product functionally complemented the yeast Δnhx1 strain. Because the yeast NHX1 protein was shown to be involved in ion sequestration (Nass and Rao 1998) and in vacuolar biogenesis (Bowers et al. 2000), the mechanistic basis of the complementation by the BvNHX1 protein is not clear. However, even the vacuolar biogenesis function of the yeast NHX1 protein is hypothesized to depend on its Na^+/H^+ exchange activity (Bowers et al. 2000). It is reasonable to conclude therefore that the expression of BvNHX1 in the yeast Δnhx1 strain provides a recovery of the salt sensitive phenotype because it functions as a Na^+/H^+ antiporter. In planta, mRNA transcript and protein levels correlated with the increase in vacuolar Na^+/H^+ antiporter activity in response to salinity treatment. It is possible that the antibodies generated against the region of BvNHX1 in this study cross-react with other putative homologues, given that the region of the protein used is conserved in members of the family in *Arabidopsis*. However, the correlation between protein and mRNA transcript abundances would support the conclusion that the protein detected by the antibody is indeed BvNHX1. The increase in the V_{max} of the Na^+/H^+ exchange with no significant change in the K_m (affinity) of the transporter for Na^+ is consistent with an increase in the number of Na^+/H^+ antiporters in NaCl-treated cells. However, the possibility that another NHX homologue is responsible for the increase in vacuolar activity (either by transcriptional or post-translational modifications) cannot be excluded.

Another interesting question remains: what is the functional significance of multiple members of the NHX gene family in plants? One function that seems clear, if not yet completely understood, is that of sodium seques-

tration. In halophytes, the accumulation of sodium in the vacuole serves to keep cytosolic concentrations of sodium at a tolerable level and to provide osmotic pressure that helps to maintain cellular water status, and ultimately, growth. Some additional NHX genes may be accounted for by expression in different tissues and/or developmental stages, although how such tissue and organ specificity is necessary remains to be seen. Other functions have been suggested for NHX genes. One of these is the regulation of vacuolar pH. The developing flower buds of morning glory change from purple to blue as the petals open. This process has been shown to depend on the activity of an NHX1 homologue in *Ipomoema nil* (Yamaguchi et al. 2001). However, it is not likely that this vacuolar pH regulation is dependent on sodium. The counter cation for proton extrusion from the vacuole is more likely to be potassium. The *Arabidopsis* NHX1 gene product does show potassium-dependent H^+ movement in isolated vacuoles and isolated tonoplast vesicles, although at a lower selectivity than for sodium (Blumwald, unpublished data; Zhang and Blumwald 2001). This suggests that other NHX genes may mediate potassium transport as well, and may have a role in potassium and pH homeostasis between the vacuole and cytoplasm.

In order to definitively connect the Na^+/H^+ antiporter activity observed at the beet tonoplast with the gene product of BvNHX1, a comparison needs to be made between the activity of membranes of untransformed cells and the membranes of cells which are derived from a stable transformation or transient transformation with a construct that inhibits expression of BvNHX1 (antisense or RNAi).

Several polypeptides whose abundance in tonoplast-enriched membranes increased in response to salt treatment were identified by photolabelling experiments with the amiloride analogue MIA (Barkla and Blumwald 1991). Further investigation revealed that the 170 kDa protein is a clathrin subunit (Apse 1998). Our results to date would indicate that the labelling of this clathrin subunit was artefactual and that this 170-kDa polypeptide is not associated with the vacuolar Na^+/H^+ antiporter.

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