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Molecular cloning and different expression of a vacuolar Na^+/H^+ antiporter gene in *Suaeda salsa* under salt stress

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Abstract

A Na^+/H^+ antiporter catalyzes the transport of Na^+ and H^+ across the tonoplast membrane. We isolated a vacuolar Na^+/H^+ antiporter cDNA (*SsNHX1*) clone from a euhalophyte, *Suaeda salsa*. The nuclear sequence contains 2262 bp with an open reading frame of 1665 bp. The deduced amino acid sequence is similar to that of *AtNHX1* and *OsNHX1* in rice, with the highest similarities within the predicted transmembrane segments and an amiloride-binding domain. Northern blot analysis shows that the expression of the *S. salsa* gene was increased by salt stress. The results suggest that the *SsNHX1* product is likely a Na^+/H^+ antiporter and may play important roles in the salt tolerance of *S. salsa.*

Additional key words: halophyte, salt tolerance, *SsNHX1*

Introduction

Sodium ions in saline soil are toxic to plants due to induction of osmotic stress and effect of excess sodium ions on cytosolic enzyme activities, photosynthesis and metabolism (Niu *et al*. 1995). Plants combat the excessive sodium in two principal ways: either by excluding Na^+ ions at the plasma membrane or by sequestering them in the large intracellular vacuole (Frommer *et al.* 1999). Sodium is compartmentalized into the vacuole through the operation of a vacuolar Na^+/H^+ antiporter, down an electrochemical proton gradient generated by the vacuolar H^+-translocating enzymes, H^+-adenosine triphosphatase (ATPase) (EC 3.6.1.35) and H^+-inorganic pyrophosphatase (PPase) (EC 3.6.1.1) (Blumwald 1987). Thus, the Na^+/H^+ antiporter can regulate the internal pH, cell volume and sodium content in the cytoplasm (Padan and Schuldiner 1996).

Na^+/H^+ antiporters are widespread in bacteria, yeast, animals and plants. In yeast, the Na^+/H^+ antiporter SOD2 is localized in the plasma membrane (Jia *et al*. 1992, Hahnenberger *et al*. 1996), while *NHX1* is found in the prevacuole membrane (Nass *et al*. 1997, 1998). In *Escherichia coli*, *NhaA*, *NhaB* and *ChaA* have been well described (Padan and Schuldiner 1996). In animals, six kinds of isoforms (*NHE1-6*) have been reported (Orlowski and Grinstein 1997). In plants, Blumwald and Poole (1985) first reported the existence of a Na^+/H^+ antiporter in tonoplast vesicles from red beet tap roots. Then in various halophytic and salt-tolerant glycophytic species, the existence of a Na^+ uptake system in the tonoplast was predicted (Barkla and Pantoja 1996, Blumwald and Gelli 1997). Recently facilitated by the *Arabidopsis thaliana* genome-sequencing project, a plant gene (*AtNHX1*) homologous to the *Saccharomyces cerevisiae NHX1* gene has been identified and characterized (Gaxiola *et al.* 1999). Overexpression of *AtNHX1* enhanced the salt tolerance of *A. thaliana*. Cell fractionation studies showed that the antiporter protein was expressed mainly in the membrane of large intracellular vacuoles (Apse *et al.* 1999). The *SOS1* (salt overly sensitive 1) gene has been identified from *A. thaliana* through positional cloning, and predicted to encode a transmembrane protein with significant similarity to plasma membrane Na^+/H^+ antiporters from bacteria and fungi (Shi *et al.* 2000).

Halophytes have NaCl tolerance mechanism different from glycophytes. Under treatment of 100 to 200 mM NaCl, their growth is accelerated with increasing Na^+ concentration (Flowers *et al.* 1977). Dicotyledonous

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halophytes accumulate NaCl in their leaves to a considerable extent to achieve an osmotic balance against the low osmotic potential of the rooting medium (Flowers *et al.* 1977, Munns *et al.* 1983). These findings suggest that the halophytes can sequester Na^+ into vacuole via a Na^+/H^+ antiporter. We isolated a Na^+/H^+ antiporter gene from a typical euhalophyte, *Suaeda salsa*. *S. salsa* is a leaf succulent euhalophyte that may have gained unique salt-tolerance mechanisms. The plant can remove sodium from the root zone and deposit it in the foliage, thus decreasing the sodium concentration in the root media by 50 % or greater (Zhang *et al.* 2001). *S. salsa* has efficient mechanisms to sequester Na^+ into the vacuoles in leaves.

Here we report the molecular cloning and characterization of a *S. salsa* gene whose product is homologous to *AtNHX1*. We show that its expression is substantially increased under NaCl stress.

Materials and methods

Plants: *Suaeda salsa* (L) Pall. seeds were placed in sand, irrigated with Hoagland solution whose composition was: 5 mM KNO_3, 2 mM $MgSO_4$, 5 mM $Ca(NO_3)_2$, 1 mM KH_2PO_4, 1 mM Fe-EDTA and micronutrients. *S. salsa* was grown under 14-h photoperiod with a photon flux density of 40 µmol $m^{-2}s^{-1}$, under 25 °C. Leaves of 6-week-old plants were used for RNA isolation. Total RNA for Northern blot was extracted from plants treated with 0, 400, and 500 mM NaCl separately for 48 h.

RNA isolation and reverse transcription - polymerase chain reaction (RT-PCR): Total RNA was isolated from *S. salsa* fleshy leaves and stems. In brief, 500 mg plant materials were ground in liquid nitrogen and extracted using *TRIZOL* (*Sargon*, Shanghai, China) reagent. With addition of 0.2 volume chloroform, after centrifugation (1 200 *g*), the supernatant was mixed with 0.5 volume isopropyl alcohol, incubated at 25 °C for 10 min, centrifuged at 4 °C. Then the RNA pellet was washed with 75 % ethanol, briefly dried and dissolved in RNase-free water. Total RNA was quantified spectrophoto-metrically. Dilutions of the RNA were electrophoresed on an RNA formaldehyde gel, the intensity of the rRNA bands was compared to confirm that equal quantities of RNA were taken for first-strand cDNA syntheses.

First-strand cDNA was synthesized from 10 µg RNA with the RNA PCR kit (*AMV*) (*TaKaRa*, Tokyo, Japan). Reverse transcription proceeded for 45 min at 45 °C.

According to the conservation domain of the transmembrane region of *NHX1* in other organisms, we designed a pair of primers, N-F: 5'-CCNCCNATHAT-HTTYAAYGCNGG-3'; N-R: 5'-YTANGCCATNAGC-ATCAT-3' (N = A+C+T+G, H = A+T+C, Y = C+T). Using those primers, the PCR cycling was as follows: 3 min at 94 °C (one cycle), 30 s at 94 °C, 1 min at 56 °C, 2 min at 72 °C (30 cycles), 10 min at 72 °C (one cycle). PCR products were analyzed by agarose gel electrophoresis.

5'- RACE and 3'- RACE: The 5' RACE was performed by 5' RACE system for rapid amplification of cDNA ends (*Version 2.0*, *Life Technologies/Gibco-BRL*, Maryland, USA). The first strand cDNA is synthesized from total RNA using *SsNHX1*-specific reverse primer, N-R-1: 5'-GAATGATACCAATGTAC-CAACG-3'. After a homopolymeric tail was added to the 3' end of the cDNA using TdT and dCTP, abridged anchor primer (5'-GGCCAACGCGTCGACTAGTA-CGGGGGGGGG-3') and N-R-2: (5'-ATGTACCAAC-GGCTCCAAAC-3') was used for PCR of dC-tailed cDNA. N-R-3: (5'-TCACCTGAAACCCCGCATTG-3') and AUAP (5'-GGCCACGCGTCGA-CTAGTAC-3') for nested amplification.

The 3' RACE was also performed by 3'RACE system of *Gibco-BRL*. The three *SsNHX1*-specific forward primers were as follows: C-F-1: 5'-TGCAAGCACTCT-GCTTGGAG-3', C-F-2: 5'-TTGGAGCAGTGACTGG-CTTG-3', C-F-3: 5'-TGGAAGGCATTCAACTGACC-3'. According to the manual, amplifications were performed.

RT-PCR of *SsNHX1* cDNA fragment: The cDNA fragment was amplified by two primers corresponding to the 5' and the 3' ends of the sequence, the forward and reverse primers are SN-F: 5'-TATCTG-AGAGCAGTCACTTGCG-3', SN-R: 5'-TAGTTTCTG-CACCAACTGCCTC-3'.

DNA sequencing and sequence analysis: Double-strand sequencing of plasmid was performed on an automated sequencer (*PE*, *Applied Biosystems*, Massachusetts, USA). Sequences were analyzed using *DNASIS* software, and databank searches were conducted through the *BLAST* program.

Northern blot analysis: Total RNA was isolated by guanidinium thioisocyanate extraction (Chomczynski and Sacci 1987). RNA amount was determined by absorbance (A_{260}), and the concentration was confirmed by electrophoresis on an RNA formaldehyde gel (Sambrook *et al.* 1989). 20 µg of total RNA was loaded per lane. The gel was then blotted onto a nylon membrane. In order to affirm uniformity in loading for RNA blots, the loaded RNAs were stained with ethidium bromide. A ^{32}P-labeled DNA probe, 400 bp fragment (3'-untranslated cDNA region) was prepared using a random primer labeling kit (*Random Primers System*, *TaKaRa*). Hybridization was performed at 50 °C, washing the membrane at room temperature.

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   1  TTT CAC AAA GAT TAT TGG ACT TCA GAA GTT TGA TTT TGT GGA GCT AGA AAG GGT TTC ACA      60
  61  TAC ATT GGA CAT TAA TTT ACT TGA ATA TAT ATA TAT TTG TTG TGG GTC TTG GAT TCG GGT      120
 121  GCA CAA AGA AAT AGG TGA ACA ATG TTG TCA CAG TTG AGC TCT TTT TTT GCA AGT AAG ATG      180
   1                              M   L   S   Q   L   S   S   F   F   A   S   K   M       13
 181  GAC ATG GTT TCG ACG TCT GAT CAT GCT TCC GTT GTT TCG ATG AAT TTG TTT GTG GCA CTG      240
  14   D   M   V   S   T   S   D   H   A   S   V   V   S   M   N   L   F   V   A   L  TM1   33
 241  TTA CGT GGC TGC ATT GTA ATT GGT CAT CTT CTC GAA GAG AAT CGC TGG ATG AAT GAA TCC      300
  34   L   R   G   C   I   V   I   G   H   L   L   E   E   N   R   W   M   N   E   S       53
 301  ATT ACA GCT TTG CTA ATA GGT TTA TCT ACT GGG ATT ATA ATC CTG CTA ATT AGT GGA GGA      360
  54   I   T   A   L   L   I   G   L   S   T   G   I   I   I   L   L   I   S   G   G  TM2   73
 361  AAG AGT TCG CAT TTG TTG GTC TTC AGT GAA GAT CTT TTC TTT ATA TAC CTC CTT CCA CCG      420
  74   K   S   S   H   L   L   V   F   S   E   D   L   F   F   I   Y   L   L   P   P  TM3   93
 421  ATT ATA TTC AAT GCG GGG TTT CAG GTG AAA AAG AAG CAA TTT TTC CGC AAC TTC ATT ACT      480
  94   I   I   F   N   A   G   F   Q   V   K   K   K   Q   F   F   R   N   F   I   T  TM4  113
 481  ATT ATT TTG TTT GGA GCC GTT GGT ACA TTG GTA TCA TTC ATA ATC ATA TCT CTT GGT TCA      540
 114   I   I   L   F   G   A   V   G   T   L   V   S   F   I   I   I   S   L   G   S       133
 541  ATA GCT ATA TTT CAA AAG ATG GAT ATT GGT TCG CTG GAG TTA GGG GAT CTT CTT GCA ATT      600
 134   I   A   I   F   Q   K   M   D   I   G   S   L   E   L   G   D   L   L   A   I  TM5  153
 601  GGT GCA ATA TTC GCT GCA ACT GAT TCA GTT TGC ACA TTG CAA GTG CTT AAT CAA GAT GAG      660
 154   G   A   I   F   A   A   T   D   S   V   C   T   L   Q   V   L   N   Q   D   E       173
 661  ACT CCA CTT CTT TAT AGT CTC GTG TTT GGT GAA GGT GTC GTC AAT GAT GCT ACA TCA GTG      720
 174   T   P   L   L   Y   S   L   V   F   G   E   G   V   V   N   D   A   T   S   V  TM6  193
 721  GTG TTG TTC AAT GCA ATT CAA AAC TTT GAC CTC ACG CAC ATT GAC CAC AGA ATT GCC TTC      780
 194   V   L   F   N   A   I   Q   N   F   D   L   T   H   I   D   H   R   I   A   F       213
 781  CAA TTT GGT GGC AAC TTT CTA TAT TTA TTT TTT GCA AGC ACT CTG CTT GGA GCA GTG ACT      840
 214   Q   F   G   G   N   F   L   Y   L   F   F   A   S   T   L   L   G   A   V   T  TM7  233
 841  GGC TTG CTA AGC GCT TAT GTC ATC AAA AAG TTG TAC TTT GGA AGG CAT TCA ACT GAC CGT      900
 234   G   L   L   S   A   Y   V   I   K   K   L   Y   F   G   R   H   S   T   D   R       253
 901  GAG GTA GCC TTA ATG ATG CTT ATG GCT TAT CTA TCG TAC ATG CTT GCT GAA CTC TTC TAT      960
 254   E   V   A   L   M   M   L   M   A   Y   L   S   Y   M   L   A   E   L   F   Y  TM8  273
 961  CTG AGC GGA ATT CTT ACA GTA TTC TTC TGT GGG ATT GTC ATG TCC CAT TAT ACA TGG CAC     1020
 274   L   S   G   I   L   T   V   F   F   C   G   I   V   M   S   H   Y   T   W   H       293
1021  AAT GTG ACG GAG AGC TCC AGA GTA ACC ACC AAG CAT GCT TTT GCA ACA CTC TCT TTT GTA     1080
 294   N   V   T   E   S   S   R   V   T   T   K   H   A   F   A   T   L   S   F   V  TM9  313
1081  GCT GAG ATC TTC ATC TTT CTA TAT GTT GGT ATG GAT GCA CTG GAT ATT GAG AAG TGG AGA     1140
 314   A   E   I   F   I   F   L   Y   V   G   M   D   A   L   D   I   E   K   W   R       333
1141  TTT GTG AGC GAT AGT CCT GGA ACA TCT GTT GCT GTG AGT TCC ATA CTG CTT GGT CTT CAC     1200
 334   F   V   S   D   S   P   G   T   S   V   A   V   S   S   I   L   L   G   L   H TM10  353
1201  ATG GTT GGG CGA GCT GCT TTT GTT TTT CCC TTC GCC TTT TTA ATG AAC TTG TCC AAG AAA     1260
 354   M   V   G   R   A   A   F   V   F   P   F   A   F   L   M   N   L   S   K   K       373
1261  TCA AAT AGT GAG AAG GTC ACC TTC AAT CAG CAG ATA GTC ATT TGG TGG GCT GGT CTC ATG     1320
 374   S   N   S   E   K   V   T   F   N   Q   Q   I   V   I   W   W   A   G   L   M TM11  393
1321  AAA AGT GCT GTC TCC GTG GCA CTT GCT TAT AAT CAG TTT TCA AGG TCA GGA CAC ACA CAG     1380
 394   K   S   A   V   S   V   A   L   A   Y   N   Q   F   S   R   S   G   H   T   Q       413
1381  CTG AGG GGA AAT GCA ATC ATG ATT ACA AGC ACC ATA ACC GTT GTC CTT TTC AGT ACG ATG     1440
 414   L   R   G   N   A   I   M   I   T   S   T   I   T   V   V   L   F   S   T   M TM12  433
1441  GTA TTT GGG TTG CTG ACA AAG CCT CTT ATA CTC TTT ATG TTG CCT CAA CCG AAA CAT TTC     1500
 434   V   F   G   L   L   T   K   P   L   I   L   F   M   L   P   Q   P   K   H   F       453
1501  ACT AGT GCA AGC ACC GTG TCA GAT TTG GGG AGT CCA AAG TCA TTC TCC TTG CCT CTT CTT     1560
 454   T   S   A   S   T   V   S   D   L   G   S   P   K   S   F   S   L   P   L   L       473
1561  GAA GAT AGA CAA GAT TCT GAA GCT GAT TTG GGC AAC GAT GAT GAA GAA GCC TAC CCC CGT     1620
 474   E   D   R   Q   D   S   E   A   D   L   G   N   D   D   E   E   A   Y   P   R       493
1621  GGG ACT ATA GCT CGA CCT ACT AGT CTT CGT ATG CTA CTA AAT GCA CCA ACT CAC ACT GTC     1680
 494   G   T   I   A   R   P   T   S   L   R   M   L   L   N   A   P   T   H   T   V       513
1681  CAT CAT TAT TGG CGC AGA TTC GAT GAT TAT TTC ATG CGG CCT GTA TTT GGT GGC CGG GGT     1740
 514   H   H   Y   W   R   R   F   D   D   Y   F   M   R   P   V   F   G   G   R   G       533
1741  TTT GTA CCT TTT GTC CCA GGT TCA CCC ACC GAA CAG AGC ATC ACT AAT TTG TCA CAG AGA     1800
 534   F   V   P   F   V   P   G   S   P   T   E   Q   S   I   T   N   L   S   Q   R       553
1801  ACA TAA GTT AGC GAT AAT TGA GGC AGT TGG TGC AGA AAC TAA TAA CTT ACA GCC CTA CAG     1860
 554   T   *                                                                           555
1861  GCA ATC TAC AAA GAC AAA AAA TGC CCT TAC CCA AGA ACG AAC AGC CCG GTG TTT GGT CTC     1920
1921  GTG GGC TTG ATG TTA AGA CTG TGC TGT ACT TCT GTT AAT AGA GAG TAA GTT ACA GAA ACC     1980
1981  ACC GAT TTA AAC ATA TCT GTA ATT TTT TAC AGC ATG GAT ATT CGA TGC ATT CTT TAA TCT     2040
2041  GGC TGT AGC TAG AAT ACT CTA GCA TGT TTT GTA GTT TCA GTC TTA CCA TTT AGG TTT TCT     2100
2101  CCT ACA TAA CCT CAA TAA GCT GTT TAG TGT GCT TAC TGC TTA CTT TAG AGC AAA CTG CAA     2160
2161  CTG TGA AAA TTG CTT ACG TCA GCG GCA CCT GTG TAA TTT ATC ATT TTT ATA ATG ATG GAG     2220
2221  CAT GAT CAT TTG CAA TCA AAT TTA CAA TAC TGT GAT TAA AAA                           2262
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Fig. 1. Nucleotide sequence of *SsNHX1* cDNA and deduced amino acid sequence of *SsNHX1*, the accession number is AF370358. Nucleotide sequences and deduced sequence of amino acid residues of the insert in the *S. salsa* Na^+/H^+ antiporter (*SsNHX1*) cDNA clone. The amino acid residues are indicated by a single letter code. Three potential glycosylation sites are in the boxes. The 12 putative transmembrane domains (TM) are underlined.

Results

Isolation of *SsNHX1*: Using the primers N-F and N-R for RT-PCR a 0.5-kb band was observed. Sequencing of this fragment showed that it contains the conserved transmembrane domain and had high homology to *AtNHX1* (approximately 81 % identity in amino acids). Using the 5'-RACE and 3'-RACE systems, two PCR products were obtained separately, the 5' product was 0.4 kb and the 3' product was 1.4 kb. With the primers corresponding to 5' and 3' ends, a 2.3-kb fragment was amplified. The fragment was cloned into pMD18 vector and sequenced (Fig. 1).

The cDNA was 2.3 kb with a 5'-untranslated region of 141 bp, an open reading frame (ORF) of 1665 bp and a 3'-untranslated region of 455 bp. The amino acid sequence deduced from the ORF showed that the cDNA encodes a protein of 554 amino acids with a calculated molecular mass of 61.2 kDa.

Structural analysis of *SsNHX1*: Hydropathy plot analysis of the sequence (by the method of Hofmann and Stoffel 1993) revealed that the N-terminal portion of *SsNHX1* is highly hydrophobic and has 12 putative transmembrane domains (Figs. 1, 2), the C-terminal portion is a highly hydrophilic tail in the product (Fig. 2). The deduced amino acid sequence (*SsNHX1*) has high similarity with *McNHX1* (88 %), and is similar to *AtNHX1* and *OsNHX1* with identity 67 - 68 %.

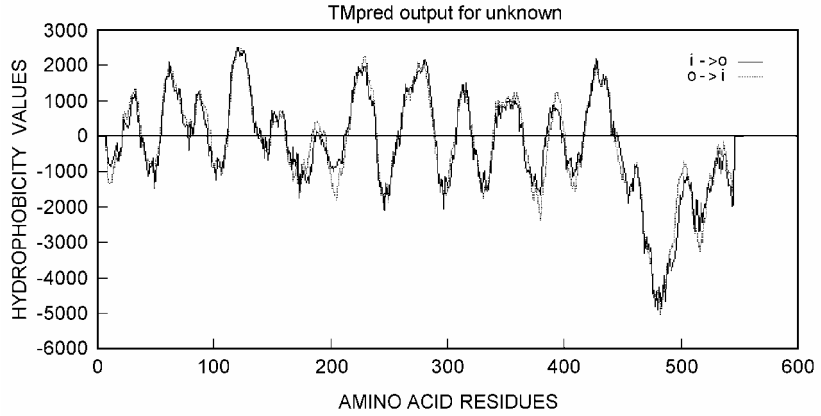

Fig. 2. Hydrophobicity plot of *SsNHX1*. The hydrophobicity values were calculated by the program TMpred available at http://www.ch.embnet.org/software/TMPRED-form.html

Based on the preliminary topological model and the known sites of glycosylation in other *NHE* isoforms, we hypothesized that the likely site(s) of N-linked glycosylation were on the loops between transmembrane segments, namely at one or more of the residues Asn-49, -292 and -367. These sites are located near the positions of the consensus N-glycosylation sites in human *NHE1* (Counillon *et al.* 1994). The results suggest that the *SsNHX1* protein is glycosylated.

In the eukaryotic Na^+/H^+ antiporter, the membrane-spanning segments are well conserved. *SsNHX1* shares high similarity with other vacuolar Na^+/H^+ antiporters, *AtNHX1*, *OsNHX1* and *InNHX1* within predicted transmembrane segments (Fig. 3). The sequence of ^{85}LFFIYLLPPI^{94} in *SsNHX1* is highly conserved within *AtNHX1*, *OsNHX1*, *NHX1* and mammalian *NHE*. In mammals, this region is identified as the binding site of amiloride which inhibits the eukaryotic Na^+/H^+ exchanger. These results indicated that the gene *SsNHX1* is a vacular-type Na^+/H^+ antiporter.

Phylogenetic analysis of different Na^+/H^+ antiporters indicated that the halophytes *Mesembryanthemum crystallinum* and *S. salsa* shared the same origin. They also shared the same origin from glycophytes, but they were different from yeast (Fig. 4).

Expression analysis of *SsNHX1*: To examine if the expression of the *SsNHX1* gene in *S. salsa* was regulated by Na^+ concentration, a piece of nylon membrane was transferred with total RNAs from plants treated for 48 h with 0, 400, or 500 mM NaCl. To examine the tissue-specific expression of *SsNHX1* under NaCl stress, the other two membranes were transferred separately with total RNAs from roots or leaves of the plants treated with 0, 400, or 500 mM NaCl. The loaded RNAs were stained with ethidium bromide to access the relative quantity in each lane. A hybridization band about 2.4 kb was observed in every lane.

The expression of *SsNHX1* was increased by NaCl treatment, both in the whole plant and in root, leaf tissues. With the Na^+ concentration increased, the mRNA amount increased also. The results showed that the expression of *SsNHX1* was significantly stimulated by salt stress in the whole plant (Fig. 5*A*). In the leaves, relative amounts of mRNA increased up to 8- and 10-fold higher than the control (0 mM) in response to 400 and 500 mM NaCl treatment (Fig. 5*B*). In the roots, the mRNA increased up to 4 to 5 times, respectively (Fig. 5*C*). On the whole, *SsNHX1* expression was up-regulated by salt stress in both roots and leaves, and the amounts of induction in leaves were larger than this in roots.

Fig. 3. Amino acid sequence alignment of *SsNHX1* with *NHXs* from other species. All sequences are from GenBank, EMBL and DDBJ databases. The accession numbers and sources of each of the other representative Na^+/H^+ antiporters are as follows: *S. salsa* (*SsNHX1*; AF370358), *A. thaliana* (*AtNHX1*; AC 009465), *Ipomoea nil* (*IoNHX1*; AB033989), *Oryza sativa* (*OsNHX1*; AB021878), *Saccharomyces cerevisiae* (*ScNHX1*; NP-010744.1). Sequences were aligned by the program *Clustalx*. Aligments are from the N terminus of each sequence. Asterisks indicate the identical amino acid residues, colons indicate amino acids that have high similarity, periods indicate amino acids that have low similarity, and dashes indicate gaps. The amiloride binding sites are shaded.

Discussion

To cope with salt stress, plants have developed the mechanisms of ion homeostasis including Na^+ extrusion system, or sequester Na^+ into the vacuole and regulate the ratio of K^+/Na^+ (Blumwald 2000a). Na^+/H^+ antiporter plays a role in the Na^+ compartmentalization. In the glycophyte *A. thaliana*, sodium ions flow through the Na^+/H^+ antiport into the prevacuoles and then into the large vacuole through a pathway of vesicles (Apse *et al.* 1999, Frommer *et al.* 1999).

S. salsa is an important euhalophyte exhibiting high degree of salt tolerance with leaf succulent character. It does not have salt glands or salt bladders on its leaves. Thus this plant must compartmentalize the excessive Na^+ in the vacuoles. Therefore, membrane-bound transport systems regulating cytosolic ion homeostasis and ion accumulation in the vacuole can be considered of crucial

importance for adaptation to saline conditions (Serrano *et al.* 1999, Hasegawa *et al.* 2000). It is an ideal plant for studying Na^+ sequestration of the vacuole. Therefore, we isolated a putative vacuolar Na^+/H^+ antiporter gene from this euhalophyte.

Structural analysis shows that the *SsNHX1* protein is predicted to have 12 transmembrane domains in its N-terminal portion and these domains are conserved in vacuolar Na^+/H^+ antiporter (Fig. 3). This suggests that *SsNHX1* contains conserved region consistent with the other vacuolar Na^+/H^+ antiporters. *SsNHX1* also has a C-terminal hydrophilic tail which is shorter than that in animals. There is a binding site of amiloride that plays as the exchange activity inhibitor. Phylogenetic analysis revealed that *SsNHX1* clusters with vacuolar Na^+/H^+ antiporters from plants such as *McNHX1, AtNHX1,* it does not cluster with Na^+/H^+ antiporters from yeast and animals (Fig. 4). All these analyses indicate that the *SsNHX1* protein may function at the tonoplast to sequester Na^+ into vacuole.

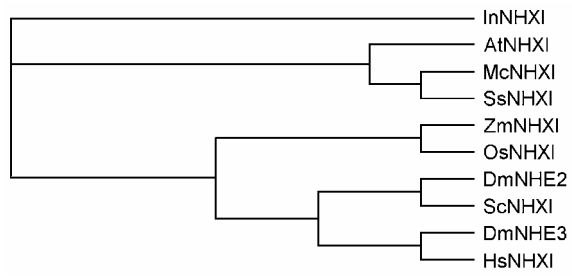

Fig. 4. Phylogenetic analysis of Na^+/H^+ antiporter proteins. The accession numbers and sources of other five Na^+/H^+ antiporters are: *Mesembryanthemum crystallinum* (*McNHX1*; AF 279671), *Zea mays* (*ZmNHX1*; AF 307944), *Drosophila melanogaster* (*DmNHE3*; AE 003614), *D. melanogaster* (*DmNHE2*; AE 003669), *Homo sapiens* (*HsNHX1*; M 81768).

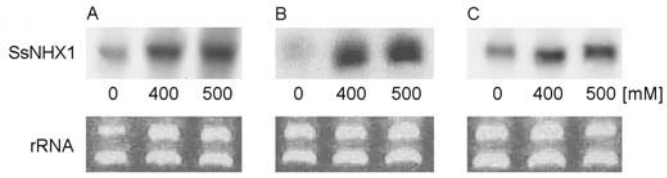

Fig. 5. Up-regulated expression of *SsNHX1* by NaCl stress. Total RNAs in the lanes were isolated from *S. salsa* with 0, 400, or 500 mM NaCl treated for 48 h. A fragment of *SsNHX1* cDNA was used as probe. The expression were monitored in the whole plant (*A*), leaves (*B*), roots (*C*). The loaded RNAs were stained with ethidium bromide (rRNA), rRNA is showen to served as a control for the same quantity of total RNAs.

Comparison of the amino acid sequence with other three plant genes showed that the variable regions were at the N-terminal (2-7) and the C-terminal (449-498, 546-554) regions. Some experiment findings have demononstrated that the structure subdivision was consistent with the partition of function (Dibrov and Fliegel 1998). The non-homologous regions can reflect the difference in Na^+/H^+ antiporter activities between halophytes and glycophytes. It will help to know why halophytes have efficient mechanisms to compart Na^+ into vacuoles.

Northern blot indicated that the *SsNHX1* gene expression was up-regulated by NaCl stress. The induced expression at 500 mM were larger than at 400 mM, and with the Na^+ concentration elevating, the induced amounts increased. This up-regulation was consistent with the role of *SsNHX1* in Na^+ tolerance. It has been known that vacuolar H^+-ATPase and H^+-PPase provided proton-motive force to drive Na^+ intracellular sequestration via Na^+/H^+ antiporter (Blumwald 1987). The expression of vacuolar H^+-ATPase gene was up-regulated by salt stress in *S. salsa* (Wang *et al.* 2000), the increase of V-ATPase would provide driving force that can sequestrate Na^+ in vacuole, to increase Na^+/H^+ antiporter activity as in *M. crystallinum* (Rataczak *et al.* 1994, Barkla *et al.* 1995).

Northern blot results also suggested that the increased ratio of *SsNHX1* expression in leaves was larger than this in roots, the *SsNHX1* expression was tissue-specific. It was coordinated with the findings in a facultative halophyte ice plant: no up-regulation of V-ATPase subunit E was seen in any root cell, even indicated down-regulation, suggests that roots are apparently unable to accumulate Na^+, and Na^+ is passed to the xylem for translocation to the leaves (Golldack and Dietz 2001).

Although functional adaptation mechanisms are likely to be largely conserved among glycophytes, halophytic organisms have evolved additional structural or distinct stress-recognition system and regulatory controls that account for their ability to withstand severe osmotic or ionic stress (Very *et al.* 1998). For a long while, the lack of progress in the characterization of the plant Na^+/H^+ antiporter has hindered our understanding of the cellular and molecular bases of salt tolerance (Blumwald 2000b). Now, most of the studies of plant Na^+/H^+ antiporter genes were focused on glycophytes (Fukuda *et al.* 1999). Based on the fact that the Na^+/H^+ antiporter works more efficiently in halophytes, so we isolated Na^+/H^+ antiporter from euhalophyte. It would be convenient for the study of regulatory controls system of the Na^+/H^+ antiporter, benefit for the study of the salt-tolerance mechanism in the whole plant.

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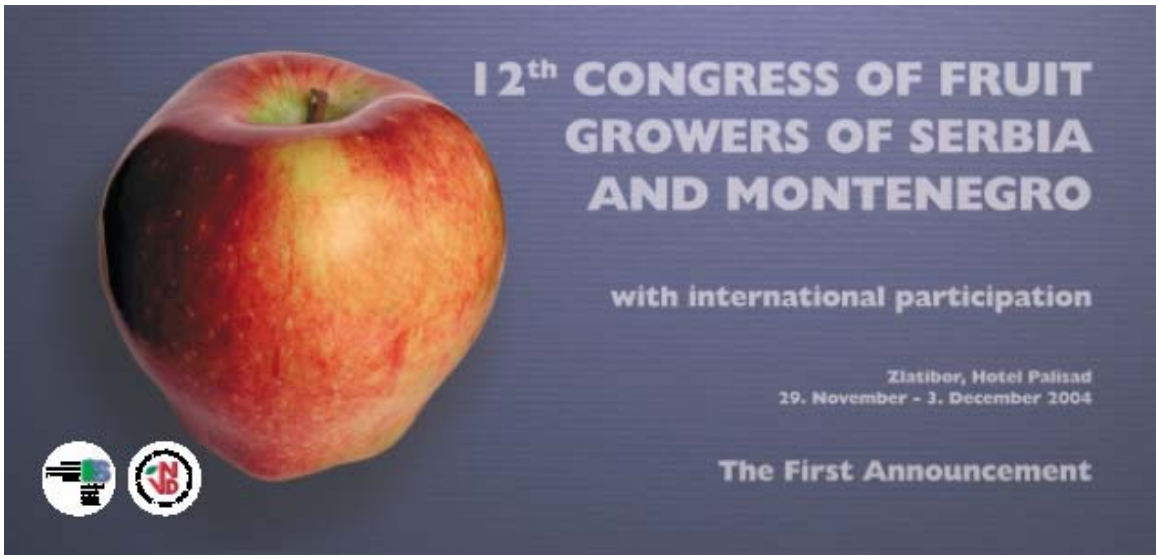

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