

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

X.-L. MA*, Q. ZHANG*, H.-Z. SHI**, J.-K. ZHU**, Y.-X. ZHAO*, C.-L. MA* and H. ZHANG*¹

Key Laboratory of Plant Stress Research, College of Life Science, Shandong Normal University, Jinan, 250014, China*

Department of Plant Sciences, University of Arizona, Tucson, AZ 85721, USA**

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

Received 17 January 2003, accepted 22 August 2003.

Acknowledgements: This work was supported by the National Natural Science Foundation of China (project 39980072), the National High Technology Research Development Project (2002AA629080) and the Development plan of the State Key Fundamental Research Project (G1999011700).

¹ Corresponding author present address: Life Science College of Shandong Normal University, Wenhua East Road 88, Jinan, 250014, Shandong Province, PR China; fax: (+86) 531 6180764, e-mail: zhangh@sdsu.edu.cn

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 $\text{Ca}(\text{NO}_3)_2$, 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 $40 \mu\text{mol m}^{-2}\text{s}^{-1}$, 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 spectrophotometrically. 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 *NHXL* in other organisms, we designed a pair of primers, N-F: 5'-CCNCCNATHATHTTYAAYGCNGG-3'; N-R: 5'-YTANGCCATNAGCATCAT-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'-GGCCAACGCGTCTGACTAGTA-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'-GGCCACGCGTCTGA-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'-TGCAAGCACTCTGCTTGGAG-3', C-F-2: 5'-TTGGAGCAGTGACTGGCTTG-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'-TATCTGAGAGCAGTCACTTGCG-3', SN-R: 5'-TAGTTTCTGCACCAACTGCCTC-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 thiocyanate 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.

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	TGT	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	TTT	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	Q	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	KCA	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	T	L	V	F	G	E	C	G	V	N	D	A	T	S	TM6 193
721	GTG	TTG	TTT	ATA	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	GCT	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	Y	TM9 313
1081	GCT	GAG	ATC	TTT	ATC	TTC	TAT	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	TTT	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	L	P	L	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

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 %.

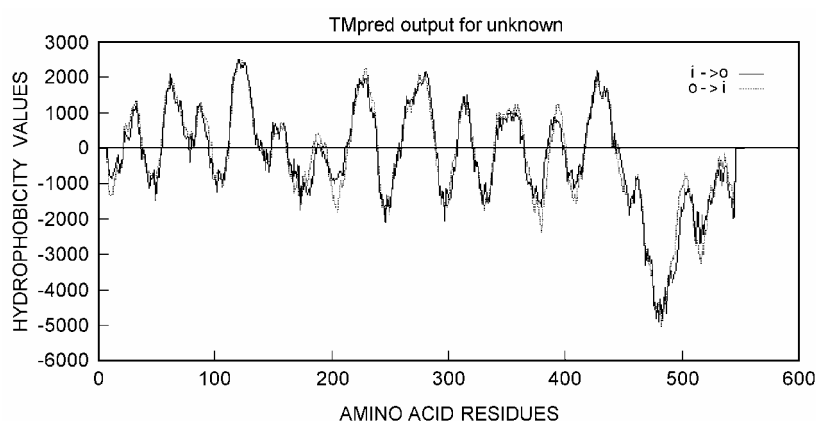


Fig. 2. Hydropathy plot of *SsNHX1*. The hydrophobicity values were calculated by the program TMPred available at <http://www.ch.embnet.org/software/TMPRED-form.html>

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 $^{85}\text{LFFIYLLPPI}^{94}$ 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 vacuolar-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. 5A). 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. 5B). In the roots, the mRNA increased up to 4 to 5 times, respectively (Fig. 5C). 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.

```

SsNHX1 MSLQLSSFFASKMDMVSTSDHASVVMNLFVALLRGCIVIGHLLEENRWMNESITALLIG
AtNHX1 MLDL-----VSKLPSTSDHASVVALNLFVALLCACIVLGHLEENRWMNESITALLIG
OsNHX1 MGMEVAA---ARLGALYTSDYASVVSINLFVALLCACIVLGHLEENRWMNESITALLIG
InNHX1 MAFGLSS---LLQNSDLFTSDHASVVMNLFVALLCACIVLGHLEENRWMNESITALLIG
*      :      ***:****:*****.***:*****:*****;*
SsNHX1 LSTGIIILLISGGKSSHLVFSDELFFIYLLPPIIFNAGFQVKKKQFFRNFTITILFGAV
AtNHX1 LGTGVITILLISGGKSSHLVFSDELFFIYLLPPIIFNAGFQVKKKQFFRNFTITILFGAV
OsNHX1 LCTGVVILLMTKGKSSHLVFSDELFFIYLLPPIIFNAGFQVKKKQFFRNFTITILFGAV
InNHX1 LCTGVVILLSSGGKSSHLVFSDELFFIYLLPPIIFNAGFQVKKKQFFVNFMTIMLFGAI
* ** : ** : : *****:*****:*****:*****:*****:*****:
SsNHX1 GTLVSFIIISLGSIAIFQKMDIGSLELGDLLAIGAIFAATDSVCTLQVLNQDETPLLYS
AtNHX1 GTIISCTIIISLGVTFQFFKKLDIGTFDLGDYLAIGAIFAATDSVCTLQVLNQDETPLLYS
OsNHX1 GTMISFFTIIISIAAIAIFSRMNIIGTLVDGFLAIGAIFSATDSVCTLQVLNQDETPFLYS
InNHX1 GTLISCSIIISFGAVKIFKHLIDFLDFGDYLAIGAIFAATDSVCTLQVLSQDETPLLYS
**:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:*
SsNHX1 VFGEVVDATSVVLFNAIQNFDLTHIDHRIAFQGGNLYLFFASTLLGAVTGLLSAYV
AtNHX1 VFGEVVDATSVVVFNAIQSFDLTHLNHEAAHLLGNFLYLFLSTLLGAATGLISAYV
OsNHX1 VFGEVVDATSVVLFNALQNFDLVHIDAADVVKFLGNFFYFLSSTFLGVFAGLLSAYI
InNHX1 VFGEVVDATSVVLFNAIQSFDMTSFDPKIGLHFIIGNFLYLFLSSTFLGVIGLLCAYI
*****:***:* **:* **:* **:* **:* **:* **:* **:* **:* **:*
SsNHX1 IKKLYFGRHSTDREVALMMLMAYLSYMLAELFYLSGILTVFFCGIVMSHYTWHNVTESSR
AtNHX1 IKKLYFGRHSTDREVALMMLMAYLSYMLAELFDLSGILTVFFCGIVMSHYTWHNVTESSR
OsNHX1 IKKLYIGRHSTDREVALMMLMAYLSYMLAELLDLSGILTVFFCGIVMSHYTWHNVTESSR
InNHX1 IKKLYFGRHSTDREVALMMLMSYLSYMAELFYLSGILTVFFCGIVMSHYTWHNVTESSR
*****:*****:*****:*****:*****:*****:*****:*****:
SsNHX1 VTTKHAFATLSFVAEIFIFLYVGMDALDIEKWRVSDSPGTSVAVSSILLGLHVMGRAAF
AtNHX1 ITTKHTFATLSFLAETFIIFLYVGMDALDIDKWRVSDDTPGTSIAVSSILMGLVMVGRAAF
OsNHX1 VTTKHAFATLSFIAETFLFLYVGMDALDIEKWEFASDRPGKSGIGISSILLGLVLIGRAAF
InNHX1 VTTRHSFATLSFVAETFIIFLYVGMDALDIEKWKVFNKSGQLSVAVSSILVGLILVGRAAF
**:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:*
SsNHX1 VFPFAFLMNLSSKSNSEKVTFNQIVIIWWAGLMKSAVSALAYNQFSRSGHTQLRGNAIM
AtNHX1 VFPLSFLSNLAKKNQSEKINFNMQVVIWWSGLMRGAVSMALAYNKFTRAGHTDVRGNAIM
OsNHX1 VFPLSFLSNLTKKAPNEKITWRQVVIWWAGLMRGAVSIALAYNKFTRSGHTQLHGNNAIM
InNHX1 VFPLSFLSNLAKKNSSDKISFRQIIIIWWAGLMRGAVSIALAYNKFTRSGHTSLHENAIM
**:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:*
SsNHX1 ITSTITVVLFTSMVFGLLTKPLILFMLPQPKHFTSAST---VSDLGSPKSPSLPLEDRQ
AtNHX1 ITSTITVCLFSTVVFGLTKPLISYLLPHQN---ATTSM---LSDDNTPKSIHIPLLD---Q
OsNHX1 ITSTITVVLFTSMVFGMMTKPLIRLLP-----ASGHP---VTSEPSPKSLHSPLLTSMQ
InNHX1 ITSTVTVVLFTSVVFGLMTKPLINLLPPHKQMPSGHSSMTTSEPSPKHFTVPLLDNQ
****:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:* **:*
SsNHX1 DSEADLGNDDEEAYPRGTIARPTSLRMLNAPHTVHHYWRWFDDYFMRPVFGGRGFVPF
AtNHX1 DSFIEPSGNH-----VPRPDSIRGFLTRPTRTVHHYWRWFDDYFMRPVFGGRGFVPF
OsNHX1 GSDLESTTN-----IVRPSSLRMLLTKPHTVHHYWRWFDDALMRPMPFGGRGFVPF
InNHX1 DSESDMITGPE-----VARPTALRMLLRTPHTVHHYWRWFDDYFMRPVFGGRGFVPF
.* : . : : ** : * : * **:* **:* **:* **:* **:* **:*
SsNHX1 VPGSPTEQSITNLSQRT
AtNHX1 VPGSPTERNPPDLKA-
OsNHX1 SPGSPTEQSHGGR----
InNHX1 VAGSPVEQSPR-----
.***.*.

```

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*. Alignments 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.

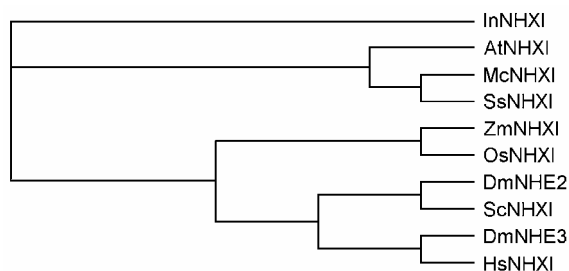


Fig. 4. Phylogenetic analysis of Na^+/H^+ antiporter proteins. The accession numbers and sources of other five Na^+/H^+ antiporters are: *Mesembryanthemum crystallinum* (*McNHX1*; AF 279671), *Zea mays* (*ZmNHX1*; AF 307944), *Drosophila melanogaster* (*DmNHE3*; AE 003614), *D. melanogaster* (*DmNHE2*;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

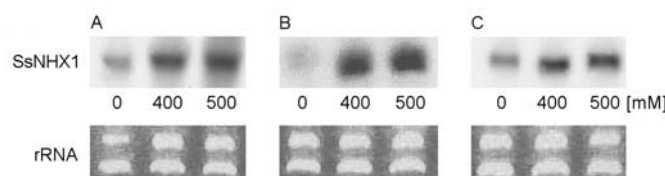


Fig. 5. Up-regulated expression of *SsNHX1* by NaCl stress. Total RNAs in the lanes were isolated from *S. salsa* with 0, 400, or 500 mM NaCl treated for 48 h. A fragment of *SsNHX1* cDNA was used as probe. The expression were monitored in the whole plant (A), leaves (B), roots (C). The loaded RNAs were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

References

- Apse, M.P., Aharon, G.S., Snedden, W.A., Blumwald, E.: Salt tolerance conferred by overexpression of a vacuolar Na⁺/H⁺ antiporter in *Arabidopsis*. - Science **285**: 1256-1258, 1999.
- Barkla, B.J., Pantoja, O.: Physiology of ion transport across the tonoplast of higher plants. - Annu. Rev. Plant Physiol. Plant mol. Biol. **47**: 159-184, 1996.
- Barkla, B.J., Zingarelli, L., Blumwald, E., Smith, J.A.C.: Tonoplast Na⁺/H⁺ antiporter activity and its energization by the vacuolar H⁺-ATPase in the halophytic plant *Mesembryanthemum crystallinum* L. - Plant Physiol. **109**: 549-556, 1995.
- Blumwald, E.: Tonoplast vesicles as a tool in the study of ion-transport at the plant vacuole. - Physiol. Plant. **69**: 731, 1987.
- Blumwald, E.: Sodium transport and salt tolerance in plants. - Curr. Opin. Cell Biol. **12**: 431-434, 2000b.
- Blumwald, E., Aharon, G.S., Apse, M.P.: Sodium transport in plant cells. - Biochim. biophys. Acta **1465**: 140-151, 2000a.
- Blumwald, E., Gelli, A.: Secondary inorganic ion transport at the tonoplast. - Adv. Bot. Res. **25**: 401, 1997.
- Blumwald, E., Poole, R.J.: Na⁺/H⁺ antiporter in isolated vesicles from storage tissue of *Beta vulgaris*. - Plant Physiol. **78**: 163-167, 1985.
- Chomczynski, P., Sacchi, N.: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. - Anal. Biochem. **162**: 156-159, 1987.
- Counillon, L., Pouyssegur, J., Reithmeier, R.: The Na⁺/H⁺ exchanger (NHE-1) contains N- and O-linked glycosylation restricted to the first N-terminal extracellular domain. - Biochemistry **33**: 10463-10469, 1994.
- Dibrov, P., Fliegel, L.: Comparative molecular analysis of Na⁺/H⁺ exchangers: a unified model for Na⁺/H⁺ antiporter? - FEBS Lett. **424**: 1-5, 1998.
- Flowers, T.J., Troke, P.F., Yeo, A.R.: The mechanism of salt tolerance in halophytes. - Annu. Rev. Plant Physiol. **28**: 89-121, 1977.
- Frommer, W.B., Ludewig, U., Rentsch, D.: Taking transgenic plants with a pinch of salt. - Science **285**: 1222-1223, 1999.
- Fukuda, A., Nakamura, A., Tanaka, Y.: Molecular cloning and expression of the Na⁺/H⁺ exchanger gene in *Oryza sativa*. - Biochim. biophys. Acta **1446**: 149-155, 1999.
- Gaxiola, R.A., Rao, R., Sherman, A., Grisafi, P., Alper, S.L., Fink, G.R.: The *Arabidopsis thaliana* proton transporters, *AtNhx1* and *Avp1*, can function in cation detoxification in yeast. - Proc. nat. Acad. Sci. USA **96**: 1480-1485, 1999.
- Golldack, D., Dietz, K.J.: Salt-induced expression of the vacuolar H⁺-ATPase in the common ice plant is developmentally controlled and tissue specific. - Plant Physiol. **125**: 1643-1654, 2001.
- Hahnenberger, K.M., Jia, Z., Young, P.G.: Functional expression of the *Schizosaccharomyces pombe* Na⁺/H⁺ antiporter gene, *sod2*, in *Saccharomyces cerevisiae*. - Proc. nat. Acad. Sci. USA **93**: 5031-5036, 1996.
- Hasegawa, P.M., Bressan, R.A., Zhu, J.K., Bohnert, H.J.: Plant cellular and molecular responses to high salinity. - Annu. Rev. Plant Physiol. Plant mol. Biol. **51**: 463-499, 2000.
- Hofmann, K., Stoffel, W.: TM base-A database of membrane spanning proteins segments. - Biol. Chem. Hoppe-Seyler **347**: 166, 1993.
- Jia, Z.P., McMullough, N., Martel, R., Hemmingsen, S., Young, P.G.: Nucleotide gene amplification at a locus encoding a putative Na⁺/H⁺ antiporter confers sodium and lithium tolerance in fission yeast. - EMBO J. **11**: 1631-1640, 1992.
- Munns, R., Greenway, H., Kirst, G.O.: Halotolerant eukaryotes. - In: Lange, O.L., Nobel, P.S., Osmond, C.B., Ziegler, H. (ed.): Encyclopedia of Plant Physiology. Vol. 12. Part C. Pp. 59-135. Springer-Verlag, Berlin 1983.
- Nass, R., Cunningham, K.W., Rao, R.: Intracellular sequestration of sodium by a novel Na⁺/H⁺ exchanger in yeast is enhanced by mutations in the plasma membrane H⁺-ATPase. - J. biol. Chem. **272**: 26145-26152, 1997.
- Nass, R., Cunningham, K.W., Rao, R.: Novel localization of a Na⁺/H⁺ exchanger in a late endosomal compartment of yeast. - J. biol. Chem. **273**: 21054-21060, 1998.
- Niu, X., Bressan, R.A., Hasegawa, P.M., Pardo, J.M.: Ion homeostasis in NaCl stress environment. - Plant Physiol. **109**: 735-742, 1995.
- Orlowski, J., Grinstein, S.: Na⁺/H⁺ exchangers of mammalian cell. - J. biol. Chem. **272**: 22373-22376, 1997.
- Padan, E., Schuldiner, S.: Bacterial Na⁺/H⁺ antiporters: molecular biology, biochemistry, and physiology. - In: Konings, W.N., Kaback, H.R., Lolkema, J.S. (ed.): Handbook of Biological Physics. Vol. 2. Pp. 501-503. Elsevier Science, Amsterdam 1996.
- Ratajczak, R., Richter, J., Lüttge, U.: Adaptation of the tonoplast V-ATPase of *Mesembryanthemum crystallinum* to salt stress, C₃-CAM transition and plant age. - Plant Cell Environ. **17**: 1101-1112, 1994.
- Sambrook, J., Fritsh, E.F., Maniatis, T.: Molecular Cloning: A Laboratory Manual. 2nd Ed. - Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989.
- Serrano, R., Mulet, J.M., Rios, G., Marquerz, J.A., Larrinoa, I.F., Leube, M.P., Mendizabal, I., Pascual-Ahuir, A., Proft, M., Ros, R., Montesinos, C.: A glimpse of the mechanisms of ion homeostasis during salt stress. - J. exp. Bot. **50**: 1023-1036, 1999.
- Shi, H., Ishitani, M., Kim, C., Zhu, J.K.: The *Arabidopsis thaliana* salt tolerance gene *SOS1* encodes a putative Na⁺/H⁺ antiporter. - Proc. nat. Acad. Sci. USA **97**: 6896-6901, 2000.
- Very, A.A., Robinson, M.F., Mansfield, T.A., Sanders, D.: Guard cell cation channels are involved in Na⁺-induced stomatal closure in a halophyte. - Plant J. **14**: 509-521, 1998.
- Wang, B.S., Ratajczak, R., Zhang, J.H.: Activity, amount and subunit composition of vacuolar-type H⁺-ATPase and H⁺-PPiase in wheat roots under severe NaCl stress. - J. Plant Physiol. **157**: 109-116, 2000.
- Zhang, L., Ma, X.L., Zhang, Q., Ma, C.L., Wang, P.P., Zhao, Y.X., Zhang, H.: Expressed sequence tags from a NaCl-treated *Suaeda salsa* cDNA library. - Gene **267**: 193-200, 2001.



Dr Radosav Cerović

Director of the Institute SERBIA – Chairman of the Organizational Board

Prof. dr Petar Mišić

Chairman of the Programme Board

The Congress will work in plenum in compliance to the following preliminary programme
PLENARY LECTURES by the invited participants and SESSIONS

1. Fruit Genetics and Breeding
2. Fruit Cultivars and Rootstocks
3. Fruit Physiology and Ecology
4. Fruit Nursery Production and Agrotechnique
5. Fruit Integrated and Organic Production
6. Fruit Protection
7. Fruit Harvest, Storage and Processing Technology
8. Economy of Fruit Production

The accompanying contents of the Congress

Promoted presentations

Exhibitions

Round table

Meeting of Scientific Pomological Society of SCG

Excursion

E-mail address of Congress Secretariat: aiserbia@eunet.yu

Phone/Fax ++ 381 11 624 626; ++ 381 11 628-398