J. Plant Biol. (2009) 52:147–153
DOI 10.1007/s12374-009-9016-z

ORIGINAL RESEARCH

Salt Tolerance is Conferred in *Arabidopsis* by Overexpression of the Vacuolar Na (+)/H (+) Antiporter Gene *SsNHX2*, an Alternative Splicing Variant of *SsNHX1*, from *Suaeda salsa*

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Received: 17 April 2008 / Revised: 19 June 2008 / Accepted: 22 November 2008 / Published online: 4 March 2009

Abstract Vacuolar Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across vacuolar membranes and compartmentalize Na^+ into vacuoles. They play important roles in cellular pH and Na^+ homeostasis. The *SsNHX1* gene was previously cloned from a typical euhalophyte, *Suaeda salsa*. Its cloning revealed another N-terminus truncated transcript, *SsNHX2*. This potentially alternative splicing variant was truncated from the 49 amino acid residues (aa) of the N terminus of SsNHX1. To compare their degree of salt tolerance, we over-expressed *SsNHX1* and *SsNHX2* in *Arabidopsis*. Southern and northern blot analyses showed that both genes had been integrated into that genome and had expressed in several lines. Two types of transgenic plants grew more vigorously than the wild type (WT) under salt stress, but no remarkable differences were found between those *SsNHX1* and *SsNHX2* transformants. Physiological analyses also supported this phenotype. Both fresh and dry weights of the transgenics as well as their accumulations of Na^+ and K^+ under salinity were much higher than that of WT, but differences were not significant between *SsNHX1* and *SsNHX2* plants for any of those parameters. These results suggest that SsNHX2 is a functional Na^+/H^+ antiporter like SsNHX1 and their levels of salt tolerance are similar.

Keywords Alternative splicing · Overexpression · Salt tolerance · SsNHX1 · SsNHX2 · *Suaeda salsa* · Vacuolar Na^+/H^+ antiporter

Plants show a "dual response" to salt stress, with an early step initiated by the more negative water potential of a salty soil solution, and a later stage due to the Na^+ toxicity that results from the relatively slower entry of Na^+ ions into leaf tissues (Munns 1993). To tolerate high salt levels, plants should be able to utilize ions for osmotic adjustment and internally distribute those ions to keep sodium away from the sites of metabolism (Wyn Jones 1981). The compartmentation of Na^+ into vacuoles, done by the vacuolar Na^+/H^+ antiporter, provides an efficient mechanism for averting the deleterious effects of Na^+ in the cytosol and maintaining osmotic balance (Glenn et al. 1999).

Genes encoding vacuole-type Na^+/H^+ antiporters have been isolated from many plant species such as *Arabidopsis thaliana* (Apse et al. 1999; Gaxiola et al. 1999), *Oryza sativa* (Fukuda et al. 1999), *Atriplex gmelini* (Hamada et al. 2001), *Mesembryanthemum crystallinum* (Chauhan et al. 2000), *Suaeda salsa* (Ma et al. 2004), *Beta vulgaris* (Xia et al. 2002), and *Gossypium hirsutum* (Wu et al. 2004). Expression of *NHX* is induced by NaCl treatment in most of the plant *NHX* genes already characterized, and their overexpression increases tolerance under saline conditions in transgenic *Arabidopsis* (Apse et al. 1999), tomato (Zhang and Blumwald 2001), *Brassica* (Zhang et al. 2001), rice (Ohta et al. 2002; Chen et al. 2007), and tobacco (Wu et al. 2004).

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Although functional analysis has been conducted on amino acid residues in mammalian and bacterial plasma membrane-bound Na^+/H^+ antiporters (Ding et al. 2006; Arkin et al. 2007), little is known about the topology, structure, and mode(s) of action for plant vacuolar Na^+/H^+ antiporters. AtNHX1, the *Arabidopsis* Na^+/H^+ antiporter, can form the common membrane topology proposed for the human Na^+/H^+ exchanger (NHE) family, even though it does not have a signal peptide found in the NHE family (Sato and Sakaguchi 2005). Using yeast as a heterologous expression system, Yamaguchi et al. (2003) have proved that deletion of the N terminus of AtNHX1 does not significantly alter the apparent V_{max} of Na^+/H^+ and K^+/H^+ transport, whereas deletion of the C terminus of AtNHX1 leads to significantly higher Na^+/H^+ and lower K^+/H^+ transport rates, with a two-fold increase in the ratio of Na^+ to K^+ transport.

Alternative splicing in *Arabidopsis* and other plants is a common mechanism for post-transcriptional modulation of gene expression and, eventually, of plant form and function (Reddy 2007). Such splicing enables the generation of proteins with different structures and functions through variations in the splicing patterns of pre-mRNA from one gene. These variants lack one or more modular domains, contain truncated domains, or fuse two transcripts (Black 2003). Genes involved are those that control growth and development (Gupta et al. 2005; Palusa et al. 2007; Lightfoot et al. 2008, Muralla et al. 2008), responses to stress (He et al. 2007), and signaling (Zhang and Gassmann 2003; Bove et al. 2008).

SsNHX1 has been cloned from a typical euhalophyte, *Suaeda salsa*. The leaves of *S. salsa* are succulent so that the vacuolar Na^+/H^+ antiporters may play important roles in its salt-tolerance mechanisms. There, *SsNHX1* is up-regulated by salt stress (Ma et al. 2004).

We over-expressed SsNHX1 and SsNHX2 in *Arabidopsis*. The latter was a variant obtained through cloning of the former, and was found to be another N terminus-truncated transcript. By comparing the responses of their transgenic plants to NaCl treatment, we observed that SsNHX2 is a functional Na^+/H^+ antiporter as SsNHX1, but the salt tolerance of SsNHX1 and SsNHX2 was similar. We hypothesized that such possible alternative splicing might be a regulatory mechanism within *S. salsa*

Materials and Methods

Gene Cloning and Plasmid Construction

Total RNA was isolated from the fleshy leaves and stems of *S. salsa* with TRIZOL Reagent (TaKaRa, Japan). First-strand cDNA was synthesized from 3 μg RNA by an RNA PCR Kit (AMV) (TaKaRa). Reverse transcription proceeded for 60 min at 42°C, and PCR amplification was performed with ExTaq DNA polymerase (TaKaRa).

SsNHX2 was amplified from cDNA template by PCR with forward, 5′-TGGTCATCTTCTCGAAGAGAATCGC; and reverse, 5′-GCCTCAATTATCGCTAACTTATGTTCTCTG primers. PCR was programmed at 94°C for 3 min; followed by 30 cycles of 94°C for 40 s, 56°C for 40 s, and 72°C for 2 min; then 72°C for 10 min. The PCR fragment was cloned into the pGEM-T easy vector (Promega, USA) and verified by sequencing. Using the pYES2 vector as a middle vector for obtaining the correct restriction enzyme site, we inserted *SsNHX2* into the *Bam*HI /*Xba*I site of binary plant vector pROK2, and the resulting plasmid was named pROK: *SsNHX2*.

The open reading frame of *SsNHX1* was amplified using cDNA as template and forward, 5′-AAGGATCCCGGGTG CACAAAGAAA; and reverse, 5′-ATGGTACCGCAC CAACTGCCATCAA primers. The forward primer included the *Bam*HI site while the reverse contained the *Kpn*I site (underlined sequences are enzyme sites). PCR was programmed at 94°C for 3 min; followed by 30 cycles of 94°C for 40 s, 56°C for 40 s, and 72°C for 2 min; and finally 72°C for 10 min. The PCR fragment was confirmed by sequence analysis, then inserted into the *Bam*HI/*Kpn*I site of pROK2. This resulting plasmid was called pROK:*SsNHX1*. The open reading frame region of *SsNHX1* was 1,665 bp long.

Plant Transformation

Plasmids pROK:*SsNHX2* and pROK:*SsNHX1* were introduced into *Agrobacterium tumefaciens* strain GV3101 and transferred into 5-week-old *Arabidopsis* 'Columbia' wild-type (WT) plants by the floral-dip method (Clough and Bent 1998). Their seeds were screened on a Murashige and Skoog medium supplemented with 30 μg ml^{-1} kanamycin.

Southern and Northern Analyses

Southern and northern blots were made with approximately 20 μg of genomic DNA and 30 μg of total RNA per track. The CTAB extraction method was used for isolating genomic DNA from T3 transgenic lines and the WT (Murray and Thompson 1980). Genomic DNA was digested with *Hin*dIII, then transferred to a Hybond N^+ nylon membrane (Amersham, Buckinghamshire, UK). Southern hybridization was performed as described by Sambrook et al. (1989). DNA probes were labeled with [^{32}P] dCTP according to the manufacturer's instructions (Sigma, St. Louis, USA). Total RNA was obtained from T3 transgenic lines and the WT via guanidium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacci 1987). The hybridization protocol was the same as for Southern blot analysis.

Salt Treatment and Measurement of Fresh and Dry Weights

Transgenic T3 plants from lines with single or multiple-copy insertions of *SsNHX1* and *SsNHX2* were evaluated for salt tolerance. Seeds of those and the WT were sown in 9-cm plastic pots containing a 2:1:1 (*v*:*v*:*v*) mixture of soil: perlite:vermiculite. Plants were grown in a greenhouse under a 16-photoperiod, at 25°C/20°C (day/night), and a relative humidity of 60%/80% (day/night). For salt-stress experiments, we applied 60 ml of Hoagland solution to each pot of 4-week-old seedlings on alternate days over the 16-day trial. The control group received no supplemental NaCl while 100, 150, or 200 mM NaCl was added to the other watering solutions. Those concentrations were increased stepwise by 50 mM every 4 days for each group, to the indicated maximum. Roots and shoots were harvested after the treatment period, and their fresh weights were recorded immediately. Dry weights were measured after tissues were dried for 48 h at 70°C.

Determination of Total Na^+ and K^+ Contents

Total potassium and sodium contents of the plants were determined at the end of the salt treatments. Dry samples were digested with HNO_3, and K^+ and Na^+ were evaluated with an atomic absorption spectrophotometer (Z-8000, Hitachi, Tokyo, Japan) (Wang and Zhao 1995).

Results

Cloning of *SsNHX1* and *SsNHX2*

When *SsNHX1* was cloned, we often found another N terminus-truncated transcript that we called *SsNHX2*. There, the 1- to 134-bp segment of the former had been deleted. SsNHX2 may be an alternative splicing variant, truncating the 49 amino acid residues (aa) from the N terminus of SsNHX1. The 50th aa of SsNHX1 (GenBank accession no. AF370358, 554 aa) was methionine, which can initiate the translation of SsNHX2, so that the latter included 505 amino acid residues.

To examine whether *SsNHX2* has Na^+/H^+ exchanging activity and if activities differ between *SsNHX1* and *SsNHX2*, we over-expressed them in *Arabidopsis*. *SsNHX1* and *SsNHX2* were PCR amplified from the cDNA template and were inserted into pROK2.

Genetic Transformation

Five-week-old ‘Columbia’ wild-type plants were infected with *Agrobacterium tumefaciens* strain GV3101 carrying the expression vectors of pROK:*SsNHX2* and pROK:*SsNHX1*. In all, 82 and 57 kanamycin-resistant plants were obtained for *SsNHX2* and *SsNHX1*, respectively. These initial plants were named T1, and their self-crossed progenies were T2. Several transgenic homozygous lines (T3) that were all resistant to kanamycin (30 μg ml^{-1}) were selected as n7–1, n8–9, n9–6, n10–6, n20–4, n21–8, n26–8, and n30–4 for *SsNHX1*; and s6–1, s8–1, s21–1, s55–7, s56–8, s62–5, s65–10, and s66–6 for *SsNHX2*. These were verified by PCR and Southern and northern blots. Afterward, n20–4, n26–8, s62–5, and s66–6 were selected for further analysis. No morphological or developmental differences were obvious between transgenic and WT plants.

Molecular Characterization of Transgenic Plants

PCR revealed intense bands corresponding in size to *SsNHX1* and *SsNHX2* fragments for our T3 kanamycin-resistant transgenics whereas nothing was obtained from the WT (data not shown).

To characterize copy numbers for the integrated foreign *SsNHX1* and *SsNHX2*, genomic DNA of the WT and T3 plants digested by *Hin*dIII was hybridized with the 750-bp fragment from the C terminus of *SsNHX1* as the gene-specific DNA probe for *SsNHX1* and *SsNHX2*. Southern blot analysis indicated that both genes had been integrated into all of the genomes of transgenic plants, although their copy numbers differed. Only n20–4 and n30–4 had integrated a single copy of *SsNHX1* while the others carried more copies. WT plants showed no hybridization signal in the Southern analysis (Fig. 1).

Northern blot analysis revealed expression of *SsNHX1* and *SsNHX2* in T3 plants from several non-segregating transgenic lines (Fig. 2). A hybridization signal was also shown in the WT, probably because the endogenous *AtNHX*s shared high identity with *SsNHX1*, and because the probe, including the conserved domains, could also combine with those endogenous *AtNHXs* (Fig. 2a). These phenomena have also been reported, for example, with confirmation via mRNA detection of transgenic rice with *AgNHX1*, where endogenous mRNA for the Na^+/H^+ antiporter gene (*OsNHX1*) is also observed (Ohta et al. 2002). We also used the 350-bp fragment from the C terminus of *SsNHX1* as a northern-blot probe (absolutely specific for *SsNHX1* and *SsNHX2*) and found no hybridization signal in the WT (Fig. 2b, c).

Growth Comparison of SsNHX1 and SsNHX2 Transformants

Transgenic T3 plants from lines with single or multiple-copy insertions of *SsNHX1* and *SsNHX2* were assessed for salt tolerance. Four independent transgenic lines—n20–4 and n26–8 for *SsNHX1*; s62–5 and s66–6 for *SsNHX2*—as well as the WT were treated with 0, 100, 150, or 200 mM

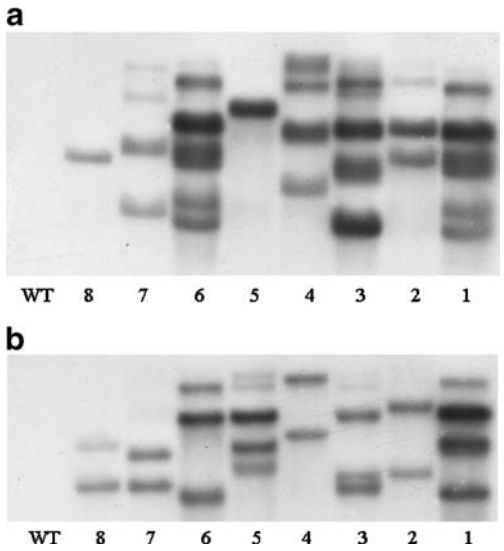

Fig. 1 Southern-blot analysis of the DNA from the WT and some transgenic plants. **a** Southern blot analysis of WT and *SsNHX1* transformants. *WT*: wild-type plants; *lanes 1–8*: transgenic lines n7–1, n8–9, n9–6, n10–6, n20–4, n21–8, n26–8, and n30–4. **b** Southern blot analysis of WT and *SsNHX2* transformants. *WT*: wild-type plants; *lanes 1–8*: transgenic lines s6–1, s8–1, s21–1, s55–7, s56–8, s62–5, s65–10, and s66–6. Genomic DNA of WT and T3 plants was digested by *Hin*dIII; fragments were resolved by gel electrophoresis and transferred to nylon membranes that were hybridized with 750-bp fragment from C terminus of *SsNHX1* as probe for *SsNHX1* and *SsNHX2*

NaCl. At that highest concentration, the WT displayed progressive chlorosis, smaller leaves, and inhibited growth whereas the transgenic plants were only slightly affected or showed more vigor in comparison. No remarkable differences were observed between *SsNHX1* and *SsNHX2* transformants (Fig. 3).

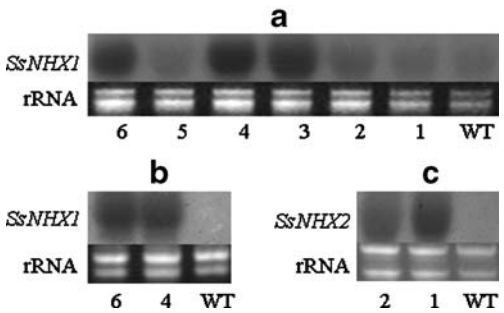

Fig. 2 Northern blot analysis of *SsNHX1* and *SsNHX2* transformants. **a**, **b** Expression of *SsNHX1* in WT and transgenic plants. *WT*: wild-type plants; *lanes 1–6*: n7–1, n9–6, n10–6, n20–4, n21–8, and n26–8. **c** Expression of *SsNHX2* in WT and transgenic plants. *WT*: wild-type plants; *lanes 1*, *2*: s62–5 and s66–6. RNA of WT and T3 plants was analyzed by RNA gel blots that were hybridized with either 750-bp fragment from C terminus of *SsNHX1* as probe for *SsNHX1* (**a**) or 350-bp fragment from C terminus of *SsNHX1* as probe for *SsNHX1* and *SsNHX2* (**b**, **c**). Northern blots were exposed to X-ray film for 7 days. Ethidium bromide-stained rRNA bands indicate loading control

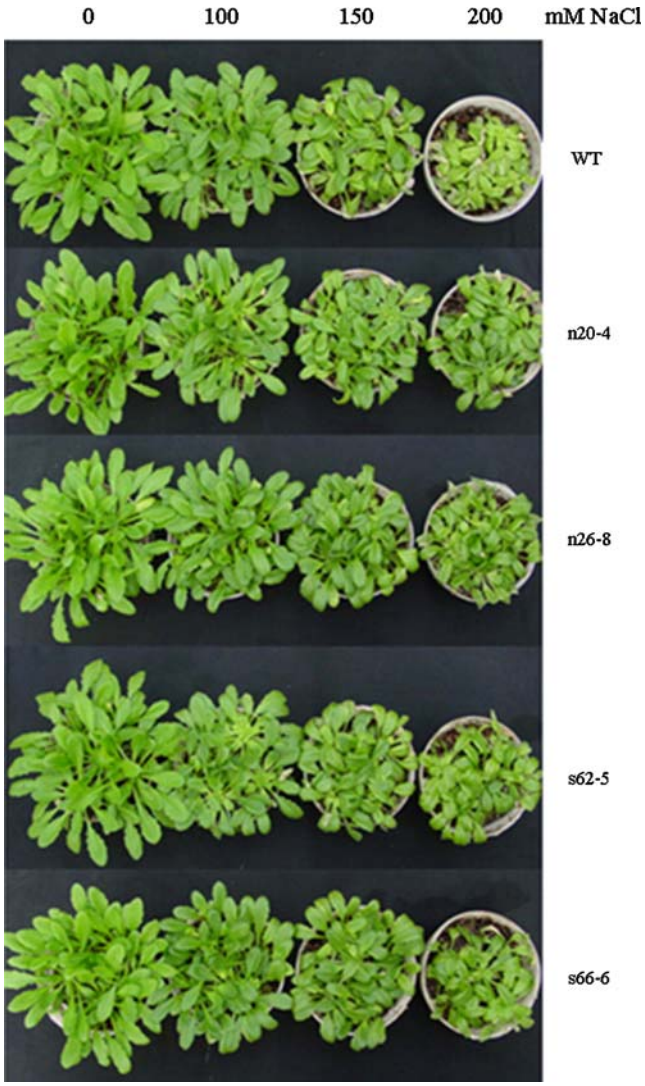

Fig. 3 Effect of salt treatment on expression. WT and homozygous transgenic plants (n20–4 and n26–8, *SsNHX1*; s62–5 and s66–6, *SsNHX2*) were treated with 0, 100, 150, or 200 mM NaCl (*left* to *right*). *Top* to *bottom*: WT, n20–4, n26–8, s62–5, and s66–6. All plants were grown in soil and watered every 3 days to induce NaCl stress

Effect of Overexpressing SsNHX1 and SsNHX2 on Fresh and Dry Weights

NaCl treatment decreased shoot fresh and dry weights in all plants, but those weights were greater in the transgenics at all concentrations (Fig. 4). Consistent with our phenotype observations, no significant differences in fresh or dry weights were found between *SsNHX1* and *SsNHX2* transformants.

Na^+ and K^+ Contents in SsNHX1 and SsNHX2 Transformants

Vacuolar Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+ across vacuolar membranes, and compartmentalize

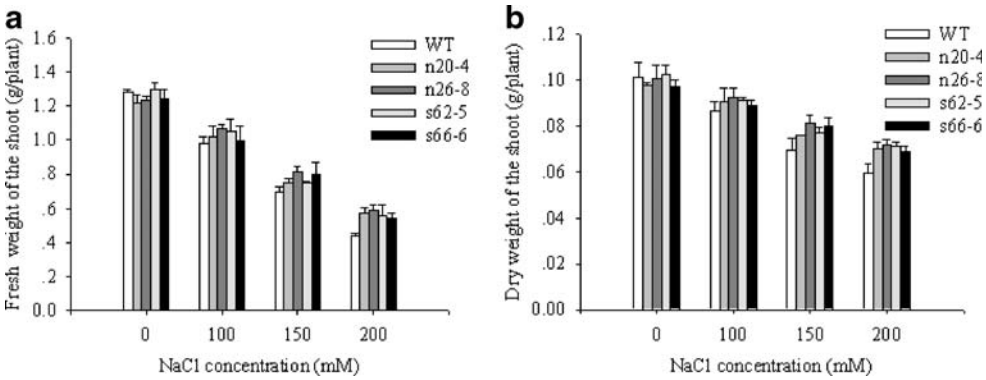

Fig. 4 Fresh (**a**) and dry weights (**b**) of shoots. Transgenic and WT plants were grown in absence or presence of NaCl. *White* and other *bars* indicate WT; n20–4, n26–8 for *SsNHX1*; s62–5, s66–6 for *SsNHX2*. Values are means ± SD (n=3)

Na^+ into vacuoles (Glenn et al. 1999). Sodium and potassium play important roles under NaCl stress. To determine if overexpression of *SsNHX1* and *SsNHX2* increase Na^+ accumulation in *Arabidopsis*, Na^+ and K^+ contents were examined in transgenic and WT plants. Without salt stress, those contents were nearly the same in both WT and transgenic plants. NaCl treatment increased cellular Na^+ levels and decreased K^+ contents in all plants, but the Na^+ and K^+ contents in the transgenic lines were higher than that in WT. Values were not significantly different between *SsNHX1* and *SsNHX2* transformants (Fig. 5).

This higher Na^+ content in transgenics under NaCl stress, together with their vigorous growth and greater fresh and dry weights, is consistent with their increased vacuolar compartmentation of Na^+. Again, no remarkable differences were found in salt tolerance between *SsNHX1* and *SsNHX2* transformants.

Discussion

Because of the sequestration of toxic sodium ions in vacuoles, the *NHX* genes in plants are mainly implicated in salt tolerance (Tester and Davenport 2003; Pardo et al. 2006) and overexpression of the *NHX* genes improved the salt tolerance of transgenic plants (Apse et al. 1999; Zhang and Blumwald 2001; Zhang et al. 2001; Ohta et al. 2002; Wu et al. 2004; Chen et al. 2007). Here, transgenic *Arabidopsis* plants of *SsNHXs* grew more vigorously than

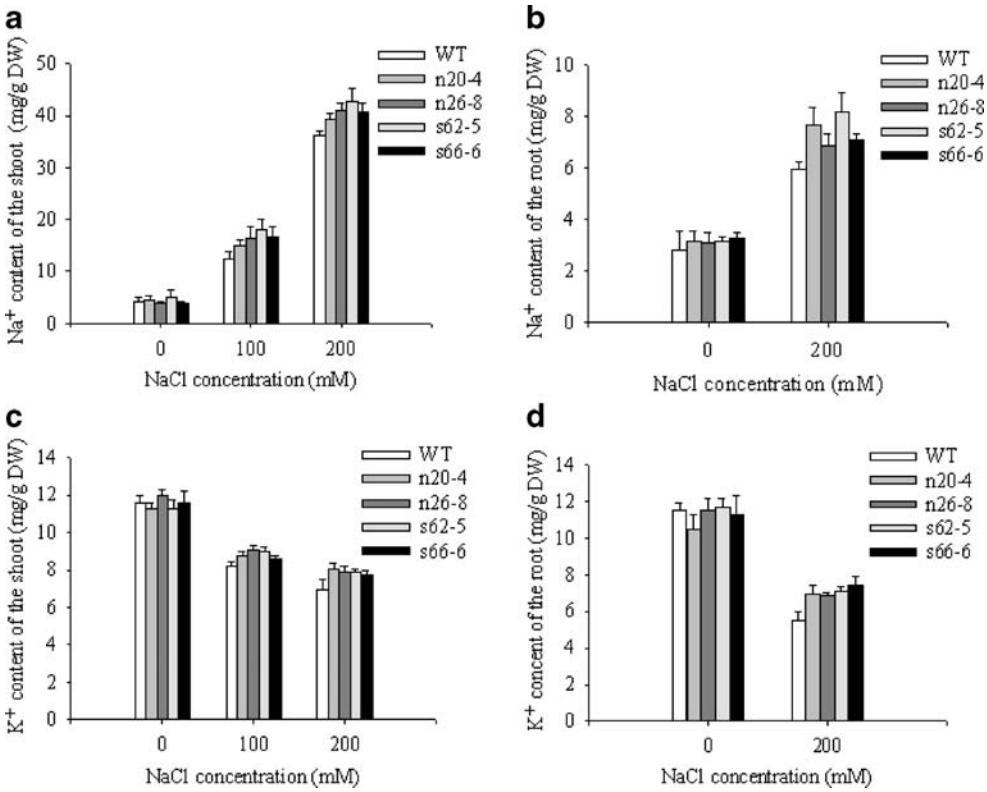

Fig. 5 Sodium and potassium contents in WT and transgenic tissues. Plants were grown in absence or presence of NaCl. **a** Na^+ in shoots; **b** Na^+ in roots; **c** K^+ in shoots; **d** K^+ in roots. *White* and other *bars* indicate WT; n20–4, n26–8 for *SsNHX1*; s62–5, s66–6 for *SsNHX2*. Values are means ± SD (n=3)

the WT after treatment with 200 mM NaCl. Likewise, their fresh and dry weights were higher under salt stress, illustrating fully the beneficial effects of *SsNHX* overexpression. As Na^+ continued to accumulate in the transgenic plants, their K^+ content also was elevated. Because the vacuolar Na^+/H^+ antiporter can also function as a K^+/H^+ antiporter (Fukuda et al. 2004; Wu et al. 2004), it is plausible that greater Na^+/H^+ antiporter activity in the tonoplast would enhance concentrations of both Na^+ and K^+ in the vacuole, reducing water potentials and improving water uptake under higher salinity.

Despite the availability of sequence information for plant *NHX* genes, little is known about the structure or function of these transporters. Using yeast as a heterologous expression and assay system, Yamaguchi et al. (2003) have found that deleting the N terminus of AtNHX1 has little effect on its activity. Consistent with that, we showed that, in *Arabidopsis*, overexpression of *SsNHX2*, the N-terminal-truncated form of *SsNHX1* from alternative splicing, had no more influence on conferring salt tolerance in transgenic plants than did overexpression of *SsNHX1*. Under salt stress, no statistically significant differences were found between *SsNHX1* and *SsNHX2* transformants in their phenotypes, fresh and dry weights, or Na^+ and K^+ contents.

The capacity for vacuolar compartmentalization of Na^+ and Cl^- is a salt-adaptation mechanism conserved in halophytes and glycophytes (Blumwald et al. 2000; Hasegawa et al. 2000), with that process being more efficient in the former. It is unknown whether greater Na^+/H^+ antiporter activities in salt-tolerant plants can be attributed to differences in the protein itself or in their regulatory mechanisms. Nevertheless, our results partially demonstrate that the role of the N terminus from Na^+/H^+ antiporters may be similar between halophytes and glycophytes.

Alternative splicing in plants is a largely unexplored area in the study of gene expression. However, genome-wide computational analyses have revealed that alternative splicing in flowering plants is far more prevalent than previously thought (Iida et al. 2004). For example, the pre-mRNAs of *Arabidopsis* genes that encode serine/arginine-rich (SR) proteins, a conserved family of splicing regulators in eukaryotes, are extensively spliced. This alternative splicing of some SR genes is controlled in a developmental and tissue-specific manner, and is altered by stresses and hormones (Palusa et al. 2007). The disease resistance gene *RPS4* from *Arabidopsis* produces multiple transcripts via alternative splicing of two regular introns flanking Exon 3 and a cryptic intron within that exon; RPS4-mediated resistance requires the combined presence of transcripts encoding both full-length and truncated open reading frames (Zhang and Gassmann 2003). Two splicing variants of *SsNHXs* exist—full-length *SsNHX1* and the N-terminal-truncated form *SsNHX2*. *Suaeda salsa* may employ alternative splicing to regulate *SsNHXs*. Here, the expression of *SsNHX1* and *SsNHX2* did not differ among the leaves, stems, and roots regardless of NaCl treatment (data not shown). We speculate that these two transcripts may depend on developmental stage or tissue type. Future studies will be needed to describe the mechanism by which these alternatives confer tolerance.

Acknowledgments This work was supported by The State Key Basic Research and Development Plan of China (973 Program; Project No. 2006CB100100).

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