

## Molecular cloning and functional characterization of a $\text{Na}^+/\text{H}^+$ antiporter gene from halophyte *Spartina anglica*

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Received: 01.03.2010

**Abstract:**  $\text{Na}^+/\text{H}^+$  antiporters have been suggested to play important roles in salt tolerance in plants. English cordgrass (*Spartina anglica*) is a gramineous halophyte with very strong salt tolerance. It possesses salt glands in its stems and leaves, through which excess salt can be excreted. In this study, a vacuolar  $\text{Na}^+/\text{H}^+$  antiporter homologous gene (temporarily named SaNHX1) was isolated from *Spartina anglica* by RT-PCR and rapid amplification of cDNA ends (RACE). Southern blot analysis suggested that there might be 2 or 3 copies of the vacuolar  $\text{Na}^+/\text{H}^+$  antiporter genes in the English cordgrass genome. Northern blot analysis showed that the expression of vacuolar  $\text{Na}^+/\text{H}^+$  antiporter genes in English cordgrass is induced by salt stress. Overexpression of SaNHX1 driven by constitutive promoter Ubi-1 in rice significantly enhanced the salt tolerance of transgenic plants, validating the function of SaNHX1 and suggesting its value for the genetic improvement of salt tolerance in plants.

**Key words:** Halophyte, salt tolerance, SaNHX1, *Spartina anglica*, transgenic rice

### Introduction

There are approximately 830 million hectares of saline-alkali soil in the world, accounting for approximately 6.5% of the land area, and 77 million hectares (approximately 4.5%) of arable land (including irrigated and dry land) are impacted by salinity (Munns et al. 1999; Anonymous 2010). Salt stress is an important abiotic stress in crop production and can dramatically reduce crop yields. Improving the salt tolerance of crops is an efficient way to solve this problem. For this purpose, it is necessary to understand the mechanisms of salt

tolerance in plants. Salt tolerance is a complex trait controlled by many genes, including those encoding functional proteins that directly protect macromolecules and membranes (e.g. LEA proteins, osmotin, antifreeze proteins, chaperones, and mRNA binding proteins) (Singh et al. 1985; Xu et al. 1996) or maintain water movement through membranes (e.g. water channel proteins and membrane transporters) (Yamada et al. 1995); enzymes that catalyze the biosynthesis of various osmoregulators (e.g. proline, betaine, and sugars) (Wood et al. 1996; Zhu et al. 1998); detoxification enzymes that

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enable normal cellular physiological or biochemical metabolism (e.g. glutathione S-transferase, soluble epoxide hydrolase, catalase, superoxide dismutase, and ascorbic peroxidase) (Dionisio-Sese 1998; Meneguzzo et al. 1999); and transcriptional factors (e.g. bZIP, MYC, MYB, and DREB) (Liu et al. 2000; Uno et al. 2000), protein kinases (e.g. MAP kinase, CDP kinase, receptor protein kinase, ribosomal protein kinase, and transcription-regulation protein kinase) (Petersen et al. 2000; Ma and Wu 2007), and proteinases (e.g. phosphoesterase and phospholipase C) (Hirayama et al. 1995) that are involved in signal transduction and gene expression regulation related to stress tolerance.

Na<sup>+</sup>/H<sup>+</sup> antiporters are functional proteins that play important roles in salt tolerance (Blumwald and Poole 1985; Ballesteros et al. 1997). Two types of Na<sup>+</sup>/H<sup>+</sup> antiporters have been found in plants. One is located in the vacuole membrane (e.g. AtNHX1 of *Arabidopsis thaliana*); the other is located in the plasma membrane (e.g. SOS1 of *Arabidopsis thaliana*). Overexpression of the AtNHX1 gene can enhance the salt tolerance of *Arabidopsis* and tomato (Apse et al. 1999; Zhang and Blumwald 2001). Similarly, overexpression of the SOS1 gene can also increase the salt tolerance of *Arabidopsis* (Shi et al. 2000, 2002). These results suggest that both vacuolar and plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporter genes would be useful for salt tolerance improvement of crops via genetic engineering.

A number of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter genes have been isolated from a wide range of plant species apart from *Arabidopsis thaliana* (Fukuda et al. 1999; Fukada-Tanaka et al. 2000; Hamada et al. 2001; Ma et al. 2004; Wu et al. 2004; Zhang et al. 2008), including some halophytes such as *Atriplex gmelini*, *Suaeda salsa*, and *Aeluropus littoralis*. Although Na<sup>+</sup>/H<sup>+</sup> antiporters isolated from various plant species all appear to be functional, cloning Na<sup>+</sup>/H<sup>+</sup> antiporter genes from halophytes would be particularly interesting because they might be more functionally efficient than those from glycophytes. English cordgrass (*Spartina anglica*) is a gramineous halophyte with a very high salt tolerance that grows on beaches. English cordgrass has powerful salt glands in its leaves and stems, through which excess salt can be excreted. This implies that Na<sup>+</sup>/H<sup>+</sup> antiporters might play important roles in the strong salt resistance of English cordgrass.

In this study, we cloned a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene (SaNHX1) from English cordgrass. We found that the expression of SaNHX1 in English cordgrass is induced by salt stress, and overexpression of SaNHX1 in rice could significantly improve the salt tolerance of the transgenic plants.

## Materials and methods

### Plant materials and growth conditions

English cordgrass (*Spartina anglica* L.) plants were collected from the beach of Luoyuan Bay, Fujian Province, China, and then planted in plastic flower pots (30 cm in diameter and 40 cm in height, with a hole at the center of the bottom) under natural spring climate conditions. The pots were filled with clay loam soil with moderate organic matter content (2.2%), a pH level of 6.6, and an electrical conductivity of 1.1 dS m<sup>-1</sup>, obtained from the paddy rice field at the campus of Fujian Agriculture and Forestry University in Fuzhou, Fujian, China. The plants were watered with tap water once a day to keep the soil wet and with 1 L of half-strength Hoagland solution (Hoagland and Arnon 1950) once a week to complement the nutrition in the soil. Rice cultivar Nipponbare (*Oryza sativa* L. subsp. *japonica*) was used as the recipient for genetic transformation.

### Isolation and sequence analysis of SaNHX1 cDNA

The cDNA ends of SaNHX1 were previously obtained with the rapid amplification of cDNA ends (RACE) method (Lan et al. 2006, 2007). Based on the available sequences of cDNA ends, a pair of specific primers (forward primer SANHXF: 5' - GCCATGGGGCCCCGGCCTGGGC - 3'; reverse primer SANHXR: 5' - TGTTCACCGTCCTCCATGGACGC - 3') was designed for amplifying a cDNA fragment covering the coding region of SaNHX1. Total RNA was isolated from leaves of English cordgrass under salt stress using the TRIzol reagent method (Invitrogen). The first-strand cDNA synthesis and RT-PCR were performed with the primer pair SANHXF/SANHXR using Invitrogen superscript II and LA Taq (Takara). The amplified fragment was recovered, cloned into the pMD18-T vector using the TA cloning kit (Takara), and sequenced. This cDNA sequence was

assembled with the 2 cDNA ends to find the full-length cDNA sequence. The amino acid sequence of the SaNHX1 protein was deduced from the coding region sequence and some general bioinformatics analyses were conducted.

Protein-protein BLAST (blastp) was used on the NCBI website (<http://www.ncbi.nlm.nih.gov/>). Multiple alignment of the N ends of SaNHX1 and some vacuolar Na<sup>+</sup>/H<sup>+</sup> exchangers (antiporters) from other species was performed with ClustalW2 (<http://www.ebi.ac.uk>), and the results were processed with BioEdit 7.0 (Hall 1999) to search for conserved regions. A phylogenetic tree of SaNHX1 and some Na<sup>+</sup>/H<sup>+</sup> exchangers from other species was constructed using MEGA4 software (Tamura et al. 2007) based on the neighbor-joining method.

The signal peptide cleavage site was predicted with SignalP 3.0 Server (<http://www.cbs.dtu.dk>) based on a combination of several artificial neural networks and hidden Markov models. Prediction of transmembrane helices and hydropathy plot analysis were performed with TMHMM Server v. 2.0 (<http://www.ch.embnet.org>) and KYTE DOOLITTLE HYDROPATHY PLOT (<http://gcat.davidson.edu>).

#### Southern and northern blot analyses

Southern blot analysis was performed following the protocol described by Sambrook et al. (1989). The genomic DNA of English cordgrass was digested with restriction endonucleases *Eco*RI and *Hind*III, separated on 0.8% agarose gel, blotted onto Hybond-N<sup>+</sup> membrane (Amersham), and hybridized against an a-<sup>32</sup>P-dCTP labeled 800-bp SaNHX1 cDNA fragment located within the coding region, followed by autoradiography.

English cordgrass plants were precultured in half-strength Hoagland solution for 5 days. NaCl was then added to the Hoagland solution to make a NaCl concentration of 400 mmol L<sup>-1</sup>. Total RNA was extracted from the leaves of the English cordgrass plants after they were exposed to the salt stress for 0, 1, 3, 6, 10, and 24 h. Northern blot analysis was performed by referring to Sambrook et al. (1989). The RNA were separated on 1% formaldehyde gel (each lane contained 30 µg total RNA) and blotted onto Hybond-N<sup>+</sup> membrane (Amersham). The same probe used in the Southern blot was used for the northern blot.

#### Vector construction, transformation, and salt stress treatment of rice

The cloned cDNA fragment containing the coding region of SaNHX1 (see above) was cut out of the pMD<sup>TM</sup>-18T simple vector and inserted into RNAi vector pTCK303 by replacing the rice intron located between the Ubi-1 promoter and NOS terminator (Figure 1). The construct was cloned in *Escherichia coli* strain DH5a and then transferred into *Agrobacterium tumefaciens* strain LBA4404, which was further used to transform rice calli induced from mature embryos of Nipponbare. Resistant calli were screened and regenerated into seedlings by predifferentiation and differentiation. Positive transgenic plants were identified by PCR amplification of the selectable marker hygromycin phosphotransferase gene.

T<sub>1</sub> seeds harvested from positive T<sub>0</sub> plants were sown in plastic flower pots (20 cm in diameter and 30 cm in height, with a hole at the center of the bottom) after pregermination (seed soaking at room temperature for 48 h plus germination at 37 °C for 24 h). The pots were filled with clay loam soil from the same paddy rice field that was used for planting the English cordgrass. When the seedlings were 20 days old, 2 rounds of treatment were conducted. In each round, plants were first irrigated with a salt solution containing 150 mmol L<sup>-1</sup> NaCl for 10 days, and then irrigated with fresh water for 5 days. Traits of the plants were investigated at the end of each round. Two kinds of controls were set. One (CK1) was Nipponbare plants that were always irrigated with fresh water; the other (CK2) was Nipponbare plants that were treated in the same way as the transgenic plants.

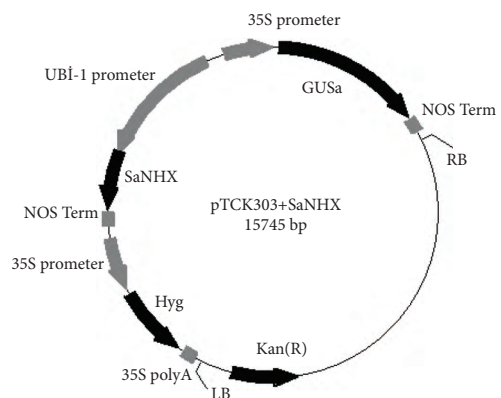


Figure 1. Construction of pTCK303+SaNHX1.

**Results**

**cDNA sequence of SaNHX1**

A 2089-bp cDNA sequence of the expected homologous gene of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter in English cordgrass (temporarily named SaNHX1) was obtained, consisting of a 95-bp 5'-untranslated region (UTR), a 1602-bp coding region, and a 392-

bp 3'-UTR (Figure 2). The deduced SaNHX1 protein sequence contained 533 amino acids (Figure 2), including 38 basic amino acids (K, R), 33 acidic amino acids (D, E), 235 hydrophobic amino acids (A, I, L, F, W, V), and 133 polar amino acids (N, C, Q, S, T, Y), with an isoelectric point of 8.563 and a calculated molecular mass of 58,656.13 Da.

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acaaaacaaggttgcatctctttttcttccgcggggatcccatccctccgccgatccgctgagctaccgcccgcagacagc
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L C A C I V L G H L L E E N R W V N E S M T A L I I G L C
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T G V V I L L T T K G K S S H I L V F S E D L F F I Y L L
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G T M I S T F T I S I G A I A I F S R M N I G A L D V G C D
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F L A I G A I F S A T D S V C T L Q V L N Q D E T P L L Y
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gatgtagccgttgactgaaattcctaggaactctctctattttctgcaagcacttccctggagatatttctggtttgctc
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V L V G R A A A F V G F P L S G L S N L T K K A D F E K I S W
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gtaggcctcctccatgtaattcaatttctcgtgctcgtgttttaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
aa
    
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Figure 2. The full-length cDNA sequence (lowercase) and deduced amino acid sequence (uppercase) of SaNHX1. Thick underlines: primer sequences, double underlines: potential glycosylation sites, wavy underlines: putative transmembrane domains, boldfaced amino acids: signal peptide, boldfaced nucleotides: poly-A tail, shadowed nucleotides: initiation and termination codons, box: Kozak sequence.

Sequence comparison and clustering analysis (Figure 3) indicated that SaNHX1 is highly similar to vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters from other plant species, as expected, especially those from monocot species such as *Aeluropus littoralis* (AlNHX1), rice (OsNHX1), and barley (HvNHX1). A noticeable feature of SaNHX1 is that it contains a putative amiloride-binding motif (FFILLPPII), which commonly exists in vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters (Figure 4). The transmembrane helix prediction and hydropathy plot analysis showed that SaNHX1

has 12 transmembrane domains dispersed in the polypeptide (Figure 2). Such a distribution pattern of transmembrane domains is a common feature of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters (Fukuda et al. 1999; Hamada et al. 2001), which is different from that of plasma membrane Na<sup>+</sup>/H<sup>+</sup> antiporters, in which transmembrane domains are mainly located in the N-terminal region (Shi et al. 2000). These results strongly suggest that the cDNA sequence acquired is quite probably a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene.

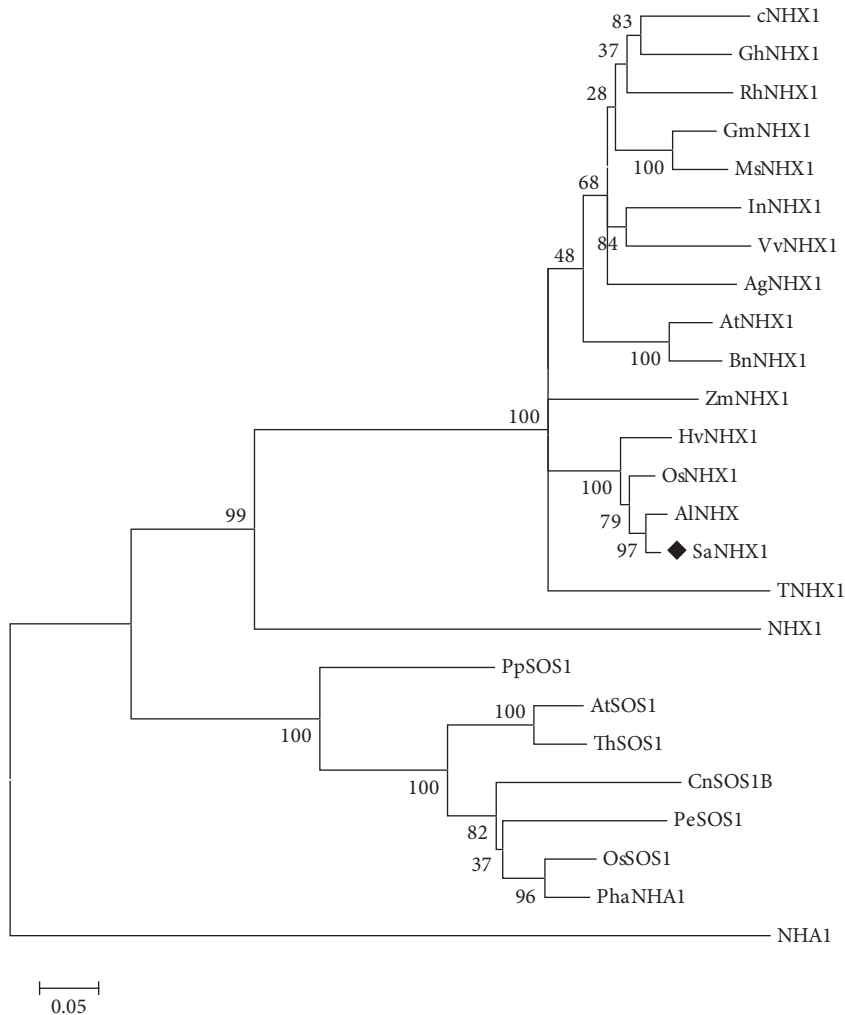


Figure 3. A phylogenetic tree of SaNHX1 and Na<sup>+</sup>/H<sup>+</sup> antiporters from different plant species and yeast. The number beside each node is the bootstrap probability (%) estimated based on 5000 sampling repetitions. The GenBank accession numbers of the protein sequences are: cNHX1, AAK27314; GhNHX1, AAM54141; RhNHX1, BAD93487; GmNHX1, AAY43006; MsNHX1, AAR19085; InNHX1, BAD91200; VvNHX1, AAV36562; AgNHX1, BAB11940; AtNHX1, Q68K14; BnNHX1, AAO38856; ZmNHX1, NP\_001105221; HvNHX1, BAC56698; OsNHX1, BAA83337; AlNHX, AAV80466; TNHX1, AAK76737; NHX1, NP\_010744; PpSOS1, CAD91921; AtSOS1, Q9LKW9; ThSOS1, ABN04857; CnSOS1B, CAL44986; PeSOS1, ABF60872; OsSOS1, AAW33875; PhaNHA1, BAF41924; NHA1, Q99271.

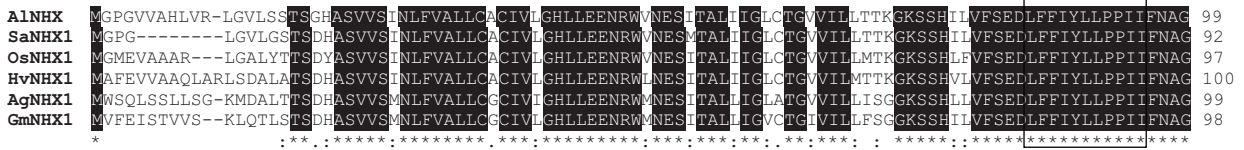


Figure 4. Alignment of SaNHX1 and some vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters from other plant species. Identical residues among the 6 proteins are boxed in black and indicated with asterisks underneath. Colons and periods indicate amino acids with high and low similarity among the proteins, respectively. Dashes indicate gaps. The box indicates an amiloride-binding motif.

In addition, based on a preliminary topological model and known glycosylation sites of other mammalian NHE isoforms, it could be deduced that one or more Asn residues (Asn-44, -287, and -362) on the loops between the transmembrane regions in SaNHX1 are probably N-linked glycosylation site(s), similar to those in rice OsNHE (Fukuda et al. 1999). Signal peptide analysis indicated that the position between the 34th and 35th amino acids in SaNHX1 might be a cleavage site, suggesting that SaNHX1 has a signal peptide comprising 34 amino acids (Figure 2).

**Copy number and expression pattern of SaNHX1**

The Southern blot analysis of the genomic DNA of English cordgrass digested by *Hind*III/*Eco*RI identified 2 or 3 bands, and there was always 1 strong band, which must result from the hybridization of the probe to the target SaNHX1 gene in each case (Figure 5). This result suggests that apart from SaNHX1,

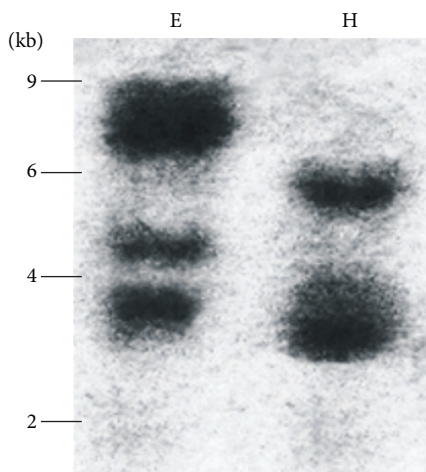


Figure 5. Southern blot analysis of SaNHX1 in English cordgrass. Lane E is genomic DNA digested with *Eco*RI; lane H is digested with *Hind*III.

there might be 1 or 2 paralogs of SaNHX1 (detected as the weaker bands in the Southern blot analysis) in the English cordgrass genome.

The northern blot analysis indicated that SaNHX1 was weakly expressed in English cordgrass plants under conditions without salt stress, but the expression of SaNHX1 quickly and significantly increased after the plants were exposed to salt stress (Figure 6). The transcription of SaNHX1 reached the highest level at 6 h after treatment and became stable afterwards. This result clearly indicates that SaNHX1 expression is upregulated by salt stress. It is necessary to point out that since the probe used for the northern blot analysis was the same as that used for the Southern blot analysis and because the probe could hybridize not only to the target SaNHX1 gene but also to its paralogs in the Southern blot analysis, the expression intensity detected by the northern blot analysis might be a mixture of SaNHX1 and its paralogs. In other words, we could not exclude the contribution from the paralogs from the expression signal detected in the northern blot analysis. Nevertheless, since the hybridization of the probe to SaNHX1 was stronger than that to the paralogs (Figure 5), we expect that the results of the northern blot analysis could still reflect the response pattern of SaNHX1 to salt stress.

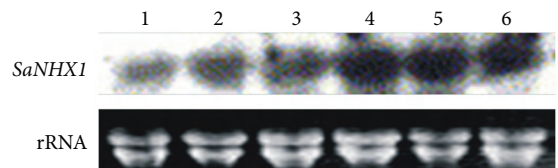


Figure 6. Northern blot analysis of SaNHX1 transcription in English cordgrass. Lane 1 is the untreated control; lanes 2-6 are treated with 400 mM NaCl for 1, 3, 6, 10, and 24 h respectively.

### Overexpression of *SaNHX1* in rice

The genetic transformation experiment produced 25 resistant clones, of which 19 regenerated into seedlings. PCR analysis indicated that all of these seedlings were positive transgenic plants, which carried the introduced *Ubi-1::SaNHX1*. Most (17, or approximately 90%) of the next generation ( $T_1$ ) lines of the 19 positive  $T_0$  plants exhibited tolerance to salt stress, among which line 3 appeared to be the most tolerant (Figure 7). In the salt stress experiment, CK2 plants began to wilt after the first round of treatment and were dead by the end of the second round of treatment (Figure 7). However, the transgenic plants were much less affected. They grew normally in the first round of treatment, similar to CK1. In the second round of treatment, they still actively grew, although the color of their leaves became darker than those of CK1 (Figure 7). These results clearly indicate that constitutive overexpression of *SaNHX1* can significantly enhance the salt tolerance of rice plants. This proves the role of *SaNHX1* in salt resistance.

### Discussion

In this study, we revealed that *SaNHX1* must have 1 or 2 paralogs in the English cordgrass genome (Figure 5). However, it is still not clear what these paralogs might be. At least 3 types of  $\text{Na}^+/\text{H}^+$  antiporters have been found in plants according to their locations in the cell, including the vacuole membrane type (e.g. *AtNHX1*; Apse et al. 1999), the plasma membrane type (e.g. *AtSOS1*; Shi et al. 2002), and the chloroplast

envelope type (e.g. *AtCHX23*; Song et al. 2004). These  $\text{Na}^+/\text{H}^+$  antiporters function independently without redundancy. For example, loss-of-function mutation of the *AtSOS1* gene in *Arabidopsis* leads to the phenotype being oversensitive to salt, which cannot be compensated for by other antiporter genes (Shi et al. 2000). In addition, these  $\text{Na}^+/\text{H}^+$  antiporters are also quite different in structure. Our sequence comparison analysis using MegAlign 5.0 in DNASTar 7.0 (<http://www.dnastar.com/>) showed that in *Arabidopsis*, the vacuole membrane  $\text{Na}^+/\text{H}^+$  antiporter *AtNHX1* (GenBank accession no.: Q68KI4) only shares 14.3% and 8.2% amino acid sequence identities with the plasma membrane  $\text{Na}^+/\text{H}^+$  antiporter *AtSOS1* (Q9LKW9) and the chloroplast envelope  $\text{Na}^+/\text{H}^+$  antiporter *AtCHX23* (NP\_172049), respectively. The sequence similarities between their cDNA coding regions are even lower, with only 1.8% between *AtNHX1* (AF106324) and *AtSOS1* (NM\_126259) and 2.0% between *AtNHX1* and *AtCHX23* (NM\_100438), respectively. Based on these findings in *Arabidopsis* and considering the conservation of  $\text{Na}^+/\text{H}^+$  antiporters in evolution, we can infer that the paralogs of *SaNHX1* identified in this study may not be the genes of the plasma membrane and chloroplast envelope  $\text{Na}^+/\text{H}^+$  antiporters, but the genes of a vacuole membrane  $\text{Na}^+/\text{H}^+$  antiporter similar to *SaNHX1*. Hence, we can conclude that there are 2 or 3 copies of the vacuole membrane  $\text{Na}^+/\text{H}^+$  antiporter gene in the English cordgrass genome (Figure 5), and their expressions as a whole are upregulated by salt stress (Figure 6).

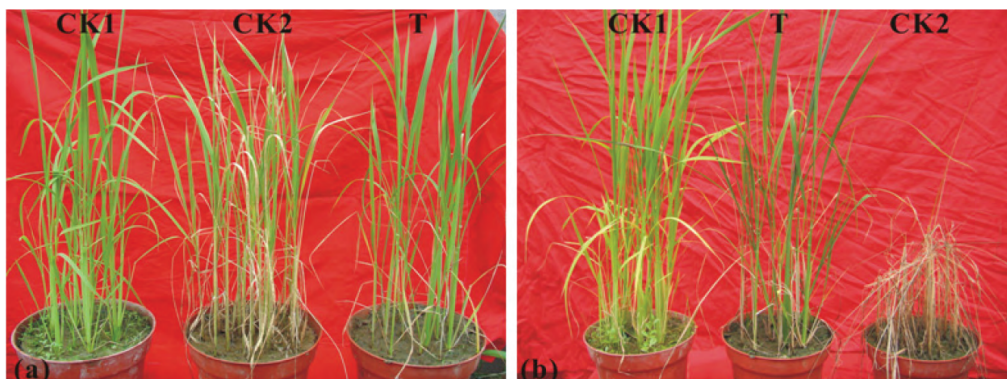


Figure 7. Overexpression of *SaNHX1* in rice: a) rice plants treated with 150 mM NaCl for 15 days, b) rice plants treated with 150 mM NaCl for 1 month. CK1: wild-type plants irrigated with fresh water, CK2: wild-type plants treated with 150 mM NaCl, T: transgenic plants treated with 150 mM NaCl.

It has been suggested that vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters are involved in sequestering Na<sup>+</sup> into vacuoles, thus preventing the toxic effects of Na<sup>+</sup> in cytoplasm and adjusting cellular osmotic pressure under salt stress. Hence, vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters should play an important role in the salt tolerance in plants. English cordgrass is a monocot halophyte with very high salt tolerance. Considering the importance of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters in salt tolerance, it is reasonable to assume that the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters from halophytes might be more active than those from glycophytes. This assumption motivated us to perform this study.

However, comparison of amino acid sequences indicated that although SaNHX1 has the highest similarity (94%) to the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter of another monocot halophyte, *Aeluropus littoralis* (AlNHX), it is also highly similar to those of rice (OsNHX1, 92%) and barley (HvNHX1, 90%). In fact, the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters of different plant species, no matter whether from glycophytes or halophytes, are generally very similar (Figure 3). This suggests that vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters are very conservative in plants, which implies that vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters should be required for the normal growth of plants. Indeed, studies have found that although the expressions of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter genes in plants are significantly induced by salt stress, they are still detectable under normal conditions (Fukuda et al. 1999; Shi and Zhu 2002; Brini et al. 2005; Zhang et al. 2008).

In addition, many studies have shown that overexpressing vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter genes from glycophytes can also significantly enhance the salt tolerance of transgenic plants (Brini et al. 2007; Chen et al. 2007; Zhao et al. 2007), suggesting

that the function of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters is also conservative in plants, consistent with the conservation of their structure. These together suggest that the vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters of halophytes are not distinctive from those of glycophytes in structure or in function. Hence, it appears that the great difference between halophytes and glycophytes in salt tolerance may not be attributable to the structural variation of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporters among plants. Nevertheless, considering that overexpression of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter genes can obviously enhance salt tolerance, and that halophytes generally grow under high salinity environments and therefore a high osmotic pressure in their root cells is required to avoid dehydration, there is a possibility that the expression of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter genes in halophytes under salt stress is stronger than that in glycophytes. This assumption appears to be supported by the findings that halophyte plants such as *Aeluropus littoralis* (Zhang et al. 2008) and *Spartina anglica* (this study) tend to possess multiple copies of a vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter gene, while glycophyte plants such as rice and wheat (Brini et al. 2005) usually have only a single copy. Another possible mechanism could be that the promoters or regulation systems of vacuolar Na<sup>+</sup>/H<sup>+</sup> antiporter genes in halophytes are more active than those in glycophytes. Further studies are needed to elucidate these issues.

### Acknowledgments

The authors thank Professor Kang Chong (Institute of Botany, Chinese Academy of Sciences) for providing the plant expression vector pTCK303. This work was supported by the Department of Science and Technology, Fujian Province, China (Grant Nos.: 2003I014, 2007N0011).

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