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ORIGINAL RESEARCH

Isolation and Characterization of a Putative Vacuolar Na^+/H^+ Antiporter Gene from *Zoysia japonica* L.

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Abstract A Na^+/H^+ antiporter gene from *Zoysia japonica* (*ZjNHX1*) which is a member of plant *NHX*-genes family was cloned by reverse-transcription-polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE) technology. The isolated cDNA is 2,421 bp in length and contains one open-reading frame (ORF) which comprises 540 amino acid residues and shows higher homology with other plant Na^+/H^+ antiporters. ZjNHX1 could partially complement the salt-sensitive phenotypes of Δ*nhx1* and Δ*ena1-4*Δ*nhx1* yeast mutants in the presence of NaCl, KCl, and LiCl. The expression of *ZjNHX1* in *Z. japonica* increased after NaCl treatment and this result accords with that of Na^+ contents determination under the same treatment. These results implied that *ZjNHX1* functions as a vacuolar Na^+/H^+ antiporter and plays an important role in salt tolerance and ion homeostasis in *Z. japonica*.

Keywords Ion homeostasis · Na^+/H^+ antiporter · Salt tolerance · *Zoysia japonica*

Abbreviations

NHX	vacuolar Na^+/H^+ antiporter
NHX	vacuolar Na^+/H^+ antiporter gene
ORF	open-reading frame
RACE	rapid amplification of cDNA ends
C_T	threshold cycle values

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Introduction

Salinity is one of the major factors effecting the growth and yield of plants. In most saline environments, NaCl is the predominant salt species, which inhibits cell division and expansion, and results in slower cell growth and smaller plants. Restriction of Na^+, active Na^+, efflux and compartmentation of Na^+ into the vacuole are the three main mechanisms function cooperatively to prevent the accumulation of Na^+ influx in plants (Padan et al. 2001).

Na^+/H^+ antiporters which are localized in plasma membrane and vacuolar membrane, catalyze the exchange of Na^+ for H^+ across membranes (Yamaguchi et al. 2003). In plants, vacuolar Na^+/H^+ antiporters can pump Na^+ into vacuolar to reduce Na^+ toxicity and maintain a high K^+/Na^+ ratio in cytosol to alleviate salt stress. Na^+/H^+ antiporters also regulate the internal pH, cell volume, and sodium level in the cytoplasm and vacuole (Apse et al. 1999). It has been proved that plant vacuolar Na^+/H^+ antiporter plays an important role in salt tolerance, ion homeostasis and development (Yamaguchi et al. 2003). The physiological activity of Na^+/H^+ antiporter in plants was firstly found in tonoplast vesicles in red beet storage tissue (Blumwald and Poole 1985), and the first Na^+/H^+ antiporter in higher plant was cloned from *Arabidopsis* (Apse et al. 1999). In recent years, a series of Na^+/H^+ antiporter coding genes have been cloned and identified from *Oryza sativa* (Fukuda et al. 1999), *Atriplex gmelini* (Hamada et al. 2001), *Beta vulgaris* (Xia et al. 2002), *Gossypium hirsutum* (Wu et al. 2004), *Rosa hybrida* (Kagami and Suzuki 2005), *Aeluropus littoralis* (Zhang et al. 2008), *Thellungiella halophila* (Wu et al. 2009), and some other glycophytes and halophytes. Overexpressing *AtNHX1* in *Arabidopsis* resulted in increasing activity of vacuolar Na^+/H^+ antiporter and the transgenic plants were able to grow in the presence of 200 mM NaCl

(Apse et al. 1999). *AtNHX1* transgenic tomato accumulated salt in foliage and was able to grow at high salt concentration (Zhang and Blumwald 2001). Treatment with high concentration of NaCl increased the transcript levels of *OsNHX1* in rice and overexpression of *OsNHX1* improved the salt tolerance of transgenic plants (Fukuda et al. 2004a). All these results demonstrated that the vacuolar Na^+/H^+ antiporters play a crucial role in the salt tolerance of various plants.

Zoysia japonica, a monocot halophyte plant, is one of the most popular warm-season type turfgrass and is widely used for home lawns, golf courses, athletic fields and parks due to its excellent characteristics which include tolerance to heat, drought and salinity (Inokuma et al. 1998). Since monocot halophytes were capable of growing on or surviving in high saline condition, they were the good materials to further understand the molecular basis of vacuolar Na^+/H^+ antiporters and to evaluate the potential applications in the field of plant salt tolerance. Cloning the salt-tolerant genes from such plant sources and investigating their characterizations and functions should be valuable for further understanding the molecular mechanism of plant salt tolerance and helpful for breeding the new cultivars and varieties for animal husbandry, turf industry, and ecological construction.

The purpose of this study is to try to isolate a novel vacuolar Na^+/H^+ antiporter gene which has not been cloned yet from *Z. japonica*, a monocot halophyte plant, and to investigate its characterizations and functions. We expected that the study might be helpful for the understanding of the salt tolerance mechanism and molecular breeding of turfgrass.

Materials and Method

Plant Materials

Z. japonica were grown on soil for 2 months under a growth chamber (16 h photoperiod, 25°C), and watering with the nutrient solution supplemented with NaCl. Salinization treatment was begun at 50 mM NaCl and raised in 50 mM steps every 3 days until the final concentration reached 200 mM. Plants were watered at 200 mM NaCl for 7 days before harvest.

Cloning of *ZjNHX1* and Sequence Analysis

Degenerate primers were designed based on the alignment of amino acid sequences of plant Na^+/H^+ antiporters (data not shown). The two corresponding degenerate primers are: NHX1F: 5′-CC(A/T)CC(G/C)AT(C/T)AT(A/C)TTCAATG CAGG(C/G/T)TTTCA-3′and NHX1R:5′-(T/A/C)ACAA CACC(C/T)TC(A/G/T)CC(A/G)AA(G/T)AC(A/C) AGACTGTA-3. Total RNA was isolated by Trizol method (Invitrogen, Carlsbad, CA, USA). Less than 500 ng RNA was used in RT-PCR reaction as specified in the RNA PCR Kit (AMV) Ver.3.0 (TaKaRa, Japan). First-strand cDNA synthesis was performed with oligo (dT) primer at 42°C for 30 min. The two degenerate primers were added for PCR amplification under the following conditions: 32 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; and an additional polymerization step at 72°C for 5 min. The amplified product was cloned into the PGM-T vector (Tiangen, China) and sequenced. Based on the sequences of this RT-PCR fragment, two additional primers were designed to amplify the full-length cDNA using the Smart™ RACE cDNA Amplification Kit (Clontech, USA): GSP-3R: 5′-CACGTTGTTCGGTGCTGTTGGGAC AATG-3′ and GSP-5R: 5′-CCTTCATCACGATATCA GCTGGCGCCAT-3′.

To perform RACE reaction, approximately 1 μg total RNA was used for first-strand cDNA synthesis (42°C for 1.5 h) using the primers provided in the Kit. For 3′-RACE, 2.5 μL of 3′-RACE-Ready cDNA was used as the template, the primer combination was GSP-3R and the UPM primer which was provided in the kit. The PCR reaction was conducted under the following conditions: 94°C for 2 min; 30 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 3 min; and an additional polymerization step at 72°C for 10 min. For 5′RACE, 2.5 μL of 5′-RACE-Ready cDNA was used as the template and the primers were GSP-5R and UPM. The PCR reaction was performed under the following conditions: 94°C for 2 min; 30 cycles of 94°C for 30 s, 63°C for 30 s, 72°C for 3 min; and an additional polymerization step at 72°C for 10 min. The amplified products was cloned into the PGM-T vector and sequenced. The sequences of 3′-RACE and 5′-RACE fragments were assembled to get the full-length cDNA, which was designated as *ZjNHX1*.

The multiple-sequence alignment and the phylogenetic tree of *ZjNHX1* were conducted using CLUSTAL X (Thompson et al. 1994) and PHYLIP program (Felsenstein 1997), respectively. A hydropathy plot of ZjNHX1 was generated using the Kyte–Doolittle method (Kyte and Doolittle 1982) with the TMpred program (http://www.ch.embnet.org/software/TMPRED_form.html).

Yeast Complementation Test

The ORF of *ZjNHX1* was amplified by PCR using primers with restriction sites suitable for subcloning into the pYPGE15 yeast expression vector. The primers were designed as NHX1YF: 5′-CGGAATTCATGGG CCCCGGCGTGGTG-3′ and NHX1YR: 5′-ACGCGTCGA CTCACCGTCCTCCATG-3′. The amplified fragment was gel purified, digested with *Eco*RI and *Sal*I, and ligated into

the pYPGE15 vector which has been digested with the same enzymes. The recombinants were transformed into *Escherichia coli DH5α* and sequenced. These constructs were used for yeast complementation test.

Three *Saccharomyces cerevisiae* mutants, *Δnhx1* (*Δnhx1::HIS3*), *Δena1-4Δnhx1*(*Δenal-4::HIS3Δnhx1::TRP1*) and *Δena1-2*(*Δenal-2::HIS3*) which were isogenic to the wild-type strain W303-1B (*MATα*, *ade2-1*, *ura3-1*, *can1-100*, *leu2-3*, *trp1-1*, *his3-11*), were used for the yeast complementation studies. Yeast strains were transformed with the *ZjNHX1* ORF-pYPGE15 or an empty pYPGE15 plasmid vector by lithium acetate method (Gietz et al. 1992) and were selected on APG (ura^-) medium. APG is a synthetic minimal medium containing 10 mM arginine, 8 mM phosphoric acid, 2% (*w*/*v*) glucose, 2 mM $MgSO_4$, 1 mM KCl, 0.2 mM $CaCl_2$, trace minerals, and vitamins (Rodriguez-Navarro and Ramos 1984).

Saturated liquid media cultures of each strain were serially diluted (1:10, 1:100, 1:1,000) with initial OD_{600}=0.8 and these dilutions were spotted onto solid APG medium at different pH values (pH4.0, adjusted with arginine) and supplemented with different concentrations of NaCl, KCl, and LiCl. All strains were grown at 28°C for 3 days.

ZjNHX1 Expression Analysis by Real-Time Quantitative PCR

Real-time PCR was performed to investigate the expression level of *ZjNHX1*. Total RNA of roots and shoots from NaCl-stressed plants and non-stressed plants were extracted, and 400 ng RNA was used for first-strand cDNA synthesis (SYBR^{R}PrimeScript™ RT-PCR Kit, TaKaRa, Japan) which was used as template for real-time PCR.

The relative expression intensity was calculated according to $2^{-\Delta\Delta C_T}$ equation (Livak and Schmittgen 2001). This method includes a house-keeping gene (*actin* from *Z. japonica*) as an internal control to measure the expression level of the target gene (*ZjNHX1*). The primer of *actin* gene was designed based on the alignment of DNA sequences of several cloned plant *actin* genes (data not shown). The two primers of the house-keeping *actin* gene used in the study were actin-F: 5′-GGTCCTCTTCCAGCCATCCTTC-3′ and actin-R: 5′-GTGCAAGGGCAGTGATCTCCTTG-3′ with an amplification length of 188 bp. The primer pair of *ZjNHX1* used was ZjNHX1-F: 5′-TCACAATATCAGCT GGCGCCATAG-3′ and ZjNHX1-R: 5′-CACAACCCCTTC ACCAAACACAAG-3′ with an amplification length of 182 bp. The amplification efficiency of *ZjNHX1* and *actin* were tested in a separate experiment according to Livak's method.

The real-time PCR analysis was carried out by using SYBR-green fluorescence in the Bio-Rad IQ5 cycler. The cycling was performed as: denaturation at 95°C for 5 s, annealing at 59.5°C for 20 s, extension at 72°C for 20 s and collecting fluorescence signal at 83°C for 20 s, repeats for up to 40 cycles. Melting curves using SYBR-green fluorescence of obtained PCR-sequences detected no hairpin or loop formation. Negative controls with no templates were conducted concurrently. Amplifications were repeated at least twice in independent experiments.

Threshold values (C_T) were calculated by the internal software of Bio-Rad IQ5 cycler according to $2^{-(\Delta C_T ZjNHX1 - \Delta C_T \text{actin})}$. ΔC_T-values were calculated by comparing C_T-values of *actin* and C_T-values of *ZjNHX1* derived from the same cDNA templates, respectively.

Measurement of Na^+ Content in Leaves and Roots

Roots and leaves of the salt stressed plants and the control plants were harvested at the end of treatment and dried at 120°C for 30 min and then 48°C for 48 h until the weight was invariable. The samples were digested with HNO_3, Na^+ content were determined using an atomic absorption spectrophotometer (Shi et al. 2002).

Results

Cloning of *ZjNHX1*

The full-length cDNA of *ZjNHX1* was obtained by RT-PCR and RACE method. The cloned cDNA of *ZjNHX1* is 2421 nucleotides long with a 5′-untranslated region of 408 nucleotides, a predicted ORF of 1,623 nucleotides and a 3′-untranslated region of 390 nucleotides. The predicted ORF encodes a protein of 540 amino acids with a calculated molecular mass of 59.4 KDa. The sequences of *ZjNHX1* cDNA have been deposited at GenBank with accession number EU333827.

Multiple alignments revealed a high degree of homology between deduced amino acid sequences of *ZjNHX1* and putative vacuolar Na^+/H^+ antiporters from other higher plants (Fig. 1). The deduced amino acid sequence of ZjNHX1 is 95%, 91%, 74% identical to that of AlNHX1, OsNHX1, and AtNHX1 respectively which are all vacuolar-type antiporters. The sequence of 'FFIYLLPPI' in ZjNHX1 is highly conserved among other Na^+/H^+ antiporters, and this region was identified as the binding site of amiloride which inhibits the activity of Na^+/H^+ antiporters (Hamada et al. 2001).

Hydropathy plot analysis (using the TMpred program) demonstrated that ZjNHX1 has 12 putative transmembrane domains (Fig. 2), which is also the typical feature of other NHX1 antiporters such as AtNHX1 (Yamaguchi et al. 2003) and OsNHX1 (Fukuda et al. 1999). The phylogenetic analysis of a number of Na^+/H^+ antiporters was generated

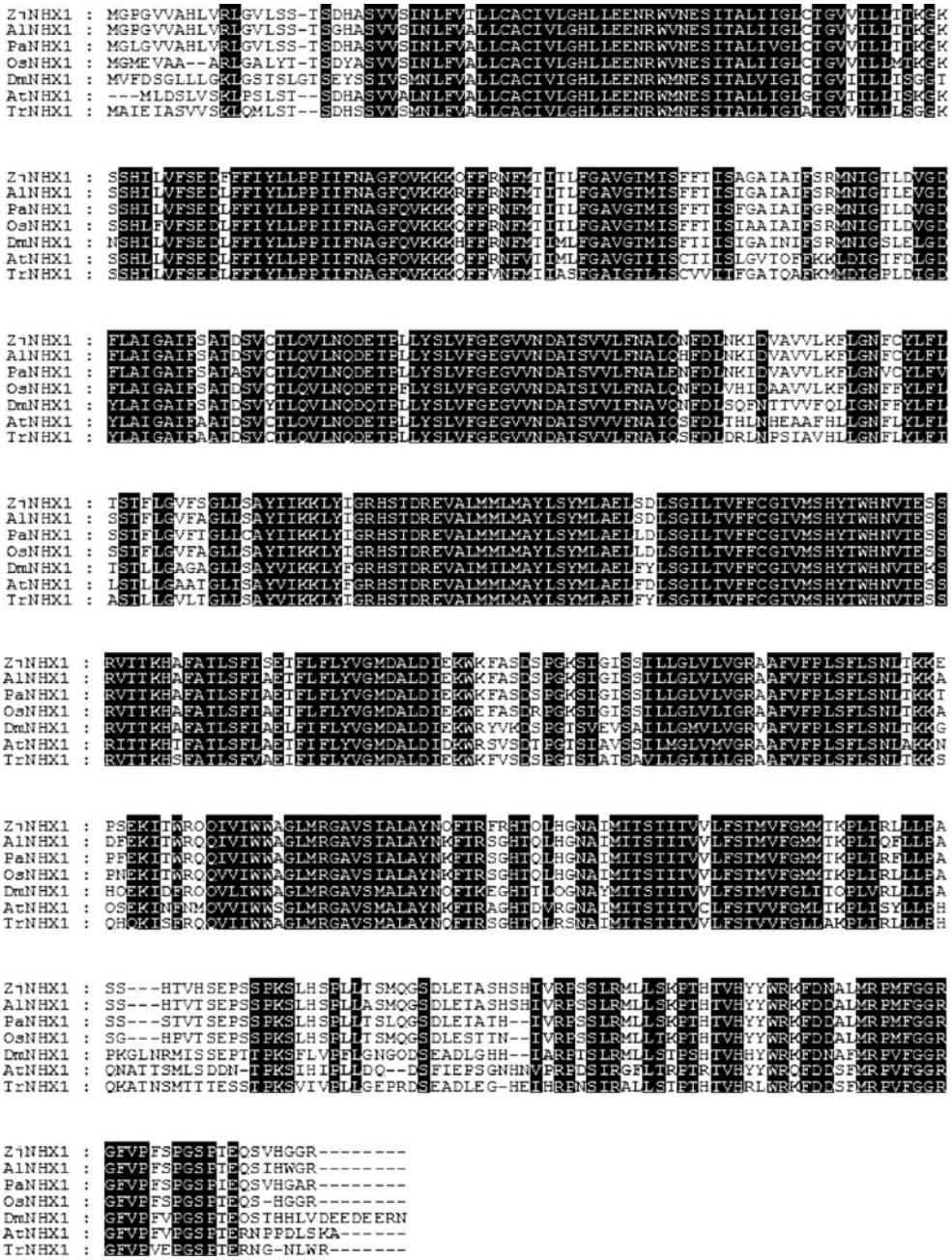

Fig. 1 Alignment of ZjNHX1 protein sequence with other cloned plant antiporters. Sequences are aligned by the program CLUSTAL X. The drop shadows indicate the identical amino acid residues. Sources of other representative Na^+/H^+ antiporters and their GenBank accession numbers are as follows: DmNHX1 (ABN71591), *Dendranthema morifolium*; TrNHX1 (ABV00895), *Trifolium repens*; AtNHX1(AAF21755), *Arabidopsis thaliana*; ZjNHX1 (ABY19311), *Z. japonica*; AlNHX1(AAV80466) *A. littoralis*; OsNHX1 (BAA83337), *O. sativa*; PaNHX1 (BAD95562), *Phragmites australis*

by CLUSTAL X and PHYLIP softwares (Fig. 3) which showed that ZjNHX1 formed a cluster with the most closely related plant vacuolar NHX homolog, which differed from the cluster of plasma membrane Na^+/H^+ antiporter such as SOS1, SOD2 and NhaA. All these results implied that the obtained *ZjNHX1* is a vacuolar-type Na^+/H^+ antiporter gene.

ZjNHX1 Complements the Growth of Salt-Sensitive Yeast Mutants

Heterologous complementation of *S. cerevisiae* mutants with plant genes provides a useful strategy for the functional assessment of plant proteins. Given the similarity in amino acid composition and function between the endosomal yeast and the plant vacuolar Na^+/H^+ antiporters, yeast mutants lacking NHX1 provide a unique tool for the assessment of the functional role of the NHX1. Studies were carried out to determine whether the *ZjNHX1* gene product could functionally complement the *Δnhx1* and *Δena1-4Δnhx1* yeast mutants by suppressing their observed phenotypes. As shown in Fig. 4a, b, the *Δnhx1* mutant was sensitive to high concentrations of NaCl (400 mM) and LiCl (100 mM), whereas the mutant harboring the full length of *ZjNHX1* cDNA grew normally and displayed a similar growth rate with the wild-type

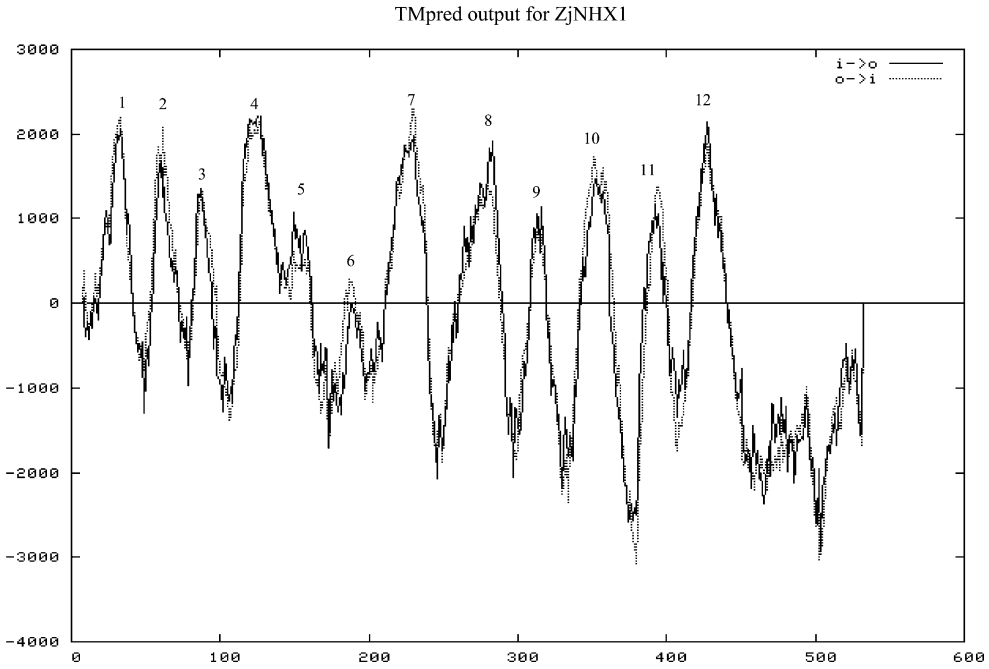

Fig. 2 Hydrophobicity plot of ZjNHX1. The hydrophobicity values were calculated by the program TMpred available at http://www.ch.embent.org/software/TMPRED_form.html

strain. Moreover, it was found that *Δnhx1* mutant was also sensitive to a high K^+ concentration in APG medium (Fig. 4c). Overexpression of ZjNHX1 in *Δnhx1* mutant rendered the strain tolerant to a 1,000 mM K^+ concentration which is to some extent similar with that of the wild-type strain. The results suggested that *ZjNHX1* improved the tolerance of *Δnhx1* mutant to the stress of NaCl, LiCl, and KCl.

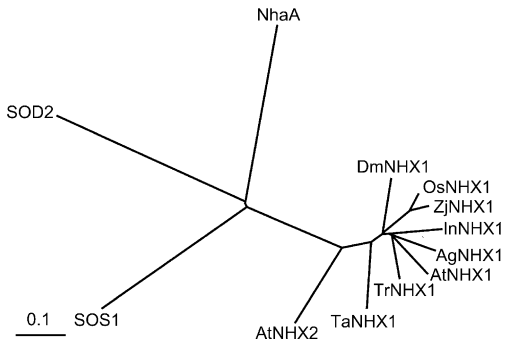

Fig. 3 Phylogenetic analysis of vacuolar Na^+/H^+ antiporters. Distances were calculated using PHYLIP (Felsenstein 1997): PRODIST was used to generate the distance matrix and FITCH was used to generate the tree. The origins of the proteins are as follows (abbreviations with GenBank accession number in brackets): AtNHX1(AAF21755), *A. thaliana*; AtNHX2(BAB08564), *A. thaliana*; AgNHX1(BAB11940), *A. gmelini*; DmNHX1(ABN71591), *D. morifolium*; InNHX1(BAB60899), *Ipomoea nil*; OsNHX1 (BAA83337), *O. sativa*; TrNHX1(ABV00895), *T. repens*; ZjNHX1 (ABY19311), *Z. japonica*; NhaA (AAA23448), *E. coli*; SOD2 (CAA77796), *Schizosaccharomyces pombe*; SOS1(NP_178307), *A. thaliana*

ZjNHX1 was also overexpressed in the *Δenal1-4Δnhx1* mutant which lacks both *S. cerevisiae* NHX1 and plasma membrane sodium efflux pump, and monitored on APG medium with a serious treatment of NaCl concentrations (0, 25, 50, and 75 mM) at pH5.5. In the treatment of 75 mM NaCl, it was found that *ZjNHX1* functionally complements the growth of yeast double mutants by suppressing its observed phenotypes (Fig. 5). All the results above indicated that ZjNHX1 functions normally in the endosomal compartmentation of yeast mutants, plays a similar role with that of the yeast Na^+/H^+ antiporter.

Expression Analysis of *ZjNHX1*

Real-time PCR was performed to investigate the expression pattern of *ZjNHX1* under salt treatment. Relative expression analysis was generated from data of target gene *ZjNHX1* and reference gene *actin* using $2^{-\Delta\Delta C_T}$ method (the amplification efficiency of *ZjNHX1* and *actin* was the same, data not shown). It was found that the *ZjNHX1* transcript was increased after salt treatment. As shown in Table 1, the steady *ZjNHX1* accumulation after salt treatment in shoots were 2.45-fold increased compared to that of the control, whereas the transcription level of *ZjNHX1* was only slightly increased (1.008-fold) in roots. This indicated a tissue-specific expression response of *ZjNHX1* to NaCl stress.

The Na^+ content in shoots and roots of the untreated and salt-treated plants were measured by atomic absorption spectrophotometer (Z-5000 polarized Zeeman). After 200 mM NaCl treatment for 14 days, it was found that Na^+ content rose up from 0.67% to 1.80% in shoots,

whereas Na^+ content remains 0.8% in roots (Fig. 6). Salt stress caused a significant sodium accumulation in shoots but only a minor change in roots. This result implied that a higher inclusion of Na^+ was compartmented by the activity of ZjNHX1 into the vacuoles of shoot cells. After salt stress, the transcript level of *ZjNHX1* in shoots rose up and compartmented more Na^+ into the vacuole. The data from sodium ion content and from the quantitative real-time PCR all supported the opinion that *ZjNHX1* expression showed a tissue-specific NaCl response pattern.

Discussion

Soil salinity and drought are the main abiotic stresses bringing damage to the growth of plants and the productivity of crops. Plants have developed the multiple mechanisms of ion homeostasis including extrusion of Na^+, intracellular sequestration Na^+ into the vacuole and the regulation of K^+/Na^+ ratio to cope with the stress (Blumwald 2000). Great progress has been made in improving plant salt-tolerance by re-establishing ion homeostasis, such as manipulating vacuolar Na^+/H^+ antiporter to produce transgenic plants since Na^+/H^+ antiporter can observably increase the plant salt tolerance. Many researches demonstrated that it is feasible to reduce the ionic stress imposed on the cell by compartmentalizing Na^+ into the vacuole. In this study, we isolated a putative tonoplast-associated Na^+/H^+ antiporter gene from *Z. japonica* named *ZjNHX1*. To our knowledge, this is the first description of full-length gene encoding the tonoplast-associated Na^+/H^+ antiporter in *Z. japonica*.

Phylogenetic analysis of antiporters from different species indicated that the cloned transcripts of *ZjNHX1* showed a high homology with the vacuolar Na^+/H^+ antiporters but shared much lower homology with either plasma membrane or mitochondrion membrane-associated Na^+/H^+ antiporters. Therefore, *ZjNHX1* may belong to the type of vacuolar Na^+/H^+ antiporter. Meanwhile, the hydrophobicity plot of *ZjNHX1* indicates that it has 12 transmembrane spanning domains which is the typical feature of plant NHX (Fukuda et al. 1999; Yamaguchi et al. 2003).

Several non-homologous regions, however, appeared at the N-terminal (2–11) and the C-terminal (449-498, 546-554) regions of ZjNHX1. Some researches have demonstrated that the structural subdivision of Na^+/H^+ antiporter accords with a partition of its function. For example, it was found that the deletion of the hydrophilic C terminus of AtNHX1 resulted in a dramatic increase in the relative rate of Na^+/H^+ transport (Yamaguchi et al. 2003). Therefore, these variant sequences of ZjNHX1 may reflect the

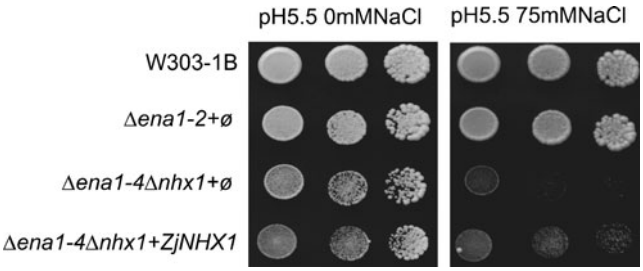

Fig. 5 Expression of ZjNHX1 in *Δena1-4Δnhx1* yeast mutants. All strains were transformed with the *ZjNHX1* ORF-pYPGE15 or an empty pYPGE15 plasmid vector and were selected on APG (ura^-) medium. Each strain were serially diluted (1:10, 1:100, 1:1,000) with initial OD_{600}=0.8 and these dilutions were spotted onto solid APG medium at pH 5.5(adjusted with arginine) and supplemented 75 mM NaCl. All strains were grown at 28°C for 3 days

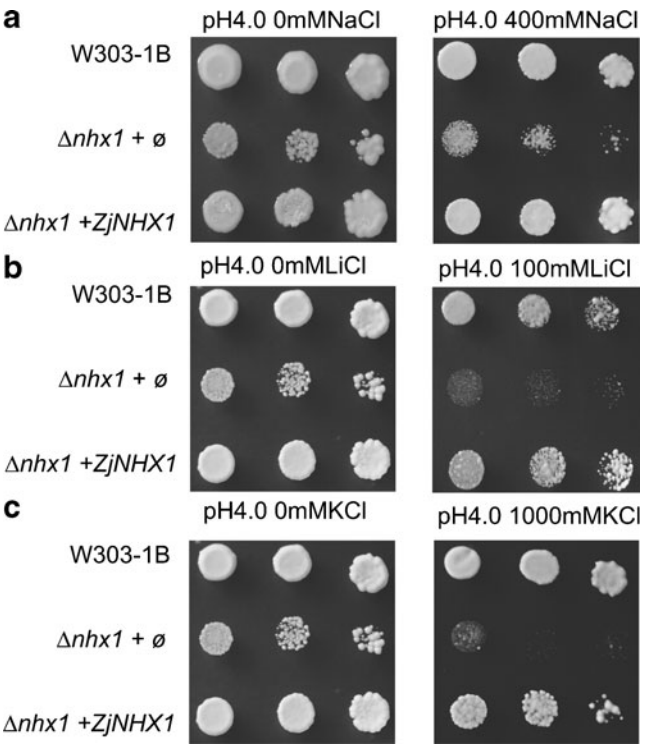

Fig. 4 Expression of ZjNHX1 in *Δnhx1* yeast mutants. All strains were transformed with the *ZjNHX1* ORF-pYPGE15 or an empty pYPGE15 plasmid vector and were selected on APG (ura^-) medium. Each strain were serially diluted (1:10, 1:100, 1:1,000) with initial OD_{600}=0.8 and these dilutions were spotted onto solid APG medium at pH 4.0 (adjusted with arginine) and grown on at 28°C for 3 days on different media. **a** Comparison of yeast cell growth upon NaCl treatment. Serial dilutions of the strains were grown on APG plates with or without 400 mM NaCl. **b** Comparison of yeast cell growth upon LiCl treatment. Serial dilutions of the same strains were grown on APG plates supplemented with or without 100 mM LiCl. **c** Comparison of yeast cell growth upon LiCl treatment. Serial dilutions of the same strains were grown on APG plates supplemented with or without 1M KCl

Table 1 The relative quantitative expression of *ZjNHX1* in each tissue by real-time PCR analysis

Tissue	Group	Average C_T	ΔC_T	$\Delta\Delta C_T$	Ratio
Shoot					
NaCl stress	T*actin*	23.67±0.05	2.35±0.07	−(1.29±0.01)	2.45 (2.43–2.46)
	T*NHX1*	26.01±0.05			
Control	C*actin*	23.11±0.07	3.64±0.08		
	C*NHX1*	26.75±0.04			
Root					
NaCl stress	T*actin*	27.88±0.09	−(2.13±0.1)	−(0.11±0.18)	1.008 (0.95–1.22)
	T*NHX1*	25.74±0.05			
Control	C*actin*	27.61±0.05	−(2.02±0.15)		
	C*NHX1*	25.59±0.15			

Zoysia japonica plants were treated with a nutrient solution supplemented with NaCl. NaCl concentration was increased gradually by 50-mM increments every three days until the final concentration reached 200 mM. Seven days later, plants were harvested. Control plants were harvested at the same age. The real-time PCR analysis was carried out using SYBR-green fluorescence in a Bio-Rad IQ5. The relative expression was calculated according to $2^{-\Delta\Delta C_T}$ method. $\Delta C_T = C_T(NHX1) - C_T(actin)$; $\Delta\Delta C_T = \Delta C_T$ (NaCl treatment) $- \Delta C_T$ (control). Values are means of three replicates±SE

difference in Na^+/H^+ antiporter activities between halophytes and glycophytes. For further understanding of the characterizations of Na^+/H^+ antiporters among different plants, more studies on the relationship between structure and function should be conducted in the future.

The function of ZjNHX1 was analyzed by yeast complementary test using the *Δnhx1* and *Δnhx1Δenal1-4* mutant strains. It was found that the *ZjNHX1* gene could suppress the sensitivity of the two yeast mutants to NaCl, KCl and LiCl (Fig. 4). The results demonstrated that ZjNHX1 has the ability to compensate partially the function of yeast NHX1. The similar studies in *OsNHX1* from rice (Fukuda et al. 2004b), *BvNHX1* from sugar beet (Xia et al. 2002) also found that those genes could partially compensate the growth of yeast *Δnhx1* mutant and *Δnhx1Δenal1-4* mutant, respectively. The compartmentalization of Na^+ into vacuoles by Na^+/H^+ antiporter not only provides an efficient way to avert the deleterious effects of Na^+ in the cytosol, but also allows plants to use NaCl as an osmoticum to maintain an osmotic potential (Blumwald et al. 2000). Our experiment with NaCl, KCl and LiCl treatment in the present study also demonstrated that ZjNHX1 has the ability to transfer Na^+, K^+, or Li^+ or other ions from the cytoplasm to vacuoles as an osmoticum to cope with the osmotic stress.

Previous reports found that the expression of plant vacuolar Na^+/H^+ antiporter was discrepant in various plant tissues. In this study, we also found that the transcript levels of *ZjNHX1* were up-regulated by salt stress. After salt treatment for 14 days, *ZjNHX1* expression was greatly increased in shoots, but only slightly increased in roots. The result was similar to that of rice, but different from the expression pattern of some salt-tolerant crops such as *barley* (Fukuda et al. 2004a) and *A. littoralis* (Zhang et al. 2008). We measured the Na^+ distributions after stress and found that Na^+ accumulation was more elevated in shoots than that in roots (Fig. 6). The results implied that the remarkably induced expression of *ZjNHX1* in shoots might accumulate more Na^+ into the vacuoles of shoot cells to alleviate the Na^+ toxicity, and therefore play an important role in salt tolerance. These data together supported the opinion that *ZjNHX1* expression showed a tissue-specific NaCl response pattern.

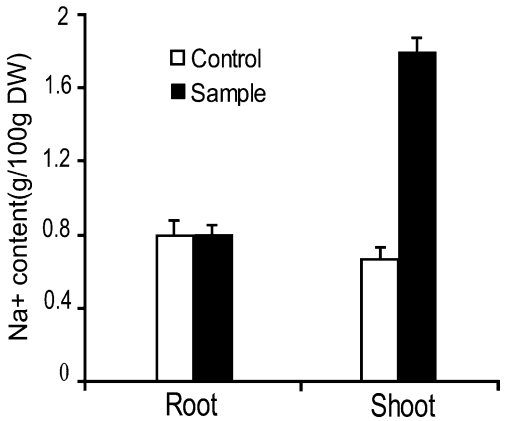

Fig. 6 Na^+ content in shoots and roots under salt stress. *Z. japonica* plants were treated with a nutrient solution supplemented with NaCl, NaCl concentration was increased gradually by 50 mM increments every 3 days until the final concentration reached 200 mM. Seven days later, plants were harvested. Control plants were harvested at the same age. Ion contents were determined using an atomic absorption spectrophotometer. Result as means±SD (n=3)

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