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Full Length Research Paper

Isolation and preliminary function analysis of a Na^+/H^+ antiporter gene from *Malus zumi*

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A full-length cDNA Na^+/H^+ antiporter gene (*MzNHX1*) was isolated from *Malus zumi* according to the homologous Na^+/H^+ antiporter gene region in plants. Sequence analysis indicated that the cDNA was 2062 bp in length, including an open reading frame (ORF) of 1629 bp, which encoded a predicted polypeptide of 542 amino acids. The MzNHX1 protein shared high identity with other reported plant vacuolar Na^+/H^+ antiporters. Southern blot analysis detected multiple copies of *MzNHX1* in the *M. zumi* genome. Northern blot analysis showed negligible expression of the gene in roots, but expression was detected in stems and leaves. To test the function of *MzNHX1*, we expressed the gene in the salt-sensitive AXT3 yeast mutant. No differences in yeast cell growth were detected given the presence or absence of *MzNHX1* on a NaCl free medium. However, on a 70 mM NaCl medium, growth in the control transformant was noticeably suppressed, and yeast overexpression of *MzNHX1* showed increased population growth rates. These results indicated that the MzNHX1 protein increased AXT3 salt tolerance. Alignments of the deduced Na^+/H^+ antiporter amino acid sequence of different plants from NCBI revealed that *MzNHX1* shared high identity (>86%) with vacuolar Na^+/H^+ antiporters, including *RhNHX1* from rose, *cNHX1* from citrus, and *AtNHX1* from *Arabidopsis thaliana.* However, *MzNHX1* shared very low identity (<10%) with plasma membrane Na^+/H^+ antiporters such as *AtSOS1* from *A. thaliana*. These results indicated that *MzNHX1* was localized to the vacuolar membrane.

Key words: *Malus zumi*, Na^+/H^+ antiporter gene, gene cloning, gene function.

INTRODUCTION

Soil salinization is one of the main limitations to crop yield and quality, which has resulted in substantial economic losses worldwide. Toxic ions, osmotic stress, and ionic or nutrition imbalances are the main causes of salt stress in plants (Liu and Wang 1998). This form of environmental pressure reduces the photosynthetic capacity of plants, increases energy consumption, accelerates senescence, suppresses growth, and in extreme cases leads to plant death. Salt-tolerant plants are able to compartmentalize sodium in the vacuole, and the Na^+/H^+ antiporter serves a vital role in this process (Blumwald and Gelli, 1987). The accumulation of Na^+ in vacuoles reduces Na^+ injury to enzymes and membranes by preventing excessive Na^+ from disrupting metabolic processes. Accumulation of Na^+ in vacuoles also functions to maintain osmotic balance in plant cells. In addition, sodium accumulation in vacuoles is one of the most efficient means to maintain high K^+/Na^+ ratios in the cytoplasm (Niu et al., 1995).

Na^+/H^+ antiporter genes have been isolated from many different plants, including rice (Fukuda et al., 1999), rape (Wang et al., 2003), New Zealand spinach (Lv et al., 2004), and Japanese morning glory (Ohnishi et al., 2005). However, research in woody plants, especially in fruit trees, is not well represented in the literature. Cultivated *Malus* species (apple) is grown worldwide, and is an important fruit crop. However, apple production is currently threatened by sodium rich soils, and in many instances, soil salts are increasing due to salinization. *Malus zumi* is a salt-resistant apple germplasm, well adapted to coastal areas and inland saline soils containing up to 0.6% salt (Gu et al., 1996). Therefore, cloning the Na^+/H^+ antiporter gene from *M. zumi* and investigating its role in salt-tolerance may provide valuable

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data to assist apple growers in developing successful salt tolerance strategies.

MATERIALS AND METHODS

Cloning the *MzNHX1* gene

Total RNA was extracted from young leaves of salt-tolerant *M. zumi* using a modified CTAB method (Zhang et al., 2005). First-strand cDNA synthesis was performed with Oligo-dT primers and Super Script II reverse transcriptase (BD). To isolate the vacuolar Na^+/H^+ antiporter gene from *M. zumi*, we designed degenerate primers based on conserved sequences in vacuolar Na^+/H^+ antiporter genes for PCR. The primer sequences were as follows:

5'-ATTGG(T/A)GC(A/C)ATITTT(G/T)CIGC-3'and 5'-TCIAT(G/A)TC CA(A/G)IGCATCC AT-3'.

Rapid amplification of cDNA ends (RACE) (BD SMART™ RACE cDNA Amplification Kit) was performed to amplify the full-length cDNA. However, only the 3'-terminal fragment was obtained. Therefore, the 5'-terminal fragment was isolated using genomic walking (Takara LA PCR™ *in vitro* Cloning Kit), the intron deleted, and spliced to other fragments. Gene specific primers were subsequently designed according to the two ends of the ORF based on the spliced sequence, and used to amplify the full-length cDNA. The re-obtained full-length cDNA was sequenced for additional confirmation.

Amino acid sequence analysis

Multiple sequence alignments and phylogenetic reconstruction were performed based on putative complete amino acid sequences using the DNAMAN program. The TMpred program was applied for hydropathy plot analysis.

Southern and Northern blot analysis

Ten micrograms of genomic DNA derived from young leaves was digested with *EcoR*I+*Pst*I, *Xba*I, or *BamH*I that had no cutting site in probe sequence and hybridized with a probe corresponding to the 3'-UTR labeled with ^{32}P-dCTP.

M. zumi subcultures were rooted, and transferred into a hydroponic culture without NaCl. The culture condition was as follows: 22 - 28℃ and RH 45 - 50% during the day, 15 - 20℃ and RH 60 -70% at night, 12 h light per day, the light intensity was 800 $mol.m^{-2}.s^{-1}$. After two weeks of cultivation, the plants were treated with 200 mM NaCl for 24 h. Total RNA was extracted from roots, stems and leaves, and hybridized with a probe corresponding to the 3'-UTR.

The analyses were performed according to standard procedures (Sambrook et al., 1992).

Yeast strains and medium

Saccharomyces cerevisiae, the salt-sensitive mutant strain AXT3 (≥ena1-4::His3 ≥nha1::LEU2 ≥nhx1::TRP1) was chosen for this study (kindly provided by Dr. José M. Pardo, Instituto de Recursos Naturalesy Agrobiologia, Spain and Dr. Wang Xue-chen,CAU, China). The strain was isogenic to W303-1B (*S. cerevisiae* MATαleu 2-3, 112 ura3-1 his3-11, 15 trp1-1 ade2-1 can1-100).

Yeast cells were grown on YPD (1% yeast extract, 2% peptone, 2% glucose), selected on SD-Ura^- (0.67% yeast nitrogen base, 2% glucose, and the appropriate amino acid), and expressed on AP (10mM arginine, 8 mM phosphoric acid, 2% glucose, 2 mM $MgSO_4$, 1 mM KCl, 0.2 mM $CaCl_2$, trace minerals and vitamins, at pH 6.7). Glucose was displaced with galactose to induce expression of the GAL promoter.

Vector construction

First-strand cDNA was used as a template to amplify the *MzNHX1* ORF with the following primers: 5'-ATAGGTACCCAATGGCGG TTCCACATTTG-3'(GGTACC, *Kpn*I site) and 5'-ACCGGATCCcc TTGCC ACTGAACATTGTTG-3'(GGATCC , *Xba*I site).

The PCR products were cloned to the pMD18-T vector (Takara). The ORF was digested with *Kpn*I and *Xba*I from the recombinant plasmid, and inserted into the yeast expression vector pYES2 (driven by a GAL promoter). The pYES2 expression vector had been digested with the same restriction enzymes. The resulting plasmid was designated pYES2-NHX1.

Expression of *MzNHX1* in the yeast mutant AXT3

The pYES2 and pYES2-NHX1 plasmids were transformed into yeast strain AXT3 using the lithium acetate method to perform functional complementation analysis. Yeast cultures saturated in liquid SD-Ura^- media were harvested and adjusted to OD_{600}=1 with sterilized double-distilled water. Ten-fold serial dilutions of the strains (10 µl) were spotted onto AP plates (1.5% Agar) containing 70 mM NaCl, 3 mM LiCl, 10 mg/L Hyg, 10 mg/L ABA, or no treatment (control), and grown at 30 for 72 h. Twenty-µl of the same strain seed culture (OD_{600}=1) were grown in 2ml of AP medium supplemented with 0, 70, 140, 210, 280, or 350 mM NaCl at 30℃ for 72 h with shaking (200 rpm). Strain growth was measured by reading absorbance at 600 nm. There were three replications for each treatment.

RESULTS AND DISCUSSION

Analysis of *MzNHX1* amino acid sequence

The *MzNHX1* cDNA was 2062 bp in length, including an open reading frame of 1629 bp, encoding 542 amino acid residues with a predicted molecular mass of 60 kDa (Figure 1).

Alignments of the deduced Na^+/H^+ antiporter amino acid sequence of different plants from NCBI revealed that the MzNHX1 shared high identity (>86%) with vacuolar Na^+/H^+ antiporters, including *RhNHX1* from rose, *cNHX1* from citrus, and *AtNHX1* from *Arabidopsis thaliana* (Figure 2). However, *MzNHX1* shared very low identity (<10%) with plasma membrane Na^+/H^+ antiporters such as *AtSOS1* from *A. thaliana* (data not shown). This result indicated that *MzNHX1* was localized to the tonoplast.

The phylogenetic tree derived from a number of Na^+/H^+ antiporter sequences is shown in Figure 3. *MzNHX1* appeared in the same group as *RhNHX1*. Two *NHX1* sequences corresponding to halophytes *AgNHX1* and *SeNHX1* were also allied. The two plasma membrane *AtSOS1* and *AtNHX8* appeared in the same group, while *MzNHX1* was nested with nine vacuolar Na^+/H^+ antiporters. This view of relationships based on antiporter sequences also suggested *MzNHX1* is localized to the

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1     ATGGCGGTTCCACATTTGAGCATGTTGATCTCAAAGTTACAAAATCTATCCACTTCGGACCACTCGTCTGTGGTTTCGATG
1      M  A  V  P  H  L  S  M  L  I  S  K  L  Q  N  L  S  T  S  D  H  S  S  V  V  S  M
82    AACCTTTTCGTGGCGCTACTTTTAGCTTGTATTGTGATCGGACATCTTCTCGAGGAGAATCGATGGGTGAATGAGTCGATC
28     N  L  F  V  A  L  L  L  A  C  I  V  I  G  H  L  L  E  E  N  R  W  V  N  E  S  I
163   ACCGCCCTTTTGATTGGTATATGTACTGGAGTAGTTATTCTTCTGATCAGTCGAGGAAAAAGTTCGCATCTTTTGGTTTTC
55     T  A  L  L  I  G  I  C  T  G  V  V  I  L  L  I  S  R  G  K  S  S  H  L  L  V  F
244   AGTGAAGATCTTTTCTTTATATGCCTCCTTCCGCCTATTATTTTTAATGCCGGGTTTCAGGTGAAAAAGAAGCAGTTCTTT
82     S  E  D  L* F* F* I* C* L* L* P* P* I* I* F  N  A  G  F  Q  V  K  K  K  Q  F  F
325   GTTAACTTCAGCACCATTGTACTGTTTGGTGCCATTGGTACATTAGTATCCTGCACTATCATATCATTAGGCGCTACACAA
109    V  N  F  S  T  I  V  L  F  G  A  I  G  T  L  V  S  C  T  I  I  S  L  G  A  T  Q
406   TTCTTTAAGAAATTGGATATCGGAACTCTGGAATTGGGGGACTTTCTTGCGATTGGTGCAATTTTTGCTGCAACGGATTCT
136    F  F  K  K  L  D  I  G  T  L  E  L  G  D  F  L  A  I  G  A  I  F  A  A  T  D  S
487   GTTTGCATGTTGCAGGTGCTCAATCAAGATGAGACACCTTTACTCTACAGTCTTGTGTTCGGGGAGGGTGTTGTTAACGAT
163    V  C  M  L  Q  V  L  N  Q  D  E  T  P  L  L  Y  S  L  V  F  G  E  G  V  V  N  D
568   GCGACATCTGTGGTTCTTTTCAATACTATTCAGAGCTTTGATCTCACCCACCTTGATTCCGGCATTGCCTTGCACTTTCTG
190    A  T  S  V  V  L  F  N  T  I  Q  S  F  D  L  T  H  L  D  S  G  I  A  L  H  F  L
649   GGAAACTTCTTTTATTTGTTTTTCGCAAGCACCATGCTAGGAGTGTTTGCAGGGCTGCTTAGTGCTTACATTATCAAAAAA
217    G  N  F  F  Y  L  F  F  A  S  T  M  L  G  V  F  A  G  L  L  S  A  Y  I  I  K  K
730   CTTTATTTTGGAAGGCACTCTACGGATCGTGAGGTTGCTCTTATGATGCTCATGGCATATCTGTCATATATACTGGCTGAA
244    L  Y  F  G  R  H  S  T  D  R  E  V  A  L  M  M  L  M  A  Y  L  S  Y  I  L  A  E
811   TTATTCTATTTGAGTGGCATTCTCACTGTGTTCTTTTGTGGGATCGTGATGTCGCATTACACTTGGCACAATGTGACTGAG
271    L  F  Y  L  S  G  I  L  T  V  F  F  C  G  I  V  M  S  H  Y  T  W  H  N  V  T  E
892   AGTTCAAGAGCTACGACCAAGCATGCTTTCGCAACCTTGTCATTTGTTGCCGAAATATTTATCTTCCTTTATGTTGGTATG
298    S  S  R  A  T  T  K  H  A  F  A  T  L  S  F  V  A  E  I  F  I  F  L  Y  V  G  M
973   GATGCCTTGGACATTGAAAAGTGGAGATTTGTAAGTGACAGTCCTGGAACATCAGTGGCAGTGAGTTCAATACTGCTAGGT
325    D  A  L  D  I  E  K  W  R  F  V  S  D  S  P  G  T  S  V  A  V  S  S  I  L  L  G
1054  CTTATTATGCTTGGAAGAGCAGCTTTCGTTTTCCCCTTATCATTCTTGTCGAACTTAACAAAGAAAAACCAACATGATAAA
352    L  I  M  L  G  R  A  A  F  V  F  P  L  S  F  L  S  N  L  T  K  K  N  Q  H  D  K
1135  ATTAGCCTCCGGCAGCAAGTTATAATATGGTGGGCTGGTCTCATGAGAGGTGCTGTGTCTATGGCACTTGCTTACAATCAG
379    I  S  L  R  Q  Q  V  I  I  W  W  A  G  L  M  R  G  A  V  S  M  A  L  A  Y  N  Q
1216  TTTACAAGGTCAGGCCACACGCAGTTGCGAGCAAATGCAATCATGATCACTAGCACGATAACTGTTGTTCTTGTCAGCACA
406    F  T  R  S  G  H  T  Q  L  R  A  N  A  I  M  I  T  S  T  I  T  V  V  L  V  S  T
1297  GTGGTTTTCGGATTGATGACGAAACCTCTTATAAGGTTCTTGCTGCCTCATTCACCAAAACAAACAACCAGCATGTTGTCG
433    V  V  F  G  L  M  T  K  P  L  I  R  F  L  L  P  H  S  P  K  Q  T  T  S  M  L  S
1378  TCAGAACCAACCTCTCCAAAATCAGTCATTGTTCCACTTCTAGGGCAGGATTCTGTAGATGATCTTGTTGGCCAAGATTTT
460    S  E  P  T  S  P  K  S  V  I  V  P  L  L  G  Q  D  S  V  D  D  L  V  G  Q  D  F
1459  CCACGGCCGGCCAGCTTACGCGATCTTCTGACAACTCCAACGCACACAGTCCATCGCTATTGGCGTAAGTTTGACAATGCA
487    P  R  P  A  S  L  R  D  L  L  T  T  P  T  H  T  V  H  R  Y  W  R  K  F  D  N  A
1540  TTCATGCGTCCAGTGTTTGGAGGCCGGGGTTTTGTTCCCTTTGTTCCCGGGTCACCAACTGAACGGGACAACAATGTTCAG
514    F  M  R  P  V  F  G  G  R  G  F  V  P  F  V  P  G  S  P  T  E  R  D  N  N  V  Q
1621  TGGCAATGAGAGGGTGGTACACCATGTGTCACTATATAAATGGTGGGATATAGATGCTAAAAAGTTAATAACTTAAAAAAT
541    W  Q  *
1702  AAAATTTCCCACTACTTCTGTTAAAATATGCGGTGTACCACTTGTCTTCCCATCATAATGAAAAATTTCTCCACTCGAACC
1783  CACTGAGCATGTGATATATGTTTCGAGGTTTGATTGTATATAGCTTGTGAAAGGTAGAAGTGTACCATAATCTATGTGTAT
1864  TTTGTTTCGATCTACTGTGTATTTTGTATGGCCTGTTATTTTATTTGCTCTAATCACCGGATTGGGGTTGTCTCTTGTCAC
1945  AAACACATCGGTTGAATCTGAGAGCTCAATGTAATGTAATGTAATAATGCTCGACAATAATCGTAATGTTGTTTGCTTTTT
2026  GTTTCCCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
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Figure 1. Nucleotide sequences and deduced amino acid residues of the *Malus zumi* Na^+/H^+ antiporter cDNA. Amino acid residues are indicated as single letters. ATG represents the translation initiation codon. The termination codon TGA is marked with asterisk. The ORF is underlined. The amino acid marked with asterisk is the amiloride-binding motif

tonoplast.

The hydropathy plot generated by TMpred indicated that *MzNHX1* contained 12 putative hydrophobic regions in its N-terminal and a hydrophilic tail in its C-terminal re-

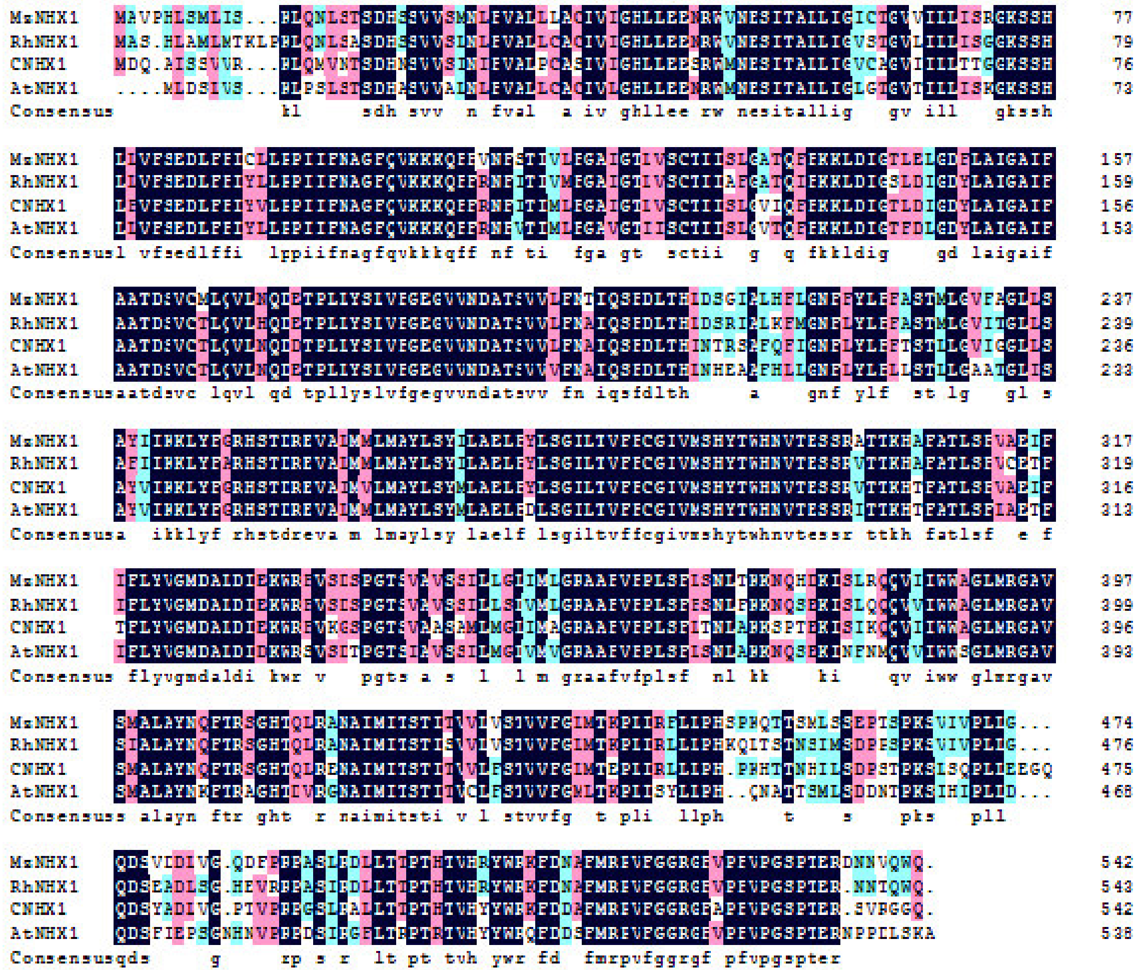

Figure 2. Na^+/H^+ antiporter amino acid sequence alignments from different plant species. The sequences were aligned using DNAMAN program. Highly conserved amino acid residues are highlighted in black, and conservative substitutions are shown in gray. The origins of the proteins are as follows: AtNHX1(AAD16946), *Arabidopsis thaliana*; RhNHX1*, Rosa hybrida*; CNHX1(AAK27314), *citrus*; MzNHX1, *Malus zumi*.

gions (Figure 4). The third transmembrane region of *MzNHX1* contained the amiloride binding domain ^{85}LFFI* LLPPII^{95} (amino acid residues marked with * in Figure 1), which is a highly conserved domain in the eukaryotic Na^+/H^+ antiporter family (Orlowski and Grinstein 1997). It has been reported that the fifth and sixth transmembrane regions are Na^+ binding sites, which play critical roles in VACUOLARtype Na^+/H^+ antiporter transport activity, but they do not appear to be transmembrane segments (Yamaguchi et al., 2003). The hydrophilic tail could regulate the activity of the Na^+/H^+ antiporter (Yamaguchi et al., 2003). These results indicated that *MzNHX1* was a vacuolar Na^+/H^+ antiporter gene.

MzNHX1 molecular analysis

Southern blot analysis showed more than one band in all lanes, which indicated that *MzNHX1* exists in multi-copies in the *M. zumi* genome (Figure 5). Results of previous studies reported a different number of *-NHX1* copies in several species, some species with multi-copies and others showing only a single copy. For example, two or three copies of *TtNHX1* were detected in the halophyte *Salicornia europaea* (Lv et al., 2004), multi-copies of *BnNHX1* in *Brassica napus* (Wang et al., 2003), and a single copy of *OsNHX1* in *Oryza sativa* (Fukuda et al., 1999).

Results of Northern blot analysis showed that *MzNHX1* was highly expressed in stems, decreased in leaves, but negligible expression was detected in roots, when all tissues were treated with 200 mM NaCl for 24h (Figure 6). The expression of *AgNHX1* from *Atriplex gmelini* in both roots and leaves increased with 100 or 400 mM NaCl for 24 h, and the amounts of mRNA in leaves was two to three times that of roots under the two treatments (Hamada et al., 2001). Similar results were found in rice where expression of *OsNHX1* increased slightly in roots

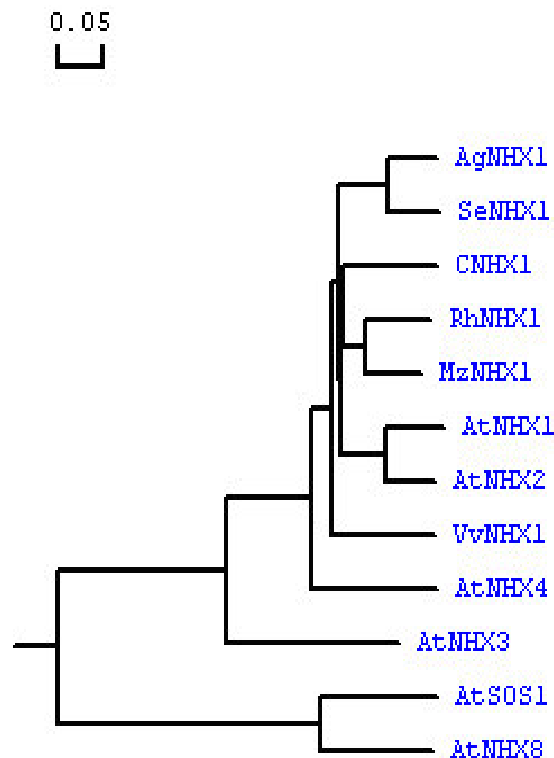

Figure 3. Phylogenetic analysis of Na^+/H^+ antiporter proteins. The phylogenetic tree was generated using DNAMAN program. The origins of the proteins are as follows: AgNHX1(BAB11940), *Atriplex gmelini*; SeNHX1(AY131235), *Salicornia europaea*; AtNHX2, 3,4,8(AAM08403, AAF08577, AAM08405, AAZ76248) and AtSOS1(AAF76139) , *Arabidopsis thaliana*; VvNHX1 (AAV36562), *Vitis vinifera*; CNHX1, RhNHX1 and MzNHX1 are as in Figure 2.

but markedly in leaves when treated with 50-200 mM NaCl for 24 h (Fukuda et al., 2004). In addition, the expression of the plasma membrane *AtSOS1* from *A. thaliana* was clearly increased in roots but only slightly in leaves following a 6 h 200 mM NaCl treatment (Shi et al., 2000). The results of these studies suggested the vacuolar Na^+/H^+ antiporter gene expressed negligibly in roots, but substantial expression was observed in stems and leaves. This expression model made plants exclude Na^+ from root cells by plasma membrane Na^+/H^+ antiporter, but sequester it to the vacuole by vacuolar Na^+/H^+ antiporter during salt resistance.

In *A. thaliana,* six *AtNHXs* were isolated and only *AtNHX*1, *AtNHX2,* and *AtNHX5* were capable of functioning similar to the *ScNHX1* gene in salt sensitive yeast mutants (An and Zhang 2006). The vacuolar Na^+/H^+ antiporter gene *InNHX1*, isolated from Japanese morning glory, controls flower color change, and cannot be induced by NaCl treatments. The gene exhibited little expression in leaves, stems and roots, but was predominantly expressed in the flower limbs at approximately 12 h prior to bloom. *InNHX2* was expressed in leaves, stems and roots, and was induced by NaCl. This gene exhibited primary control of salt tolerance in *Ipomoea* (Ohnishi et al., 2005). Different members of the Na^+/H^+ antiporter gene family have different functions, and the type of expression is related to its specific role. If additional Na^+/H^+ antiporter genes from *M. zumi* are isolated, and the function of these genes characterized, an enhanced understanding of salt tolerance mechanisms, not only in *Malus*, but in other plants can be elucidated.

Complementation of yeast with the *MzNHX1* gene

Yeast is an easily grown single celled organism with a short generation time and a well-understood expression control system. Therefore, it is suitable for use in functional genomics studies. In addition, because of many conserved genes between yeast, animals and plants, the yeast expression system can be readily applied to gene function research of animals and plants (Xing et al., 2006). Therefore, we did preliminary function analysis of *MzNHX1* using yeast mutant.

To test the function of *MzNHX1*, we expressed the gene in the salt-sensitive AXT3 yeast mutant (Figure 7). No differences in yeast cell growth were detected given the presence or absence of *MzNHX1* on a NaCl free medium. However, on a 70 mM NaCl medium, growth in the control transformant was noticeably suppressed, and yeast with overexpression of *MzNHX1* showed increased population growth rates. The results indicated that the MzNHX1 protein increased AXT3 salt tolerance.

To specifically test *MzNHX1* response to NaCl, we treated transgenic yeast with 3 mM LiCl, 10 mg/L Hyg, and 10 mg/L ABA (Figure 8). The results indicated that the mutant was hypersensitive to LiCl, but overexpression of *MzNHX1* can, to a certain extent, increase its tolerance to LiCl (Figure 8b). Therefore, *MzNHX1* did not respond specifically to NaCl stress, and likely serves important roles in other cellular processes (Hamada et al., 2001). Yeast cells are hypersensitive to the toxic cation hygromycin, which accumulates intracellularly in response to an electrochemical proton gradient (Darley et al., 2000). Gaxiola et al. (1999) reported that *ScNHX1* affected the pH or membrane potential of vacuolar compartments, and was integral in hygromycin compartmentalization into vacuoles. *MzNHX1* also suppressed hygromycin sensitivity of AXT3 (Figure 8c), indicating that the MzNHX1 protein had the same function as ScNHX1 in yeast.

MzNHX1 showed no specificity to NaCl stress, and also no response to ABA treatment (Figure 8d). Relevant research in Na^+/H^+ antiporter genes in other species helps to explain these results. *AtSOS1* expression was specifically up-regulated by salt stress but not cold stress or ABA (Shi HZ et al., 2000). The expression of *cNHX1* was induced by salt stress and heat shock, but not by ethylene or UV irradiation (Porat et al., 2002). Two Na^+/H^+ antiporter genes (*InNHX1* and *InNHX2*) isolated from Japanese morning glory (*Ipomoea nil*) were expressed,

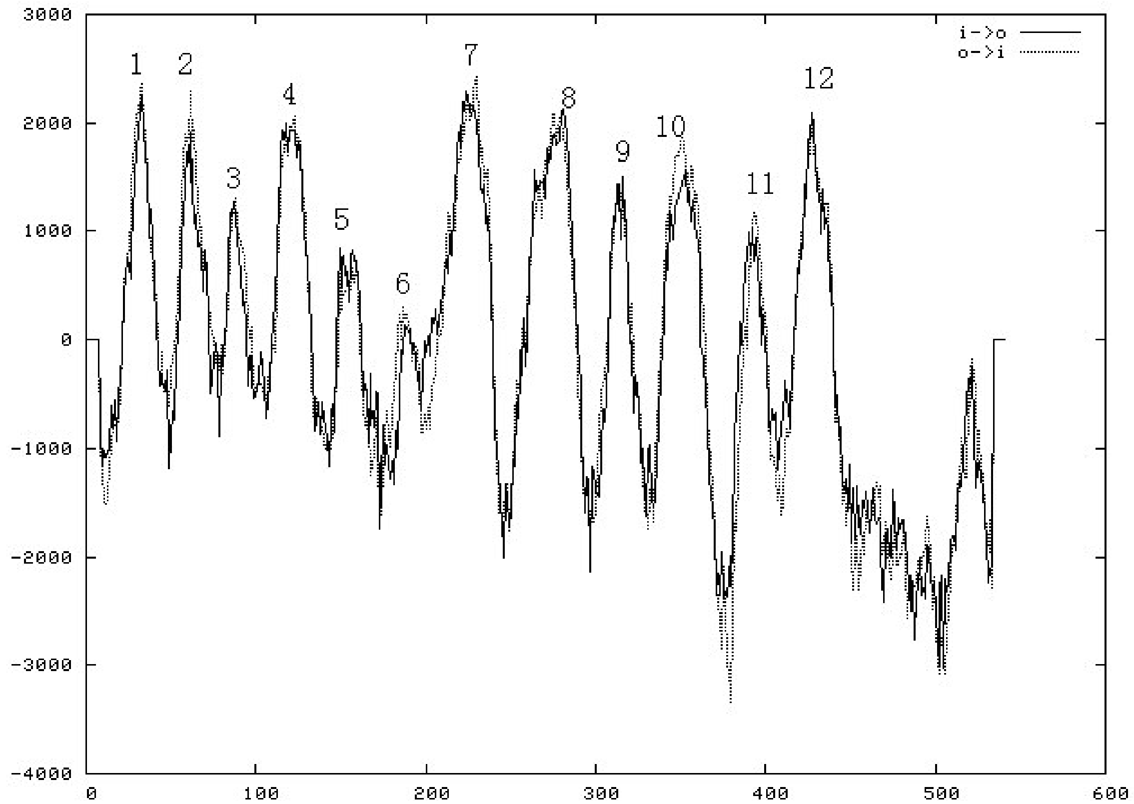

Figure 4. Hydropathy plot analysis of predicted *MzNHX1* polypeptide. The figure was genetated by TMpred program. The putative transmember domains are indicated by numbers.

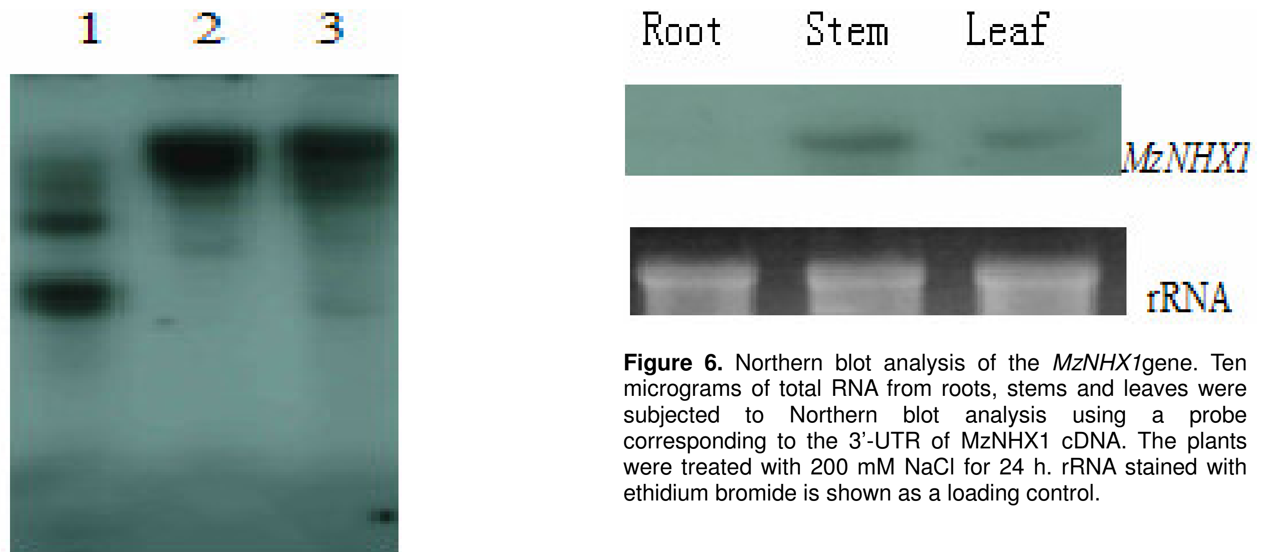

Figure 5. Southern blot analysis of the *MzNHX1* gene. Ten micrograms of genomic DNA from young leaves were digested with *EcoR*I+*Pst*I(1), *Xba*I(2), or *BamH*I(3), separated by gel electrophoresis, transferred to a nylon membrane, and hybridized with a probe corresponding to the 3'-UTR of MzNHX1 cDNA.

Figure 6. Northern blot analysis of the *MzNHX1*gene. Ten micrograms of total RNA from roots, stems and leaves were subjected to Northern blot analysis using a probe corresponding to the 3'-UTR of MzNHX1 cDNA. The plants were treated with 200 mM NaCl for 24 h. rRNA stained with ethidium bromide is shown as a loading control.

but a different model of expression was identified. In *I. nil*, NaCl can induce the expression of *InNHX2* but not *In NHX1* (Ohnishi et al., 2005). All of these reports revealed that different expression models for Na^+/H^+ antiporter genes from different species or different members of Na^+/H^+ antiporter gene family exist.

In a liquid medium in the absence of NaCl, yeast cells without *MzNHX1* grew as well as those with *MzNHX1*.

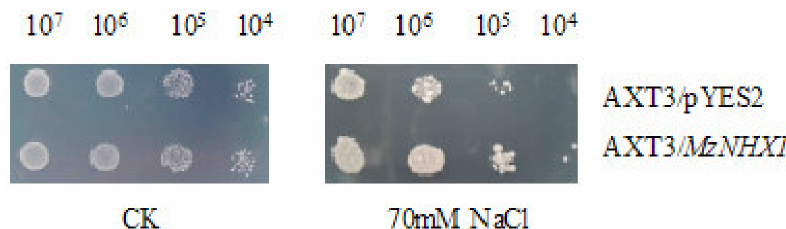

Figure 7. *MzNHX1* suppressed the salt-sensitive phenotype of a yeast nhx1 mutant. The pYES2 vector, and the pYES2-NHX1 vector, which contained *MzNHX1* were introduced into the salt-sensitive AXT3 yeast mutant. Ten-fold serial dilutions were spotted onto AP media supplemented in either the presence or absence of 70 mM NaCl. Plates were incubated at 30°C for 3 days.

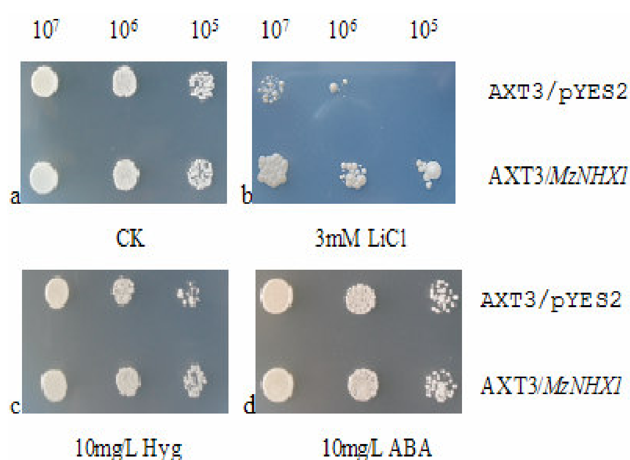

Figure 8. Effect of LiCl, Hyg and ABA on the salt-sensitive AXT3 yeast mutant, either with or without *MzNHX1*. Ten-fold serial dilutions are as in Figure 7. Strains (10 µl) were spotted onto AP plates (1.5% Agar) containing 3 mM LiCl, 10 mg/L Hyg, 10 mg/L ABA or no treatment (control), and grown at 30°C for 72 h.

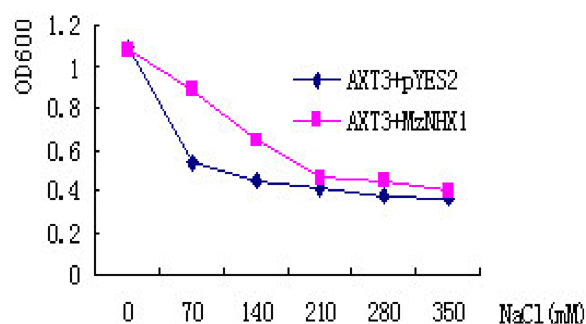

Figure 9. Affect of NaCl on transgenic yeast growth. Twenty microliters of the same seed culture strain (OD_{600}=1) was grown in 2 ml of AP medium supplemented with 0, 70, 140, 210, 280, and 350 mM NaCl at 30°C for 72 h with shaking (200 rpm). Strain growth was measured by reading absorbance at 600nm.

The growth of the two strains was suppressed with an increasing concentration of NaCl in the medium (Figure 9). However, mutant *MzNHX1* expression markedly increased NaCl tolerance at relatively low NaCl concentrations. When the NaCl concentration reached or exceeded 210 mM, the growth of both yeast strains was substantially suppressed, with no obvious differences between the two strains. These results suggested that *MzNHX1* played some role in salt tolerance, at least below certain NaCl concentrations. The introduction of vacuolar *MzNHX1* can only partially complement salt sensitivity of the yeast mutant, due to the fact that the mutant lacks Na^+-ATPase ScNHA1 (plasma membrane type), and ScNHX1 (vacuolar type). This result is of some significance on apple-planting because the *MzNHX1* protein increased the yeast mutant salt-resistance at a NaCl concentration of 140 mM, which is equivalent to a salt content of 0.8%. The upper limit of soil salt content to establish an apple orchard is 0.3%.

After all, yeast exhibits limitations in plant gene function research because it is one of the most simple of single celled eukaryotes. The results of this study revealed *MzNHX1* functions effectively in yeast. However, additional research in model plant systems is of necessity to better characterize *MzNHX1* function and to gain further insights into the salt-tolerant capabilities conferred by this gene.

Conclusion

A full-length cDNA Na^+/H^+ antiporter gene (*MzNHX1*) was isolated from *M. zumi*. The cDNA was 2062 bp in length, including an open reading frame (ORF) of 1629 bp, which encoded a predicted polypeptide of 542 amino acids. The MzNHX1 protein shared high identity with other reported plant vacuolar Na^+/H^+ antiporters and was localized to the vacuolar membrane. There were multiple copies of *Mz NHX1* in the *M. zumi* genome. There was negligible expression of the gene in roots, but expression was detected in stems and leaves. The expression of *Mz NHX1* could increase salt tolerance of salt-sensitive AXT3 yeast mutant.

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