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Full Length Research Paper

Isolation of *Arachis hypogaea* Na^+/H^+ antiporter and its expression analysis under salt stress

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The plant Na^+/H^+ antiporter gene plays a major role in salt tolerance. One Na^+/H^+ antiporter (*AhNHX1*) gene was isolated from peanut (*Arachis hypogaea*) in the present work. The full-length cDNA of *AhNHX1* was 2,331 bp, which contains a complete ORF of 1,620 bp. The deduced protein sequence contains 546 amino acids with a molecular mass of 58.8 KDa and a pI of 7.23. The amino acid sequence of the *AhNHX1* shares high similarity (shows more than 81.89% identity) with those of the previously isolated *NHX1* from other plants. Meanwhile, the *AhNHX1* protein has twelve putative transmembrane domains, and the conserved amiloride-binding sites (^{84}LFFIYLLPPI^{93}) were found in its N-terminal. Also, the expression of *AhNHX1* was tissue-specific under different level NaCl treatments. Under 50 and 100 mM NaCl treatments, its expression showed higher in roots and lower in stems and leaves relative to control plants, respectively. In addition, its expression was time-specific, such that under 150 mM NaCl treatment, its expression usually increased and reached to a stable level in roots and leaves after 24 h but in stems after 48 h, respectively. These results implied that the *AhNHX1* plays an important role under salt stress in peanut.

Key words: *Arachis hypogaea*, *AhNHX1*, salt tolerance, gene expression patterns.

INTRODUCTION

As a major environmental factor, salt stress usually induces drop in crop yield. The injury induced by salt stress to plant mainly includes water deficit resulting in osmotic stress and ionic poison. To cope with salt stress, plants have developed various adaptation mechanisms (Bohnert et al., 1995), such as osmotic adjustment, ionic compartmentation and some macromolecule protein induced by hyperhaline stress, and so on. The important mechanisms for plants to cope with salt stress are Na^+ extrusion and Na^+ compartmentation, among which Na^+/H^+ antiporters play a major role in internal pH and Na^+ homeostasis (Padan et al., 2001; Wiebe et al., 2001). Na^+/H^+ antiporters have been widely found in both prokaryotic and eukaryotic cells (Padan et al., 2001; Blumwald et al., 2000). Na^+ extrusion depends on the plasma membrane Na^+/H^+ antiporter energized by the proton gradient, which is generated by the plasma membrane H^+-ATPase. As the key factor for plant cells to salt tolerance, Na^+ compartmentation enable Na^+ to get into the tonoplast from the cytoplasm to protect cells from salinity stress (Blumwald et al., 2000). More also, the tonoplast Na^+/H^+ antiporters (NHX1) contributes to Na^+ compartmentation, which is energized by the proton gradient generated by the tonoplast H^+-ATPase and H^+-Ppase (Michelet and Boutry, 1995).

Na^+/H^+ antiporter activity in the tonoplast was first reported in the storage tissue of *Beta vulgaris* roots (Blumwald and Poole, 1985). A vacuolar Na^+/H^+ antiporter gene (*AtNHX1*) with 12 typical transmembrane segments in its amino acid sequence was firstly isolated from *Arabidopsis thaliana* (Gaxiola et al., 1999). In recent

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Table 1. PCR primers and annealing temperature.

Primer name	Primer sequence (5'→3')	Annealing temperature (˚C)	Usage
P1	CTTTATATACCTTCTGCCGCCT	54	Partial cDNA cloning
P2	ACAGCACCTCTCATAAGACCAG		
GS3'P outer	CGTTCTTATCCAACTTGGCC	62	3' cDNA cloning
GS3'P inner	TTGGTGGGCTGGTCTTATGAG		
GS5'P outer	CACCAATTGCTAGATAGTCCCCT	55	5' cDNA cloning
GS5'P inner	TTGTTAAGACTCCTGGCTGTG		
expr-F	GGCATAATGTGACAGAGAG	55	Expression
expr-R	CTGATGTTCCAGGTCTATC		
18srRNA-F	ATTCCTAGTAAGCGCGAGTCATCAG	57	Expression
18srRNA-R	CAATGATCCTTCCGCAGGTTCAC		

years, a series of vacuolar Na^+/H^+ antiporter genes have been cloned from different plants, such as *Oryza sativa* (Fukuda et al., 1999), *Atriplex gmelini* (Hamada et al., 2001), *Beta vulgaris* (Xia et al., 2002), *Brassica napus* (Wang et al., 2003), *Suaeda salsa* (Ma et al., 2004), *Vitis vinifera* (Hanana et al., 2007), *Glycine max* (Li et al., 2006), *Aeluropus littoralis* (Zhang et al., 2008), *Lotus tenuis* (Teakle et al., 2010), *Trifolium repens* (Tang et al., 2010), and so on. It has been shown that over expressing vacuolar Na^+/H^+ antiporter could improve tolerance to salt stress obviously in *A. thaliana* (Apse et al., 1999), *Brassica campestris* and *Lycopersicum esculentum* (Zhang et al., 2001), *Zea mays* (Yin et al., 2004) and *O. sativa* (Fukuda et al., 2004).

The area of the saline-alkaline land in China is very large, and effects of salt stress on peanut have been seldom mentioned except the damaging mechanisms of salt stress to peanut photosynthetic apparatus (Qin et al., 2011). In the present work, we tried to further recognize the related salt-resistant mechanisms of peanut through cloning of the new vacuolar Na^+/H^+ antiporter gene from peanut, analyzing its structure and the molecular characterization, and studying its expression patterns under salt stress. It seemed that the *AhNHX1* plays an important role in peanut under salt stress.

MATERIALS AND METHODS

Plant materials and treatments

Peanut (*Arachis hypogaea*) cultivar "Linhua 5" was used as materials for gene clone and expression analysis. Seeds were cultivated in sand in greenhouse and irrigated with Hoagland solution. The experiments were conducted when the third compound leaf was fully expanded. To induce salt stress, some seedlings were incubated in fresh Hoagland medium with 0, 50, 100, 150, 200 and 250 mM NaCl for 72 h, respectively while others were treated in fresh Hoagland medium with 150 mM NaCl for 0, 24, 48, 72 and 96 h, respectively. Roots, stems and leaves were harvested at the end of the stress, frozen immediately in liquid nitrogen for at least 3 h, and then stored at -80°C for RNA extraction and cDNA synthesis.

Total RNA extraction and cDNA synthesis

Total RNA was isolated by using TRIzol Reagent (Invitrogen, Inc. USA) according to the manufacturer's instructions, and then treated with DNase I to remove DNA contamination. First-strand cDNAs were synthesized with ~2 µg total RNAs by using a cDNA synthesis kit (Promega) according to the manufacturer's protocol.

Isolation and sequencing of *AhNHX1*

To clone *AhNHX1* gene, primers were designed (Table 1) according to the sequences of *NHX1* genes from *Galega orientalis* (GenBank accession no. EU340284), *Glycine max* (GenBank accession no. AY972078) and *Robinia pseudoacacia* (GenBank accession no. EF675631). Firstly a fragment of c.a. 933 bp was amplified from cDNA prepared from peanut leaves by using primers P1 and P2 (Table 1). Comparison with sequences in the databases revealed that the fragment codes for an internal region of the NHX1 homologous gene and lacks 5′ and 3′ sequences. Hence, to isolate the complete 5′ and 3′ regions of this gene, 5′-RACE and 3′ region amplifications were carried out using two pairs of gene-specific primers of the 5′ and 3′ full RACE Kit (TaKaRa, Dalian, China) (Table 1). The full length sequence of the gene was joined by these 3 parts. The recombinant plasmid was transformed into competent *Escherichia coli* DH5α cells. At least four positive recombinant clones were sequenced with ABI 3730 sequencer.

Software for bioinformatics analysis

Nucleotide acid sequence analysis, ORF searching and amino acid deduction were performed with DNAMAN version 6.0 (http://www.lynnon.com/). The protein prediction and analysis were performed by using the Conserved Domain Architecture Retrieval Tool of BLAST at the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov/BLAST), the Computer pI/Mw (http://au.expasy.org/tools/pi_tool.html), Protscale (http://www.expasy.org/ cgi-bin/protscale.pl), TMHMM (http://www.cbs.dtu.dk/services/ TMHMM -2.0/), SignalP 3.0 (http://genome. cbs.dtu.dk/services/SignalP) and PSORT II (http://psort.ims.u –tokyo.ac.jp/form2.html), respectively. Also, a phylogenetic tree was constructed by using software Mega 4.0 with neighbor-joining method.

Real-time quantitative PCR (qPCR)

Real-time PCR was performed using SYBR green PCR master mix (Takara, Dalian, China) in optical 96-well reaction plates (Applied Biosystems) on an Eppendorf Mastercycler system. The SYBR Green fluorescent dye was used to detect the synthesized dsDNA. The total reaction volume of 20 µL contained 10 µL 2 × Power SYBR Green Master Mix Reagent (Applied Biosystems), 2 µL of diluted cDNA and 1 µL of each gene-specific primer(10 µM). PCR conditions: 95°C for 2 min; 40 cycles of 95°C for 20 s, 55°C for 30 s, 72°C for 20 s; and then the melting curve. The data were expressed as the final cycle number necessary to reach a threshold fluorescence value (Ct). Data were normalized by the $2^{-\Delta Ct}$ method and 18S-RNA was used as control. Primers for *AhNHX1* and 18s rRNA mRNA amplification were expr-F, expr-R and 18srRNA-F and 18srRNA-R (Table 1), respectively. Five repeated trials were done in independent biological replicates.

Statistical analysis

For qPCR analysis, the mRNA level of *AhNHX1* gene was shown as mean ± standard error of the mean (SEM). Group data for multiple comparisons were analyzed by ANOVA with the GLM procedure, followed by Duncan's multiple range test to test for differences (SAS, 1989). When $P<0.05$, the difference was considered as significant.

RESULTS

Isolation and characterization of *AhNHX1*

The full sequence of peanut *NHX1* (*AhNHX1*) cDNA was cloned successfully in the present work. The cDNA of *AhNHX1* (GenBank accession no. HM590627) has 2,331 bp, while its 5′ untranslated region (UTR), open reading fame (ORF), 3′ UTR and polyA tail was 450, 1,620, 261 and 22 bp, respectively. Additionally, its ORF encodes 539 amino acids, with a molecular mass of 5.9 kDa and a theoretical pI of 6.20 (Figure 1). Multiple sequence alignments showed a high homology of amino acid sequence between *AhNHX1* and tonoplast Na^+/H^+ antiporters of other high plants. The amino acid sequence of *AhNHX1* showed more than 81.89% identities to that of *Glycine max* (*GmNHX1*), *Robinia pseudoacacia* (*RpNHX1*), *Galega orientalis* (*GoNHX1*), *Trifolium repens* (*TrNHX1*), *Lotus tenuis* (*LtNHX1*), *Medicago sativa* (*MsNHX1*) and *Caragana korshinskii* (*CkNHX1*), respectively (Figure 2). The sequence of ^{84}LFFIYLLPPI^{93} (shadow region in Figure 1) is identified as the binding site of amiloride, which inhibits the activity of Na^+/H^+ exchanger (Hamada et al., 2001).

In addition, two potential glycosylation sites were also found in *AhNHX1* (Asn-50 and -293). These results indicate that the *AhNHX1* protein is also glycosylated. The signal peptide prediction showed that the N-terminal 1-40 amino acids form the signal peptide, and the maximum cleavage site was 0.014 between pos. 40 (Gly) and 41 (His). Hydropathy analysis showed that *AhNXH1* protein was a hydrophobic protein because the AhNHX1 protein has a typical hydrophobic region in N-terminal portion and a highly hydrophilic tail in the C-terminal portion, and its grand average of hydrophilicity was 0.536. Morealso, the protein contained 12 putative trans-membrane domains (Figure 2). The ratio of AhNHX1 protein in the plasma membrane was 82.6%, and the ratio of α-helices, random coils, little extended strands and β-turns in its secondary structure was 52.21, 29.31, 15.96 and 3.53%, respectively.

Furthermore, to study phylogenetic relationship between *AhNHX1* and known Na^+/H^+ antiporters of plant, phylogenetic tree was constructed by comparing full-length amino acids of *AhNHX1* with those of *NHX1* from other plant species (Figure 3). *AhNHX1* was found to be close to *Papilionaceae* group (*GoNHX1*, *TrNHX1*, *MsNHX1*, *CkNHX1*, *GmNHX1*, *LtNHX1*, and *RpNHX1*).

Expression patterns of *AhNHX1* gene under salt stress

In the roots, relative abundance of *AnNHX1* from peanut seedlings under 50 and 100 mM NaCl treatment gradually increased up to 2.4- and 3.4-fold more than that of the control (0 mM), while the expression of *AnNHX1* showed the lowest under 150 mM NaCl treatment among salt stresses after 72 h. The expressions of *AhNHX1* in roots had no obvious difference among 50, 200 and 250 mM NaCl treatment. However, the expression levels of *AhNHX1* in roots showed no significant difference under salt stress ($P>0.05$) (Figure 4A). Moreover, the expressions of *AhNHX1* in stems and leaves were significantly inhibited under all salt stresses relative to the control ($P<0.05$) (Figure 4A). The relative abundance of *AhNHX1* mRNA were about 206-fold higher in stems in the control than that in the seedlings under 200 mM NaCl stress, and the relative abundance of *AhNHX1* in the leaves decreased about 60-fold in the seedlings under 150 mM NaCl stress relative to that in the control ($P<0.05$).

The time course of *AhNHX1* expressions under 150 mM NaCl salt stress were also detected (Figure 4B). The relative abundance of *AhNHX1* in roots showed the highest at 24 h ($P<0.01$) and decreased sharply at 48, 72 and 96 h. After 0 and 24 h during 150 mM NaCl stress, the expressions of *AhNHX1* in stems reached the peak at 48 h ($P<0.05$) and then decreased at 72 and 96 h. However, its expressions in leaves decreased gradually from 24 to 72 h and increased at 96 h, although its expressions were not significant relative to the control ($P>0.05$).

DISCUSSION

With the development of the molecular biological technology, many plant salt-responsive genes have been studied (Fukuda et al., 1999; Hanana et al., 2007; Zhang

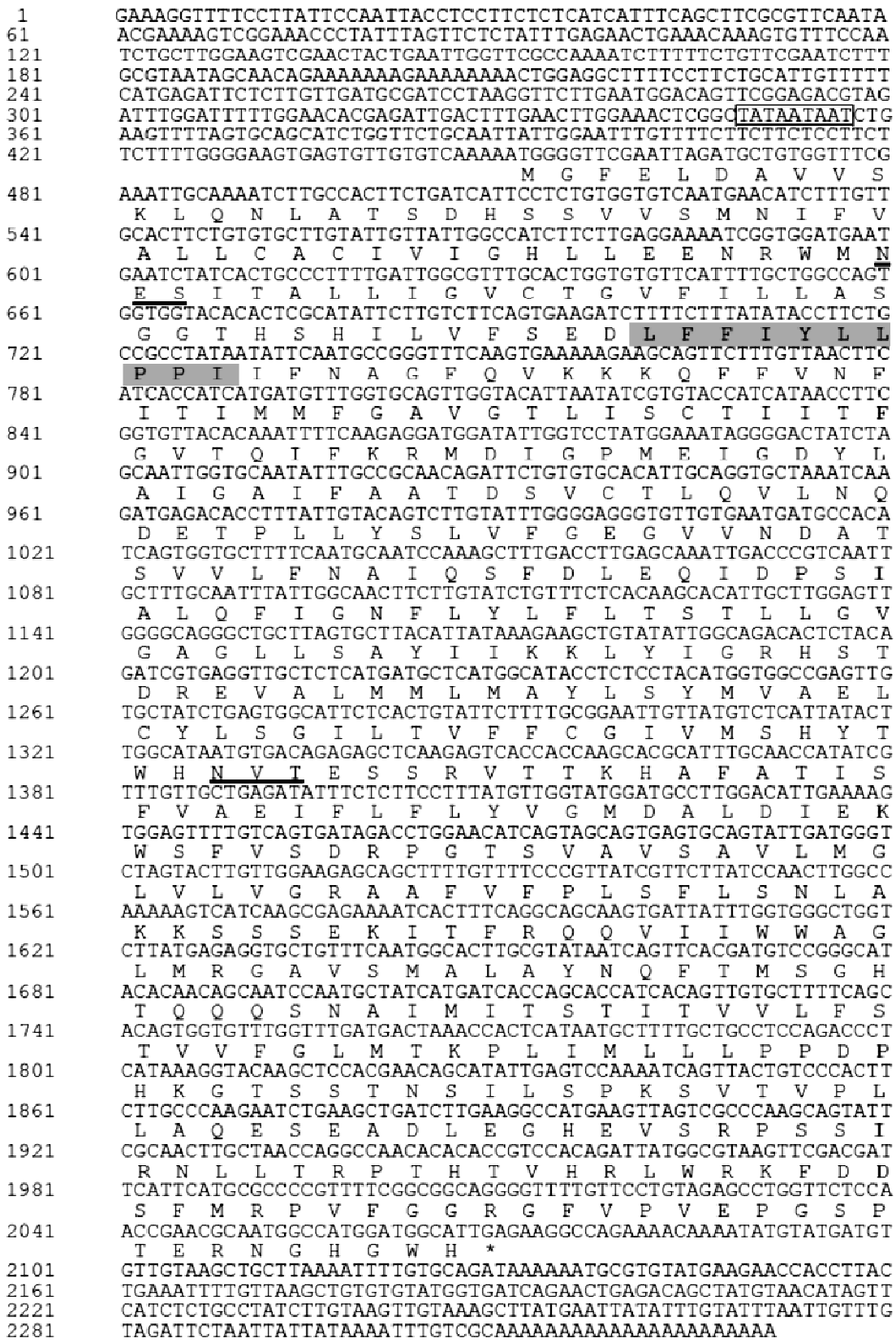

Figure 1. Nucleotide and deduced amino acid sequence of *AhNHX1* cDNA. A box shows TATA box of promoter sequence. Two potential glycosylation sites in *AhNHX1* are underlined. The binding site of amiloride that inhibited eukaryotic Na^+/H^+ antiporters is shaded.

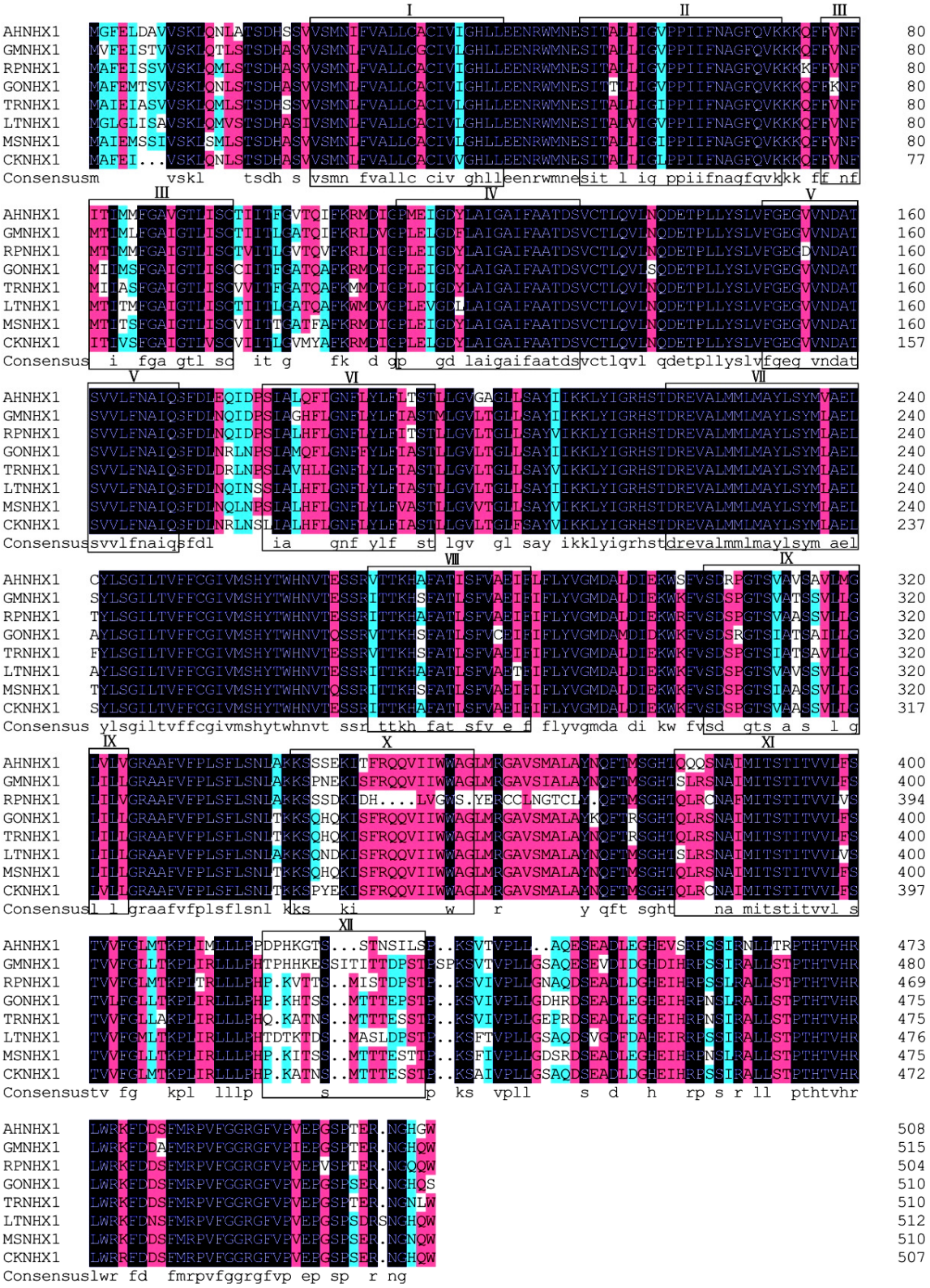

Figure 2. Comparison of *AhNHX1* with other plants amino acid sequences of Na^+/H^+ antiporters and the predicted transmembrane of *AhNHX1* protein. *AhNHX1* (HM590627), *Arachis hypogaea*; *GmNHX1* (AY972078), *Glycine max*; *RpNHX1* (EF675631), *Robinia pseudoacacia*; *GoNHX1* (EU340284), *Galega orientalis*; *TrNHX1* (EU109427), *Trifolium repens*; *LtNHX1* (EU727217), *Lotus tenuis*; *MsNHX1* (GU265772), *Medicago sativa*; *CkNHX1* (DQ812099), *Caragana korshinskii.*

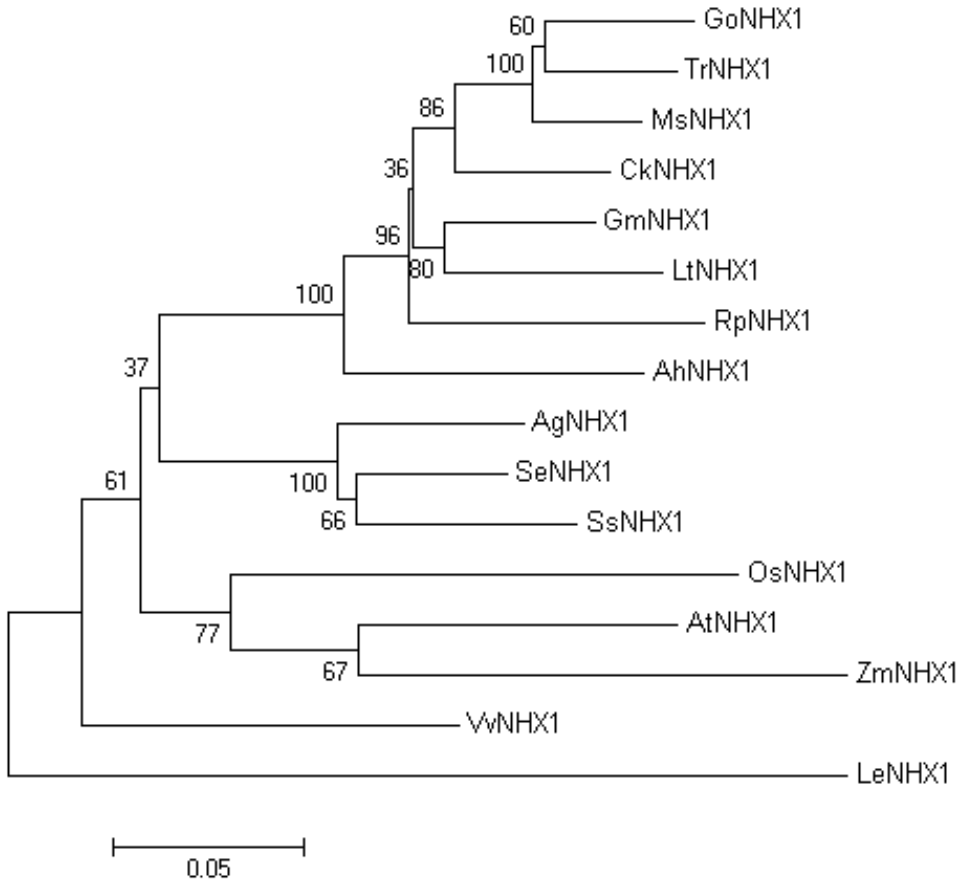

Figure 3. Phylogenetic tree of *AhNHX1* animo acid and other plant species. *AtNHX1*(AY685183), *Arabidopsis Thaliana*; *ZmNHX1*(AY270036); *Zea mays*; *OsNHX1*(AY324877), *Oryza sativa*; *AhNHX1*(HM590627), *Arachis hypogaea*; *GoNHX1*(EU340284), *Galega orientalis*; *TrNHX1*(EU109427), *Trifolium repens*; *CkNHX1*(DQ812099), *Caragana korshinskii*; *GmNHX1*(AY972078), *Glycine max*; *LtNHX1*(EU727217), *Lotus tenuis*; *RpNHX1*(EF675631), *Robinia pseudoacacia*; *VvNHX1*(AY634283), *Vitis vinifera*; *SsNHX1*(AF370358), *Suaeda salsa*; *SeHNX1*(AY131235), *Salicornia europaea*; *AgNHX1*(AB038492), *Atriplex gmelini*; *LeNHX1*(AJ306630), *Lycopersicon esculentum*; *MsNHX1*(GU265772), *Medicago sativa*.

et al., 2009; Tang et al., 2010; Zörb et al., 2005; Guan et al., 2011; Wang et al., 2011), among which Na^+/H^+ antiporters play a key role in the maintenance of osmotic balance (Rausch et al., 1996), with the vacuolar Na^+/H^+ antiporter gene becoming one of the most top genes for plant salt-tolerance research. The *NHX1* genes encode vacuolar Na^+/H^+ exchangers that catalyze the exchange of Na^+ for H^+ across the membrane in the vacuoles. In plants, the transport of Na^+ into the vacuoles is promoted by the NHX1 proteins that are energized by the H^+ gradient generated by the vacuolar H^+-ATPase and H^+-pyrophosphatase (Blumwald et al., 2000). To investigate the function of Na^+/H^+ antiporter in peanut salt tolerance, a full-length cDNA of vacuolar Na^+/H^+ antiporter was cloned from peanut and named *AhNHX1*. The putative amino acid sequence of *AhNHX1* has the conserved "^{84}LFFIYLLPPI^{93}" amiloride drug-binding site and two N-glycosylation sites at (Asn^{50} and Asn^{293}), respectively (Figure 1). The amino acid sequence of *AhNHX1* has high similarity to that of *GmNHX1*, *RpNHX1*, *GoNHX1*, *TrNHX1*, *LtNHX1*, *MsNHX1* and *CkNHX1* (Figure 2), which implied that the AhNHX1 is a vacuolar-type protein and its coding gene belongs to the gene family of vacuolar Na^+/H^+ antiporters.

The structure of Na^+/H^+ antiporters has been well characterized. Yamaguchi et al. (2003) reported that the topology of *AtNHX1* with 9 transmembrane segments and 3 hydrophobic regions do not span the tonoplast membrane, but seems to be membrane associated. The hydrophobicity plot of *AhNHX1* showed that it has 12 transmembrane segments, and TM5 and TM6 seemed not to cross the vacuolar membrane, which is very similar to some other Na^+/H^+ antiporters, such as *AtNHX1*, *GmNHX1*, *OsNHX1*, etc. (Figure 2). Additionally, several non-homologous regions appeared at the N-terminal towards the cytoplasm and the C-terminal regions with a

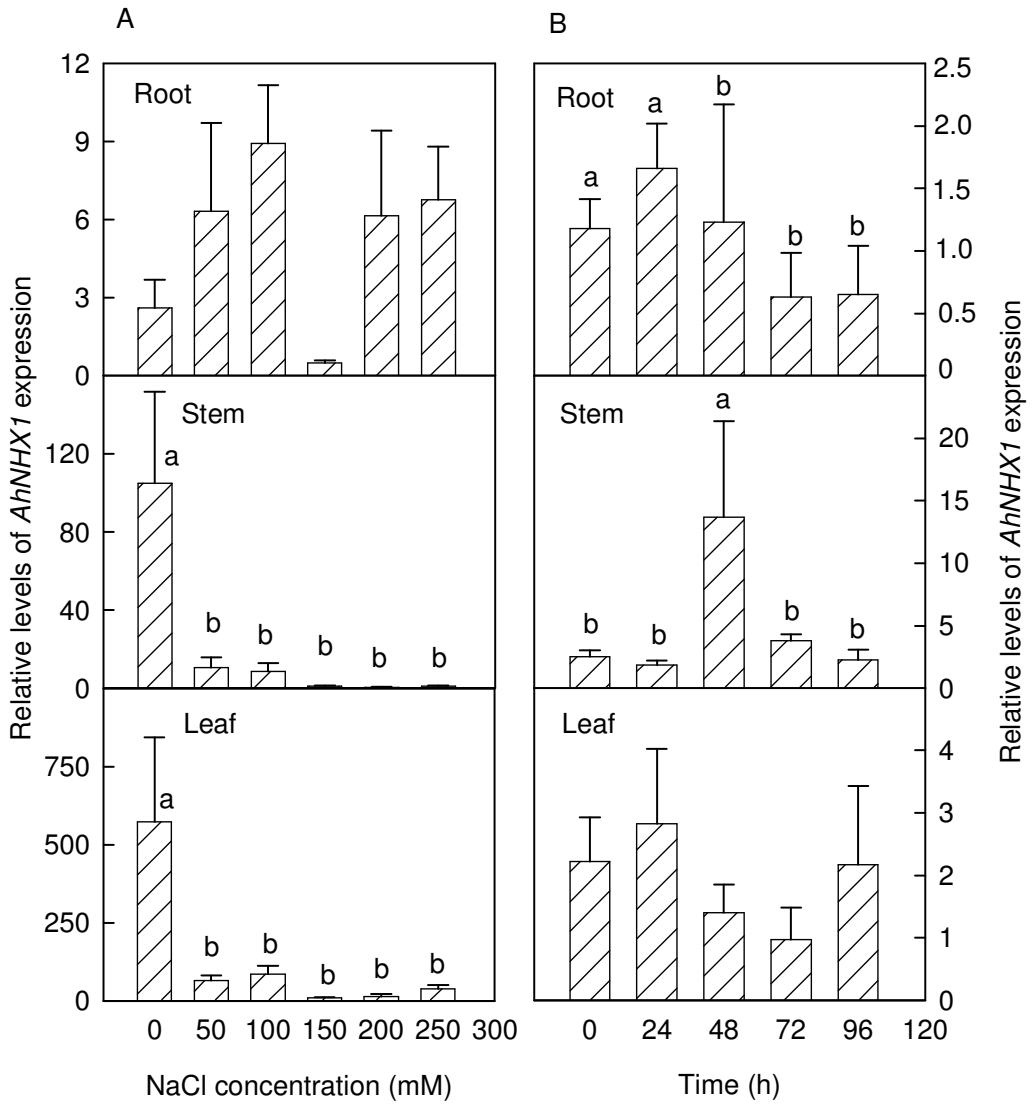

Figure 4. Real time RT-PCR analysis of the *AhNHX1* gene under NaCl stress condition. A, Different NaCl concentration stress for 72 h; B, 150 mM NaCl stress for different time. Bars with different small or capital letters are significantly different at $P<0.05$ or 0.01.

long hydrophilic tail almost entirely residing in the vacuolar lumen. It has been demonstrated that the vacuolar localization of the C terminus of Na^+/H^+ antiporter is related to regulation of the antiporter activity and cation selectivity (Yamaguchi et al., 2003, 2005). Most of variable regions are between amino acid residues 428 to 469.

Both constitutive- and induction expression of *NHX1* gene have been found in plants, for example, the expressions of *AtNHX1* (Gaxiola et al., 1999) and *BvNHX1* (Xia et al., 2002) gene belong to constitutive expression, and the expression of *NHX1* in barley root tonoplast belongs to induction expression (Garbarino and Dupont, 1989). From our result, it seems that the expression of *AhNHX1* belongs to constitutive expression, which expression could be detected apparently in roots, stems and leaves of both control and stress plants (Figure 4). Unlike *AhNHX1*, some *NHX1* genes expression in other plants showed tissue-specific, for example, Purple *NHX1* gene expressed abundantly in petals about 12 h before flowering, but it appears to be scarcely in roots, stems and leaves (Yamaguchi et al., 2001).

It has also been reported that NHX proteins in plants have been directly associated with the accumulation/ sequestration of sodium in the vacuole (Apse et al., 1999, Zhang et al., 2001). However, the ability of a plant to sustain growth under high salinity conditions is not directly correlated with the presence of a gene coding for the antiporter but is more likely to be correlated with the antiporter activity (Apse et al., 1999). *NHX1* gene from different plants showed different response to salt stress.

AtNHX1 expression could be upregulated by salt stress in leaves but not in roots (Quintero et al., 2000). More also, *CmNHX1* expression increased in roots but decreased in leaves with increasing NaCl concentration (Wang et al., 2011). Many researches on *NHX1* gene expression induced by salt stress were less than 24 h (Quintero et al., 2000; Wang et al., 2002; Wang et al., 2011; Zhang et al., 2009). In the present work, *AhNHX1* expression was assessed after 72 h salt stresses with different NaCl concentration. Its expression could be upregulated by salt stress except for 150 mM NaCl treatment in roots, but depressed in leaves and stems (Figure 4A).

The depression of *AhNHX1* in leaves and stems might be related to a prolonged stress time since some *NHX1* gene could reach expressing peak less than 24h under salt stress (Quintero et al., 2000; Wang et al., 2011). As to this depression of *AhNHX1* expression in roots induced by 150 mM NaCl treatment, its mechanism still remains unclear. Additionally, *NHX1* expression in plants was time-specific under salt stress (Wang et al., 2011), *VvNHX1* expression only showed high levels at the veraison and post-veraison stages (Hanana et al., 2007). During the time course, *AhNHX1* expression showed the highest in roots and leaves after 24 h salt stress and in stems after 48 h salt stress, respectively (Figure 4B). These results therefore implied that the response mechanisms of different peanut tissues to salt stress are different, and roots were more resistant to salt stress relative to stems and leaves. It also implied that the *AhNHX1* gene might be a functional candidate gene for peanut salt tolerance to be utilized in transgenic plants.

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