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Isolation and molecular characterization of a novel Na^+/H^+ antiporter gene, *AlNHX2*, from *Aeluropus littoralis* and comparison of AlNHX1 and AlNHX2

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Abstract

Halophytes have high tolerance against salinity and it is expected that these plants have special proteins that allow them to thrive under salinity conditions. Hence, to understand molecular aspects of vacuolar Na^+/H^+ antiporter, which has a possible role in salt tolerance in halophytic plants, a novel Na^+/H^+ antiporter gene, *AlNHX2*, was isolated and characterized by rapid amplification of cDNA ends (RACE) technique. The results revealed that *AlNHX2* is expressed in leaves, stems and roots and its expression in leaves is 1.767 and 1.269 times higher than stems and roots, respectively. This gene has an ORF with 1617 bp in length, a 3'-UTR region with 245 pb, and a 5'-UTR region with 187 bp which encodes a 538 amino acid protein shared a high homology with those putative vacuolar Na^+/H^+ antiporters of higher plants. Putative phosphorylation sites within AlNHX1 and AlNHX2 were determined using the prediction software, and the binding of 14-3-3 protein to specific domains within AlNHX1 and AlNHX2 was predicted. The results also revealed this binding is induced by a protein kinase-mediated phosphorylation of a specific Thr or Ser residue in these domains. The results also revealed that the activities of AlNHX1 and AlNHX2 are regulated by PKC, p38MAPK and GSK3. In addition, the findings reported here show the interaction of CaM protein with C-terminal of AlNHX2. Autoinhibitory domain at C-terminal region of AlNHX2 that can suppress the protein activity under normal growth conditions was also found.

Keywords: Gene isolation, Gouan, Halophytic plant, Salinity stress, Vacuolar exchanger.
Abbreviations: CTAB_ cetyltrimethylammonium bromide; ExPASy_Expert Protein Analysis System; GSK3_Glycogen synthase kinase 3; MuLV Reverse Transcriptase_ Moloney murine leukemia virus reverse transcriptase; NHX_Na^+/H^+ antiporter; PKC_Protein kinase C; p38MAPK_p38 mitogen-activated protein kinases; SIB_Swiss Institute of Bioinformatics.

Introduction

In natural ecosystems as well as agricultural systems, plants face a variety of biotic and abiotic stresses. Salt stress is a widespread challenge met by plants in these systems. Salt stress has recently been one of the main topics that draw global attention, because it can limit plant growth, development and productivity (Batool et al., 2014). Salt affected lands in all climatic regions and saline soils can be found at different altitudes. When plant exposed to high salinity, various physiological activities such as photosynthesis and protein synthesis are affected (Parida and Das, 2005). Some plants develop mechanisms using energy either to exclude toxic ions such as Na^+ from their cells or compartmentalization in the vacuole (Parida and Das, 2005). Removal of sodium ion from the cytosol is done by plasma membrane or vacuolar Na^+/H^+ antiporters (Apse et al., 1999). Na^+/H^+ antiporters are integral membrane proteins residing in the plasma membrane or endomembranes of the cell such as tonoplast (Yamaguchi et al., 2003). Although their action appears to be simple, they have been implicated in various functions. The genes encoding Na^+/H^+ antiporters are important in salt tolerance and there are different reports showing that their expressions were regulated by salt stress (Shi and Zhu, 2002; Yokoi et al., 2002). It has also been reported that these proteins have critical roles in other processes including the regulation of cellular value, regulation of intracellular pH, tolerance to Li^+, tolerance to antibiotics such as hygromycin (Orlowski and Grinstein, 1997; Fukuda et al., 2004).

In plants, the vacuolar Na^+/H^+ antiporters are considered to form a multigene family. Six full-length gene transcripts *ZmNHX1-6* from *Zea mays* L. were identified (Zörb et al., 2005). Two cDNA clones (*ZomNHX1* and *ZomNHX2*) encoding vacuolar Na^+/H^+ antiporter were isolated from *Zostera marina* L. (Alemzadeh, 2010 a,b). Also, the *Arabidopsis AtNHX* family comprises six genes (Yokoi et al., 2002). It has been predicted that there are two or three copies of *AlNHX* present in *Aeluropus littoralis* genome (Zhang et al., 2008). It has been shown that the expression of different isoforms in a species have an organ-specific pattern (Yokoi et al., 2002; Zörb et al., 2005). Although the NHX proteins function to facilitate Na^+ ion compartmentalization, one or some of them have a major function (Yokoi et al., 2002).

It is very important that the cellular processes are regulated, and one of the main ways to regulate these processes is protein phosphorylation that is catalyzed by protein kinases (Hunter, 1998). Protein phosphorylation is done at serine, threonine and/or tyrosine residues that affects multitude of cellular processes (Blom et al., 1999). Using bioinformatics tools, for some eukaryotic proteins, predicted sites that are possibly regulated by phosphorylation (Blom et al., 1999). Since determinants of phosphorylation sites probably are no longer than around ten amino acid residues, most alignment tools will not be useful for detecting these sites due to a huge

number of irrelevant hits in the protein databases (Blom et al., 1999).

The first member of the NHX gene family from *A. littoraslis*, a monocot halophyte, *AlNHX1*, was cloned by Zhang et al. (2008). In this study, the second isoform, *AlNHX2*, was isolated and characterized from this halophytic plant and the expression pattern of *AlNHX2* was investigated in different organs. In addition, the phosphorylation sites in AlNHX1 and AlNHX2 proteins were predicted and compared using bioinformatics tools.

Results and Discussion

Cloning of the AlNHX2, a vacuolar Na^+/H^+ antiporter gene from A. littoralis and sequence analysis

An alignment of amino acid sequences of vacuolar Na^+/H^+ antiporter from various plants revealed two highly conserved regions. Based on the two consensus regions, degenerate primers were designed as described in materials and methods. As expected, a 500 bp fragment was amplified by PCR from the *A. littoralis* genomic DNA and its nucleotide sequence was determined. In addition, a 300 bp fragment was amplified when a PCR reaction was carried out using cDNAs prepared from RNA extracted from leaves and its nucleotide sequence was determined. As expected, the nucleotide sequences related to a vacuolar Na^+/H^+ antiporter, but theses sequences differed from that of *AlNHX1* indicating that this is a new isoform, *AlNHX2*. Then, the full-length of the vacuolar Na^+/H^+ antiporter coding region was obtained using RACE technique. The approximately 1450 and 750 bp fragments were obtained in PCR reactions for 3' and 5' ends, respectively. These fragments were cloned and their nucleotide sequences were completely determined. The sequences of these fragments revealed that they corresponded to *AlNHX2* gene. The sequence analysis revealed that *AlNHX2* expresses a transcript with 2049 bp in length with an open reading frame (ORF) of 1617 bp encoding 538 amino acid residues and a 5'-UTR of 187 bp and 3'-UTR of 245 bp (Fig 1).

Comparison of the *AlNHX1* and *AlNHX2* nucleotide sequences revealed that *AlNHX2* encoding a novel vacuolar Na^+/H^+ antiporter in *A. littoralis* with around 73% identity to *AlNHX1*. The deduced protein AlNHX2 showed close to 75% amino acid sequence identity with AlNHX1 (Fig 2). Comparison of the nucleotide sequences of 5'- and 3'-UTRs of *AlNHX1* and *2* revealed that their 5'-UTR regions are close to 26% identical while their 3'-UTR regions are only around 12% identical. These results showed that two different isoforms can be very well distinct from each other by specific primers or probes for 5'- or 3'-UTR regions. It has been reported for other genes that the expression of one isoform can be distinguishable from those of other isoforms when 3'-UTR region was used as probe (Fukuhara et al., 1996).

A small upstream open reading frame (uORF), MD, was found upstream of *AlNHX2* at -176 position from ATG initiation codon (Fig 1). This uORF has been also reported from other vacuolar Na^+/H^+ antiporters (Alemzadeh, 2010a). This uORF was not found in the upstream of *AlNHX1*. It has been proposed that this structure is used to regulate the translation of downstream gene (Arango et al., 2003).

Analysis of the hydrophilicity and hydrophobicity profiles of the amino acid sequence of AlNHX2 showed that this molecule consists of 10 transmembrane domains connected by hydrophilic loops and has a cytoplasmic C- terminal region composed of 104 amino acid residues (Fig 2 and 3). The N-and C-terminal domains of AlNHX2 protrude into the cytoplasm. The predicted molecular weight of the protein is 58.8 kDa.

In the third transmembrane domain of AlNHX2, like AlNHX1, contained an amiloride-binding motif, FFIYLLPPII, that was common to vacuolar Na^+/H^+ antiporters (Fig 2). In addition, amino acid sequence identity was also found between transmembrane domains of AlNHX1 and 2. The results showed a range of identity from 34.375 to 100% between transmembrane domains (Table 2). The sequence identity analysis showed that only transmembrane domain III among AlNHX1 and 2 was 100% identical. As mentioned above, the third transmembrane domain contained an amiloride-binding motif that was common to vacuolar Na^+/H^+ antiporters.

The number of isoforms for vacuolar Na^+/H^+ antiporter in A. littoralis

It has been predicted earlier that there were two or three copies of *AlNHX* gene present i*n A. littoralis* genome (Zhang et al., 2008), which was different from other plants such as maize, six isogenes (Zörb et al., 2005), and *Arabidopsis*, six isogenes (Yokoi et al., 2002). Different studies indicated that the isolated and sequenced genes from *A. littoralis* have a high homology with *Triticum aestivum* L. genes. It has been reported that there are three isogenes (*TaNHX1*, AY040245; *TaNHX2*, AY040246; *TaNHX3*, AY461512) for vacuolar Na^+/H^+ antiporter in *T. aestivum* L. (Brini et al., 2005). In this study, 20 clones were sequenced and all of them related to *AlNHX1* or *AlNHX2*. There was no other isoforms for vacuolar Na^+/H^+ antiporter found. It can be suggested that there are probably only two isoforms for vacuolar Na^+/H^+ antiporter in *A. littoralis* genome which is consistent with the results reported by Zhang et al. (2008).

Phylogenetic analysis

Previous phylogenetic analysis of different plant Na^+/H^+ antiporters showed two distinct subgroups corresponding to endomembranes and plasma membrane transporters (Brett et al., 2005). Multiple alignment of the predicted amino acid sequences of vacuolar Na^+/H^+ antiporter from various plants was used to derive a phylogeny tree. On the basis of this dendrogram, it was revealed that AlNHX1 and AlNHX2 were not most closely related to each other and located in different groups (Fig 4). AlNHX2 together with other monocot species such as maize, rice, barley and wheat was located in the same group and was most closely related to ZmNHX1 (*Zea mays*) and SiNHX1 (*Setaria italica*) (Fig 4).

AlNHX2 expresses in leaves, stems and roots

To determine the organ-specific expression of *AlNHX2* in *A. littoralis* plant, semiquantitative RT-PCR was carried out with specific primers, using cDNAs prepared from total RNA from leaves, stems and roots, separately. Although, *AlNHX2* was detected to be expressed in all organs, but the expression levels varied in different organs. The expression level of *AlNHX2* ranked in the sequence of leaves>roots>stems (Fig 5). It was reported that *AlNHX1* expressed in *A. littoralis* leaves and roots and its expression level in leaves was also higher than in roots (Zhang et al., 2008). It is interesting that both isoforms are relatively expressed at higher level in leaves while, in previous studies have been suggested a different expression pattern for different isoforms in the same plant (Yokoi et al., 2002; Zörb et al., 2005).

Table 1. The list of primers used in this study.

Primer name	Sequence	TM (°C)
PrimerF	5´-CCDCCHATYATYGGAGGGTC-3´	58.1
PrimerR	5'-GATGTRGCATYGTTNGTGGG-3'	57.9
Alant2R	5'- GAGTGATTATAACAAAGGACACCAG -3'	62.1
Alant2F	5'- TTCAAGAAACTTGATGTCGGTC -3'	58.4
GeneRacerTM 5'	5'- CGACTGGAGCACGAGGACACTGA -3'	62.6
GeneRacerTM 3'	5'- GCTGTCAACGATACGCTACGTAACG -3'	59.4
AlF	3' -TGGTCACTAACGGGACGAAT -5'	57.7
ActinAlF	5'- CGTACAACTCCATCATGAAGTG-3'	61.96
ActinAlR	5'- CAAACACTGTACTTTCTCTCCG-3'	60.35

TGGACACTGAC***ATGGACTGA***AGGAGTAGAAAACCTCTCACATCTCTCCACGAATCTCTCACCGAACCCACT
CGCCCAGATTTTTGCCCCCGCGATTCAGATCGCCGGATCGATCCACAAGCTCGCGGAATCCGCAGCGGCGT
GGAGGCAGAGATTGCCGGGGGCTGTGGATCTGCTGAAGTGGCGAC**ATG**GGGCTAGATTTGGCAGCCCTTCT
CAAATCCGGCCGGCTCGCGGTCGCGGACTACGACTCCATCGTCTCTATCAACATCTTCATCGCGCTGCTTT
GCGGCTGCATTGTCATTGGCCACCTGCTGGAAGGGAACAAATGGGTGAACGAGCCCCTCACCGCGCTCGTC
ATGGGGCTCATCACTGGCGGTGTGATTTTGCTGGTCACTAACGGGACGAATTCACGCATTCTGGTGTTCAG
CGAGGATCTGTTTTTCATCTACTTGCTTCCACCGATCATCTTCAATGCCGGGTTTCAAGTAAAGAAAAAGC
AATTTTTCCGCAACTTCATCACAATTATTTTATTTGGTGCTGTTGGGACA*CTGGTGTCCTTTGTTATAATC*
*ACTC*TTGGTGCTATGGGACT*GTTCAAGAAACTTGATGTCGGTC*CACACCATCTTGCGGACTATCTTGCAAT
TGGTGCTATTTTCTCTGCAACAGATTCTGTTTGCACTTTGCAGGTGCTTAATCAGGATGAAACGCCGCTGC
TCTATAGTCTAGTTTTTGGTGAAGGTGTTGTTAATGATGCTACATCTGTTGTGCTCTTCAATGCAATCGAA
AACCTTGATATTGGACATTTTGATGCTCTTGTTCTATTAAACTTCATTGGAAAATTCCTCTACCTCTTCTT
CACCAGCACCATTCTTGGGGTAGCTGCTGGGTTGCTTAGTGCCTACATTATCAAGAAGCTTTGTTTTGCAA
GACATTCAACCGATAGAGAAGTTGCTATCATGATTCTCATGGCATACCTTTCATACATGTTGTCTGTGCTG
ATGGATCTGAGTGGCATTCTCACTGTTTTTTTCTGTGGGATAGTAATGTCACATTACACTTGGCACAATGT
GACAGAAAGCTCTAGAGTTACTACAAAGCATACTTTTGCAACTTTATCGTTCATTGCAGAAATTTTTCTCT
TTCTCTATGTCGGGATGGATGCATTGGACATTGAAAAATGGAGATTAGCTAGTAGCAGTCCTACAAAACCA
ATCGCTTTAAGTGCAATTATACTGGGCCTGATTATGGTTGGACGAGCGGCATTTATATTCCCTTTGTCTTT
CCTATCCAACCTAAGTAAAAAAGAGGCACGTCCAAAGATCTCCTTCAGGCAACAGGTAATCATATGGTGGG
CTGGTCTTATGAGAGGAGCAGTATCAATTGCACTTGCATATAACAAGTTTACAGCATCTGGTCATACTGTG
GTGCGAGTTAACGCTATCATGATCACCAGCACAGTCATTGTTGTTTTGTTCAACACAATGGTTTTCGGAAT
GATGACAAAGCCATTGATCCAATTTCTGCTTCGGGCGACAAGTCATAGGGTCACCTCTGATCCGTCTTCCC
CAAAATGCCTTCATTCTCCCCAACTAGCAAGTATGCAGGGTAAAGATCTGGAGAGGGCCTCACACTCACAT
AAAGTGAGGCCTTCAAGTCTGCGCATGCTCCTCAGCAAGCGGACTAACCCCGTCCATTATAACAGACGAAA
GATCGACGACGCTTTAAAGCGGATGGATTTCGGGGGGCGGGGAGCGGTGATATTCTCTTGGGCATCTCCAA
CGGAGCAGAGCATACATTGGGGCAGA**TAA**AGGACTTTTTTGTGTAAATTTTCCGACAAATACCTTTCTTTT
TTCATTTGTAGTATATGTTGGGTCTTAGGTTTCAAGCAGATAGTTTCGGTAGAGTTACTTAAGCTGGTATA
TTCGCTTGGAGCCACTCCAACTGTACAATATTTGATCATTTTTATGTAAAGAGTTTTATAGGTAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAACACTGTCATGCCGTTACGTAGCGA

Fig 1. The nucleotide sequence of *AlNHX2* which expresses a transcript with 2048 bp in length. Start and stop codons are shown in bold and gray. 5'-UTR (187 bp) and 3'-UTR (245 bp) are underlined. A small upstream open reading frame (uORF), MD, in upstream of *AlNHX2* at -176 position from ATG initiation codon are shown in bold and italic.

Phosphorylation sites predictions in AlNHX1 and AlNHX2

Protein phosphorylation by protein kinases at serine, threonine and/or tyrosine residues can affect various aspects of target protein such as regulation of interaction between target protein with regulator proteins. Considering the large number of potential phosphoacceptor residues in AlNHX1 (contains 104 serine, tyrosine and threonine residues, 19%)/AlNHX2 (contains 89 serine, tyrosine and threonine residues, 17%), it would be helpful to identify the most probable sites before conducting experiments in site-directed mutagenesis. The amino acid sequences of AlNHX1 and AlNHX2 are 74% identical, which makes it possible to compare putative sites in one protein with putative sites at the same position in the other one. Several of the putative phosphorylation sites are completely conserved in both proteins (Table 3). We concentrated on phosphorylation sites in both molecules with a strong prediction score above 0.9. Several phosphorylation sites were observed, especially in the last third of amino acid residues of these proteins, that some of them probably have important role in protein-protein interactions.

It has been previously reported that the activity of plant vacuolar Na^+/H^+ antiporter is regulated by interaction with of a 14-3-3 protein with its respective motif (Vasekina et al., 2005). It was also revealed that interaction of 14-3-3 with target proteins is mediated by the phosphorylation of serine or threonine residue in the motif RSxS/T(p)xP where x and S/T(p) represent any amino acid, and phosphoserine or phosphothreonine, respectively (Muslin et al., 1996). Interestingly, AlNHX1 and AlNHX2 contain this motif in the sequence of ASDS(p)PG at position 338 and SSPT(p)KP at position 338, respectively, which are located in the region of the cytoplasmic loop between the transmembrane domains VIII and IX. AlNHX1 was predicted to be phosphorylated by p38MAPK/GSK3/CKI at the Ser 338 residue. p38MAPK, p38 mitogen-activated protein kinases, are a class of mitogen-activated protein kinases that are responsive to stress stimuli such as osmotic stress (Nebreda and Porras, 2000). GSK3, Glycogen synthase kinase 3, is a serine/threonine protein kinase that mediates the addition of phosphate molecules onto serine and threonine amino acid residues. GSK3 has recently been the subject of much research because it has been implicated in a number of stresses. Originally identified in mammals as a cytoplasmic modulator

Table 2. Alignment of the deduced amino acid sequences of transmembrane domains of AlNHX1 and AlNHX2. Sequences were aligned by the Clustal Omega program.

Protein	No. of domain	Sequence of domain	identity
AlNHX1	I	ASVVSINLFVALLCACIVLGHLL	64.00%
AlNHX2		IVSINIFIALLCGCIVIGHLLEG	
AlNHX1	II	VNESITALIIGLCTGVVILLTT	65.217%
AlNHX2		VNEPLTALVMGLITGGVILLVTN	
AlNHX1	III	ILVFSEDLFFIYLLPPIIFNAGF	100.00%
AlNHX2		ILVFSEDLFFIYLLPPIIFNAGF	
AlNHX1	IV	MTITLFGAVGTMISFFTISIGAI	58.33%
AlNHX2		FITIILFGAVGTLVSFVIITLGA	
AlNHX1	V	EVGDFLAIGAIFSATDSVCTLQV	73.08%
AlNHX2		DYLAIGAIFSATDSVCTLQVLNQ	
AlNHX1	VI	FLGNFCYLFLSSTFLGVFAGLLS	62.50%
AlNHX2		NFIGKFLYLFFTSTILGVAAGLL	
AlNHX1	VII	MAYLSYMLAELSDLSGILTVFFC	86.96%
AlNHX2		MAYLSYMLSVLMDLSGILTVFFC	
AlNHX1	VIII	HAFATLSFIAETFLFLYVGMDAL	86.96%
AlNHX2		TFATLSFIAEIFLFLYVGMDAL	
AlNHX1	IX	GISSILLGLVLVGRAAFVFPLSF	66.67%
AlNHX2		LSAIILGLIMVGRAAFIFPLSFL	
AlNHX1	X	STITVVLFSTMVFGMMTKPLIQF	34.38%
AlNHX2		VRVNAIMITSTVIVVLFNTMVFG	

Fig 2. Alignment of the deduced amino acid sequences of AlNHX1 and AlNHX2. Sequences were aligned by the Clustal Omega program. Asterisks indicate the identical amino acid residues, periods indicate amino acids that have low similarity, and dashes indicate gaps. Ten putative transmembrane domains are shown in gray color. The amiloride binding motif is framed in the transmembrane domain III region are underlined and bold. IQ motif (IQxxxRxxxxR) was underlined.

of glycogen metabolism, GSK3 is now recognized as a central regulator of an array of cellular events in plants. Plant GSK3s are encoded by a multigene family, and different GSK3 proteins have various functions and some of them have critical roles in the response to salinity (Santo et al., 2012). It has been showed than salt stress upregulated transcripts of some GSK3 family members in different species (Chen et al., 2003; Koh et al., 2007). AlNHX2 was predicted to be phosphorylated by PKC at the Thr 338 residue. PKC, also known as protein kinase C, is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol (DAG) or calcium ions. Hence, PKC enzymes play important roles in several signal transduction cascades. Previous studies showed that PKC is involved in gene expression, signal transduction and regulation of the activities of many proteins. It has been showed that some salt-induced

Table 3. Predicted phosphorylation sites in AlNHX1/AlNHX2.

Protein	Position	NetPhos Score	Acc. Res.	Kinase	Sequence
AlNHX1	158	0.935	S	CKI	GAIFSATDS
AlNHX2	156	0.935	S	CKI	GAIFSATDS
AlNHX1	250	0.996	S	PKA	IGRHSTDRE
AlNHX2	248	0.976	S	PKA	FARHSTDRE
AlNHX1	302	0958	T	PKC	SSRVTTKHA
AlNHX2	300	0.974	T	PKC	SSRVTTKHT
AlNHX1	303	0.945	T	PKG	SRVTTKHAF
AlNHX2	301	0901	T	PKG	SRVTTKHTF
AlNHX1	338	0.968	S	p38MAPK/ GSK3	FASDSPGKS
AlNHX2	336	0.972	S	p38MAPK/ GSK3	LASSSPTKP
AlNHX1	460	0.998	S	p38MAPK/ GSK3/ cdk5	SEPSSPKSL
AlNHX2	453	0.998	S	GSK3/ cdk5	SDPSSPKCL
AlNHX1	490	0.995	S	RSK/ PKA	VRPSSLRML
AlNHX2	483	0.995	S	RSK/ PKA	VRPSSLRML
AlNHX1	530	0.995	S	p38MAPK/ GSK3/ cdk5	FSPGSPTEQ
AlNHX2	523	0.989	S	p38MAPK	FSWASPTEQ

NetPhos score is the output score from the ensemble of neural networks trained on that acceptor residue type. The sequence shows the context of the acceptor residue ± four residues.

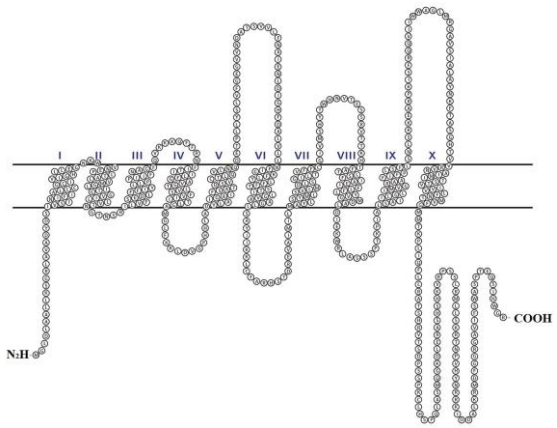

Fig 3. Proposed topological model of AlNHX2. Roman numbers indicate putative transmembrane domains.

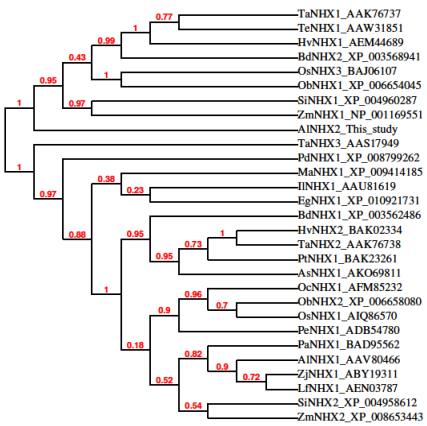

Fig 4. The phylogenetic analysis of AlNHX2 with closely related vacuolar Na^+/H^+ antiporters from various plant species. The GeneBank accession numbers are followed with Na^+/H^+ antiporter names.

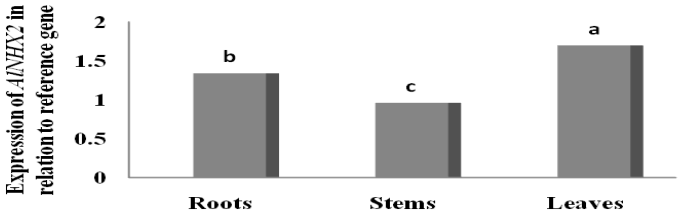

Fig 5. Semiquantitative analysis of the expression level of *AlNHX2* in roots, stems and leaves of *A. littoralis*. Column with the same letters are not significantly different at the 5% level using Duncan's multiple range test.

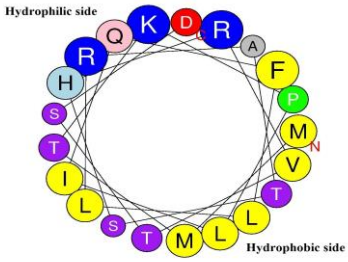

Fig 6. Helical-wheel model of 435-to-455-aa region of AlNHX2. Hydrophilic and hydrophobic sides are shown.

proteins are stimulated by phosphorylation with PKC (Vashisht et al., 2005). There is no report, to our knowledge, to show the activity of vacuolar Na^+/H^+ antiporter is regulated by these kinase proteins. PKC, p38MAPK and GSK3 also phosphorylate other amino acid residues in these proteins. In C-terminal of both proteins, p38MAPK phosphorylates AlNHX1 at Ser 530 residue and AlNHX2 at Ser 523 residue that can play an important role in regulation by regulatory proteins.

Calmodulin (CaM) is recognized as a calcium sensor that regulators a diverse group cellular proteins through binding with them (Rhoads and Friedberg, 1997). Calmodulin can bind with high affinity to a small α-helical region of target proteins. The calmodulin binding site in these proteins is IQ motif with a consensus sequence: IQxxxRxxxxR where x represents any amino acid (Rhoads and Friedberg, 1997). The IQ motif, IQFLLRATSHR, was found in AlNHX2 at position 436 in C-terminal region, but it was not found in AlNHX1 (Fig. 2). Known IQ placed in a stretch of 17-25 amino acid residues having a positively charged amphiphilic region and a tendency to form an α-helix structure upon binding to CaM (Snedden and Fromm, 2001). We have used helical wheel analysis to characterize the amphiphilic region around IQ motif within the AlNHX2. The stretch of AlNHX2 amino acids from Met 435 to Asp 455 has the potential to form a positively charged amphiphilic α-helix characteristic of a CaM-binding region (Fig 6). It has been reported that the interaction between CaM and target protein is Ca^+ and pH dependent, and the binding of Ca^+-CaM to target molecule C terminus decrease with the increase of pH (Yamaguchi et al., 2004). It has also been shown that at physiological conditions CaM would be bound to target protein C terminus and change the activity of protein (Yamaguchi et al., 2004). It was previously suggested that, binding of CaM to vacuolar Na^+/H^+ antiporter repressed the ions exchange activity in wild-type plants under normal growth conditions (Apse et al., 1999). It can be suggested that there is an autoinhibitory domain at C-terminal region of some vacuolar Na^+/H^+ antiporter, such as AlNHX2, that there is located within corresponding part of C-terminal region. The autoinhibitory domain was previously reported for other transmembrane proteins such as H^+-ATPase pump (Palmgren et al., 1991). An empirical study is necessary to assess the validity of this hypothesis.

In summary, *AlNHX2* is a novel vacuolar Na^+/H^+ antiporter from halophyte *A. littoralis*, which is expressed in leaves, stems and roots. Putative phosphorylation sites were determined using the prediction software, it was predicted that the binding of 14-3-3 protein to specific domains within AlNHX1 and 2 and this binding is induced by a protein kinase-mediated phosphorylation of a specific Thr or Ser residue in these domains. In addition, the findings reported here are evidence for the interaction of CaM protein with C-terminal of AlNHX2, but not with AlNHX1. It can be suggested that AlNHX2 play an important role in salt tolerance mechanisms in halophyte *A. littoralis* and can be

considered as a suitable candidate gene to improve salt tolerance of sensitive crops such as wheat.

Materials and Methods

Plant growth

A. littoralis seeds were supplied by Pakan Seed Research Center, Isfahan, Iran. The seeds were surface sterilized by soaking in 1% (v/v) sodium hypochlorite for 20 min and rinsed several times with distilled water. The surface-sterilized seeds were kept at 16º C for 72 h to germinate uniformly and the germinated seeds sown in pots filled with mixture of sand and grit (1:1) under green house conditions (with 25/16º C day/night temperature, 16 h light/ 8 h dark photoperiod). Plants were regularly irrigated with 100 ml of MS nutrient solution, pH 5.8, (Murashige and Skoog, 1962) every three days.

Primer design

Amino acid sequences of vacuolar Na^+/H^+ antiporter in various plant species were collected from GenBank. Multiple alignment of these sequences was carried out by CLUSTALW program to find conserved regions among them. A pair of degenerate primers, primerF and primerR, was designed by Oligo Software (Table 1), on the basis of conserved regions. All primers were used to isolate a full-length cDNA of *AlNHX2* and the primers used for semiquantitative RT-PCR are given in table 1.

Genomic DNA and total RNA extraction and preparing cDNAs

Leaves, stems and roots were sampled, frozen in liquid nitrogen and stored at -80 °C until used. Genomic DNA was extracted from leaves using modified CTAB method (Alemzadeh et al., 2006). Total RNA was extracted from leaves, stems and roots using RNA Extraction Kit (Vivantis, Malaysia). Subsequently, extracted RNA was treated with RNase-free DNase I (Fermentas, Germany) to remove genomic DNA contamination. The quality of RNA was assessed by electrophoresis on 1% agarose gel. First-strand cDNAs were prepared from total RNA by RevertAid M-MuLV Reverse Transcriptase (Fermentas, Germany), with oligo (dT) primer.

Cloning of PCR products and rapid amplification of cDNA ends (RACE)

The amplified products were cloned into the pTZ57R/T vector using the TA cloning kit (Fermentas, Germany) and various clones sequenced on both strands to confirm the sequence.

The full-length of *AlNHX2* was obtained using GeneRacer™ Kit (Invitrogen, USA) according to the manufacturer's instructions. To obtain 5' end, the first-strand cDNA was amplified using Alant2R and GeneRacerTM 5' primers. To obtain 3' end, the first-strand cDNA was amplified using Alant2F and GeneRacerTM 3' primers.

Semiquantitative analysis of AlNHX2

The expression of *AlHMA2* was investigated in leaves, stems and roots of *A. littoralis* using specific primers by semiquantitative RT-PCR. The cDNAs were amplified by specific primers for *AlNHX2* (Alant2R and AlF) and specific primers for a gene encoding actin (ActinAlF and ActinAlR) as reference gene (table 1). PCR reactions in a final volume of 25 µl reaction mixture containing 10 mM tris- HCl (pH 8.3), 50 mM KCl, 1.5 $MgCl_2$, 200 µM dNTPs, 1 µl diluted cDNAs, 0.3 µM of each primer and 1 unit Taq DNA polymerase were carried out under the following conditions: 5 min at 95 °C, followed by 28 cycles at 95°C, 30 s at 60°C, and 1 min at 72°C, with a final extension step of 7 min at 72°C. The PCR product was then separated on 1% agarose gel containing ethidium bromide and band density was measured using TotalLab Software.

PCR amplifications

PCR reactions in a final volume of 20 µl reaction containing 1 µg genomic DNA as template and suitable primers were carried out as mentioned above under the same conditions with suitable annealing temperature. The PCR product was separated on 1% agarose gel containing ethidium bromide.

Bioinformatics analysis

The homology comparison of DNA sequences and deduced amino acid sequences was carried out using CLASTALW program on the DNA Data Bank of Japan, DDBJ (http://clustalw.ddbj.nig.ac.jp/top-e.html). The percentage of amino acid identity was determined by Clustal Omega program (http://www.uniprot.org/align/). The nucleotide sequences were translated to amino acid sequences using translate tool on the SIB Bioinformatics Resource Porta, ExPASy (http://web.expasy.org/translate/). The molecular weight was computed by compute PI/MW tool, ExPASy (http://web.expasy.org/compute_pi/). The transmembrane domains of AlNHX2 were also analyzed with SOSUI online program (http://bp.nuap.nagoya-u.ac.jp/sosui). The deduced protein AlNHX2 was subjected to analysis of homology and phylogenesis by Phylogenetic tree was drawn using Phylogeny.fr program (Dereeper et al., 2008). The helical wheel representation was drawn using the Heliquest software (http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParamsV2.py). The phosphorylation sites were predicted using NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/). The kinase specific eukaryotic protein phosphorylation sites were predicted using NetPhos 1.0 (http://www.cbs.dtu.dk/services/NetPhosK/).

Conclusion

Salinity is a widespread challenge affecting plant production worldwide that understanding of how plants cope with salinity and maintain an optimal balance between sodium uptake and its compartmentation is fundamental to our knowledge of how plant can adapt to salt stress. Our research work has shown that there are some specific domains within NHXs for binding of 14-3-3 protein, and also it was cleared that the activities of NHXs are regulated by PKC, p38MAPK and GSK3. Now, we gain a better understanding of how antiporter proteins are regulated by other proteins.

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