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BIO-INFORMATIC ANALYSIS OF A VACUOLAR Na^+/H^+ ANTIPORTER (*ALaNHX*) FROM THE SALT RESISTANT GRASS *AELUROPUS LAGOPOIDES*

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Abstract

Sodium-hydrogen antiporter (NHX) protein regulates the trans-membrane transport of Na^+ in higher plants. Vacuolar-NHX is a type of NHX protein located on tonoplast and minimizes the accumulation of Na^+ in cytoplasm by compartmentalizing into vacuole especially in salt tolerant plants. In *Aeluropus lagopoides*, *AlaNHX* [NCBI: GU199336, Vacuolar-NHX] plays a vital role for efficient Na^+ sequestration into the vacuole and helps in plant survival in saline areas. Therefore, sequence analysis, structural analysis and modeling of AlaNHX were performed through bioinformatics tools. Homology of *AlaNHX* was 99% similar with the Na^+/H^+ antiporter of *Aeluropus littoralis*. Sequence of *AlaNHX* consisted of 2353 bp including 337 bp of un-translated regions (UTR) at 5′ and 393 at 3′ end. In addition, *AlaNHX* have an "open reading frame" (ORF) of 1623 bp which translated into 59.4 KDa protein containing 540 amino acids (Leucine + Serine contributed in 22% of peptide chain). AlaNHX protein consists of 10 transmembrane domains (TMD; 3 primary and 7 secondary protein structural type) and a long (95 amino acids) carboxyl terminal end in cytoplasmic region. In addition, 3, 5, 7 and 8 TMD regions of AlaNHX were highly conserved. Different glycosylation, phosphorylation and myristoylation sites were also found in AlaNHX protein. Three-dimensional (3D) structure analysis revealed that this protein may be classified as stable and of hydrophobic nature containing a significant proportion of alpha helices. In this study, a three-dimensional structure of AlaNHX protein was predicted by using in-silico3D homology modeling technique. This study provides baseline information for understanding the importance of NHX protein structure in salinity resistance of grasses. This information could help in improving salinity tolerance in salt sensitive grasses through genetic engineering.

Key words: *Aeluropus lagopoides*; Homology modeling; Na^+/H^+ antiporter; Structural analysis.

Introduction

Soil salinity is one of the major environmental constrain that limit plant growth and productivity by affecting various physiological, biochemical and molecular processes (Deinlein *et al*., 2014). High sodium concentration which is a characteristic feature of saline areas is lethal for plant survival. However, plants have the ability to reduce Na^+ toxicity but with varying degree. The ability to compartmentalize Na^+ in vacuole is a common mechanism for both halophytes (salt resistant) and glycophytes (salt sensitive), however, an efficient and well synchronized mechanism of Na^+ transport supports halophytes to survive under extreme saline conditions (Ahmed *et al*., 2013; Oh *et al*., 2009; Zhu, 2001; Katschnig *et al*., 2014). Plant controls net Na^+ influx by sequestering excess cytosolic Na^+ into the vacuole (Ahmed *et al*., 2013). Transmembrane proteins plays a vital role to regulate Na^+ flux either directly by Na^+/H^+ antiporters or indirectly through H-ATPase and H-PPase. The Sodium/Hydrogen antiporters reduce Na^+ accumulation in cytoplasm by moving Na^+ across the tonoplast and dumping in the vacuole (Ahmed *et al*., 2013; Oh *et al*., 2009). In addition, Na^+/H^+ antiporter helps in regulating cytoplasmic pH and control cell volume (Bassil *et al*., 2011). Due to major role of Na^+/H^+ antiporter gene in salinity tolerance and plant development, some low salt-tolerant transgenic lines of crops like tomato, brassica, rice, maize, wheat, tobacco etc. are claimed to be developed with over-expression of *NHX* genes (Rauf *et al*., 2014).

In higher plants, Na^+/H^+ antiporter gene was first cloned from Arabidopsis (Apse *et al*., 1999) and its physiological functions were initially observed in storage tissues of *Beta vulgaris* (Blumwald & Poole, 1985). According to genetic databank (NCBI), around 84 genes encoding vacuolar Na^+/H^+ transporters have been isolated from various taxa of eudicots [e.g. *Aegiceras*, *Kalidium*, *Haloxylon*, *Zygophyllum*, *Suaeda*, *Atriplex*, *Pyrus*, *Harrisia*, *Echinopsis*, *Populus*, *Arabidopsis*, *Bruguiera*] and 18 from monocots [*Oryza*, *Zea*, *Aeluropus*, *Triticum*, *Hordeum*]. The Na^+/H^+ antiporter consists of a single polypeptide chain of molecular mass around 56 to 100 KDa. This membrane bound protein contains 10-12 transmembrane domains (TMD) at amino-terminus and a large cytoplasmic region at carboxyl terminus. Among TMDs, 6 and 7 domains are reported to be highly conserved in this family with little similarity in the tail region of carboxyl terminus. In the last two decades, significant progress has been made in molecular modelling through computational treatments of biological molecules to better understand the evolution but not enough attention in the perspective of abiotic stresses.

Most conventional crops belong to family Poaceae and have salt sensitive nature at the same time when salinity problem and food demand is alarmingly increasing (Levetin & Mc Mahon, 2008). In such scenario, it is necessary to improve our knowledge about the genetic resources of halophytes which regulate Na^+ flux. *Aeluropus lagopoides* (L.) Trin. ex thw. is a high salt tolerant, C_4 perennial grass, growing along coastal salt marshes. It is a wild relative of wheat which makes

it an attractive model plant to study the mechanism of salinity tolerance (Razavi *et al*., 2006). Several ecological and physiological studies on *A. lagopoides* have been conducted which indicated that plant could in 300 to 450 mM NaCl (Ahmed *et al*., 2013; Sobhanian *et al*., 2010). This plant is also reported to reduce Na^+ toxicity both by secreting through aerial parts and sequestering in vacuole by altering the expression of *AlaNHX* (*VNHX*), *PM-NHX* (*SOS1*) and *H^+-ATPase* (Ahmed *et al*., 2013). However, the detailed bio-informatics of vacuolar Na^+/H^+ antiporter of *A. lagopoides* is not available. Therefore, this study was conducted to generate a 3D structure of the *AlaNHX* using homology modeling: a bio-informatics tool to provide base line information that may help in improving salt tolerance of other plant species.

Materials and Methods

Plant material and growth condition: Seeds of *A. lagopoides* (L.) Trin. ex Thw. were collected from salt marshes of Karachi coast, Pakistan (24°52□21.87□□N, 66°51□24.58□□E). Seeds were sown in plastic pots filled with pre-mix soil (Katou Sangyo Co., Japan) and placed in a growth room adjusted at 25°C. Each pot contained only one individual which was sub-irrigated with 1/2 strength Hoagland solution along with 400 mM NaCl for 2 days. Similar sized plants were selected for RNA isolation.

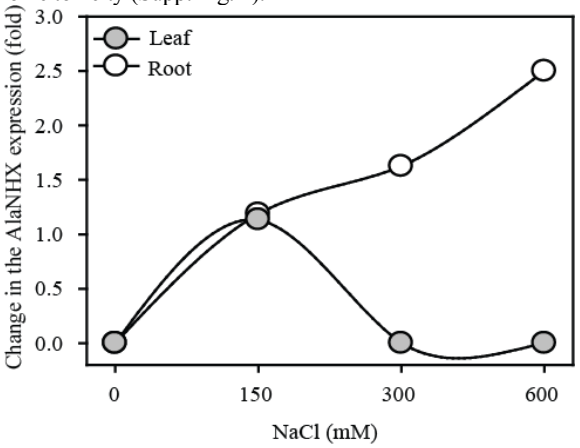
RNA isolation and cDNA synthesis: Total RNA was isolated from seedlings by using RNAqueous Kit (Ambion). First-strand of cDNA was synthesized from 1 µg RNA (DNA free) by using the protocol of cDNA Takara RNA-PCR Kit (AMV; Ver 3.0) and PCR was performed using with a pair of primers: (P1: 5′TTC ATC TAC CTG CTC CCG CCC ATC AT3′; P2: 5′CCA CAG AAG AAC ACG GTT AGA ATA CC3′), which were designed on the conserved regions of previously reported Na^+/H^+ antiporter from other plants [National Center for Biotechnology Information- (NCBI) records]. PCR product was cloned through TA cloning kit (Takara) and during process pGEM-T vector was transformed into *Escherichia coli* DH5a. After cloning, plasmid was extracted and used for sequencing. The full length sequence of *AlaNHX* gene was determined by rapid amplification of cDNA ends (RACE) as mentioned in the protocol of GeneRacer™ RACE Ready cDNA Kit Manual. The 3′ region was obtained using primers (P3: 5′GTTGTGAATGATGCCACGTC3′; M13-primer: 5′GTTTTCCCAGTCACGAC3′). The 5′ region was obtained using the primer pair (P4: 5′GAGAGCAGGAGATCCCAATC3′; P5: 5′CCACAGAAGAACACGGTTAGAATACC3′) (for more details: http://www.ncbi.nlm.nih.gov/nuccore/GU199336.1). After amplification of 3′ and 5′ regions, fragments were sequenced by ABI PRISM 310 automated sequencer and assembled to provide full length of targeted cDNA. Amplification was performed in two separate replicates while consensus sequences of 3′ and 5′ regions were obtained by alignment of five sequences of the same fragment.

Bioinformatics analysis of *AlaNHX* gene: Targeted gene sequence was analyzed using different online tools. The sequence similarity search was performed using BLAST (Basic Local Alignment Search Tool available at NCBI website, http://www.ncbi.nlm.nih.gov). Sequence alignment was done by using DNA-Dynamo software. Polygenetic analysis was performed by neighbor-joining method using NCBI program to compare the targeted sequence with closely allied homologous genes isolated earlier. The full length sequence was submitted to Gene Bank [NCBI: GU199336]. Sequence, hydrophobicity plot and two dimensional models of alpha helical transmembrane of protein were predicted by using online available SOSUI engine ver. 1.11 (http://bp.nuap.nagoya-u.ac.jp/sosui) (Hirokawa *et al*., 1998). The subcellular localization and conserved domain of AlNHX protein was predicted with the help of WoLFPSORT (http://pfam.xfam.org/family/PF00999) (Horton *et al*., 2007) and Pfam (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) (Sonnhammer *et al*., 1998) servers, respectively. The presence of regulatory binding sites was determined through ScanProsite tool (http://prosite.expasy.org/scanprosite/) (De Castro *et al*., 2006). Secondary structure based on 3-state and 8-state was predicted and the final 3D model was constructed by using online services of Raptorx (http://raptorx.uchicago.edu/) (Källberg *et al*., 2012). The quality of predicted 3D structure model was evaluated by *P*-value, Score, uGDT and GDT, uSeqID and SeqID. The disorder prediction for the entire target sequence was also performed.

Results and Discussion

In this study, a full length cDNA was isolated from *A. lagopoides* grown under saline conditions (400 mM NaCl). The cDNA was 2353 bp long in which the 5□ and 3□ untranslated region consisted of 337 and 393 bp in length (Fig. 1). Poly-A domain was found at the end of 3□ untranslated region which is a characteristic feature of mRNA. The predicted open reading frame was 1623 bp long (338-1960 bp of full length cDNA) which encoded protein of 540 amino acids (Fig. 1). Molecular mass of predicted protein and the calculated theoretical isoelectric point (pI) was 59.4 KDa and 8.5, respectively. Based on the blast analysis, this predicted protein had 99% homology with the *AlNHX* [Na^+/H^+ antiporter of *Aeluropus littoralis* – NCBI: AAV80466.1, AY825361.1]. Whereas, the conserved domain analysis using Pfam server showed that predicted protein belonged to the sodium-hydrogen exchanger family (Pfam Clan: CL0064 and *E*-value: 1.4×10^{-55}). Analysis through WoLFPSORT server showed that the targeted protein was the nearest neighbor (Identity: 73%) of vacuolar membrane protein (At5g27150.1). These results suggested that the isolated protein was a vacuolar-Na^+/H^+ antiporter channel of *A. lagopoides* and submitted in NCBI gene bank with the name of *AlaNHX* [NCBI: GU199336]. In addition, the information obtained from bio-informatics studies of targeted gene supports the link between increasing expression of *AlaNHX* and Na^+ content in *A. lagopides*. The

higher expression of *AlaNHX* appear to be involved in Na^+ sequestration in vacuole of plant cell thus helping in reduced ionic toxicity (Supp. Fig. 1).



Supplementary Fig. 1 Change in the expression of *AlaNHX* in *A. lagopoides* (data taken from Ahmed *et al*., 2013).

The phylogenetic relationship between related NHX proteins from different plants was also analyzed (Fig. 2). The AlaNHX protein showed high similarity with previously reported NHX proteins in the following order: *Aeluropus littoralis*, *Zoysia japonica* and *Phragmites australis*. An un-rooted dendrogram showed two clusters, one contained NHX sequences isolated from monocots and the other one had the NHX sequences of dicots only. The AlaNHX protein was presented in the cluster consisted of grass species (Fig. 2). Furthermore, cluster analysis showed that AlaNHX was phylogenetically closer to the salt resistant grasses (*Aeluropus littoralis*, *Zoysia japonica*, *Phragmites australis*) than the salt sensitive species (*Zea mays*, *Sorghum bicolor*, *Oryza saltiva, Hordeum vulgare*, *Triticum aestivum*) (Fig. 2).

A hydropathy plot (SOSUI program) indicated that the AlaNHX consisted of 10 putative hydrophobic regions in which transmembrane domains 1, 2 and 6 had primary protein structures while other domains represented secondary structures (Fig. 3, Table 1). Both amino- and carboxyl- terminus of AlaNHX protein were cytoplasmic (25 and 105 amino acids, respectively). The 3′ and 5′ regions of *AlaNHX* could get the signals from cytoplasmic side and regulate the activity of antiporter protein. The region of 85 to 95 (LFFIYLLPPII) amino acids in 3 TMD was highly conserved and identified as amiloride binding site (in mammals) which inhibits the activity of eukaryotic Na^+/H^+ antiporter (Figs. 1 and 3). The presence of amiloride region confirmed that AlaNHX was a vacuolar Na^+/H^+ antiporter. In addition, the down regulation of *AlaNHX* (particularly in plant shoot) treated with high salinity (> 150 mM NaCl) could be possible by such motif type.

Some other possible conserved regions were also found at 5, 7 and 8 TMDs (Fig. 1). The PROSITE results showed the presence of various regulatory sites in AlaNHX protein including three potential N-glycosylation sites, nine N-myristoylations sites, five casein kinase II phosphorylation sites, eight protein kinase C phosphorylation sites and a leucine zipper pattern site (Table 2). Presence of those regulatory sites may help in the structure maintenance and efficient activity of AlaNHX protein. The cytoplasmic region has little similarity throughout the family. The hydrophobicity value (0.559) showed a majority of non-polar amino acids in AlaNHX protein. Protein sequence revealed a dominance of 12% Leucine (69) and 10% Serine (55) amino acids of total protein (Table 3). The contribution of most easily oxidize-able amino acids (cysteine, histidine, methionine, tryptophan and tyrosine) was only 10% in AlaNHX, which could be beneficial in improving the stability of targeted protein under stress. These findings suggested that the targeted vacuolar-NHX protein of *A. lagopoides* was hydrophobic in nature. The total number of positively (Arginine + Lysine) and negatively (Aspartic acid + Glutamic acid) charged residues were 38 and 33, respectively (Table 3). Secondary structure analysis revealed that AlaNHX protein had mixed secondary structures i.e. α-helix, extended strand in β ladder, hydrogen bonded turn and bend coil (Figs. 4 and 5). However, the analysis showed that alpha helices dominated (71%) among secondary structure elements, followed by random coils (26%) and extended strands (2%). The high percentage of helices in the structure makes the protein more flexible for folding, which might increase protein interactions (Roy *et al*., 2011). Protein analysis confirmed a higher stability of AlaNHX (instability index (II): 35.20 and Aliphatic index: 109.02).A total of 435 amino acids out of 540 (80% residues) were identical between query and templates (4bwz: A) and constructed a best 3D model with *P*-value 2.54×10^{-7} (Overall uGDT (GDT): 253 (58); uSeqId (SeqId): 55(13); Score: 338). The predicted model of AlaNHX protein pre-dominantly consisted of neutral and non-polar amino acids (Figs. 4 and 5).

Table 1. The feature of possible transmembrane domains in AlaNHX protein predicted by SOSUI program.

S. No.	N-terminal	Transmembrane region	C-terminal	Structure type	Length
1.	22	ASVVSINLFVALLCACIVLGHLL	44	Primary	23
2.	50	VNESITALIIGLCTGVVILLTT	71	Primary	22
3.	78	ILVFSEDLFFIYLLPPIIFNAGF	100	Secondary	23
4.	112	MTITLFGAVGTMISFFTISIGAI	134	Secondary	23
5.	146	EVGDFLAIGAIFSATDSVCTLQV	168	Secondary	23
6.	215	FLGNFCYLFLSSTFLGVFAGLLS	237	Primary	23
7.	261	MAYLSYMLAELSDLSGILTVFFC	283	Secondary	23
8.	305	HAFATLSFIAETFLFLYVGMDAL	327	Secondary	23

9.	344	GISSILLGLVLVGRAAFVFPLSF	366	Secondary	23
10.	423	STITVVLFSTMVFGMMTKPLIQF	445	Secondary	23

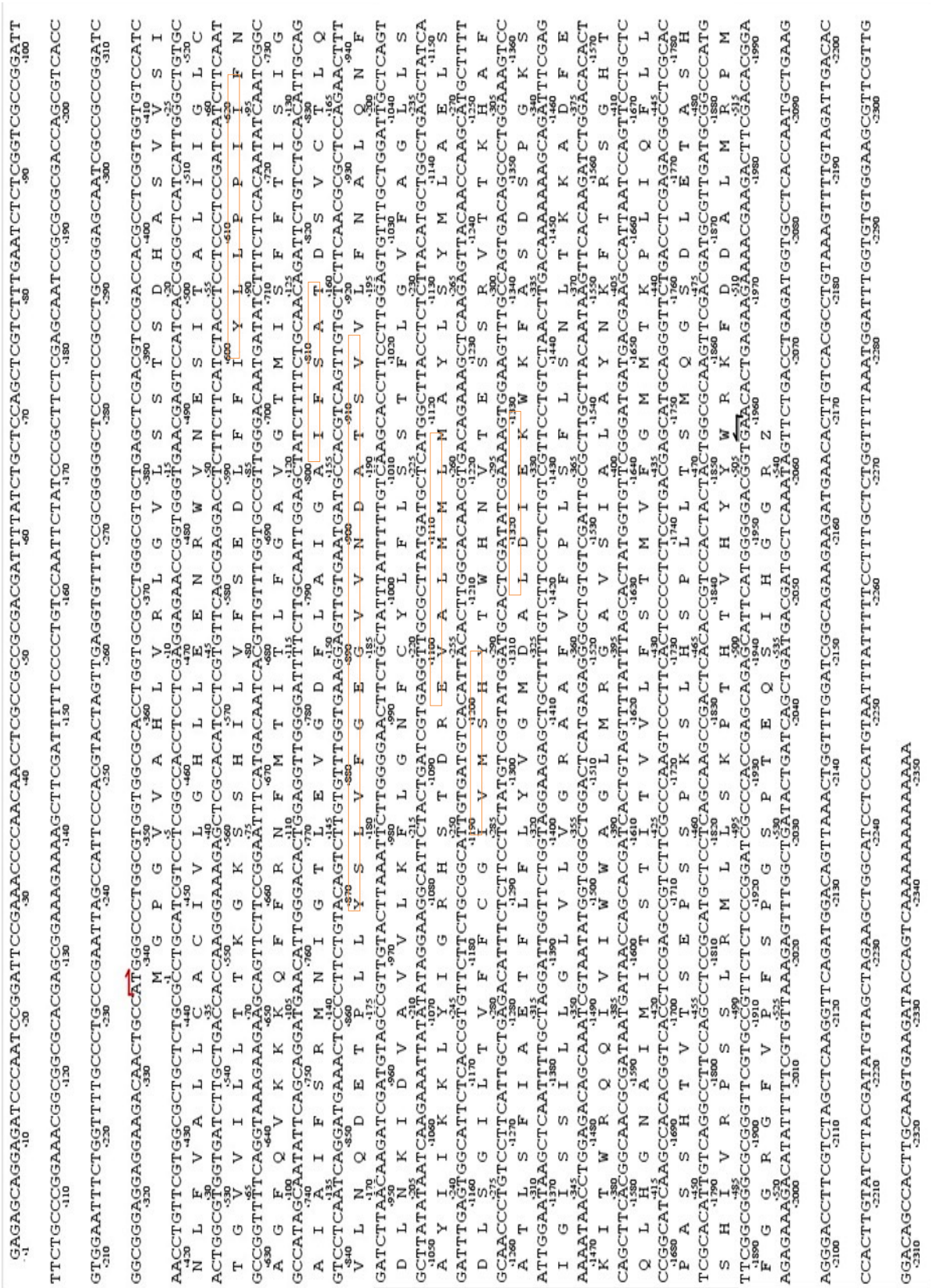


Fig. 1. Nucleotide and the deduced amino acid sequence of Na^+/H^+ antiporter of *A. lagopoides* (*AlaNHX*). The DNA sequence is numbered on above the deduced amino acid sequence while amino acid is numbered on the below side. A possible "open reading frame region (ORF)" is shown by arrows while the possible conserved regions of this NHX protein are represented in boxes.

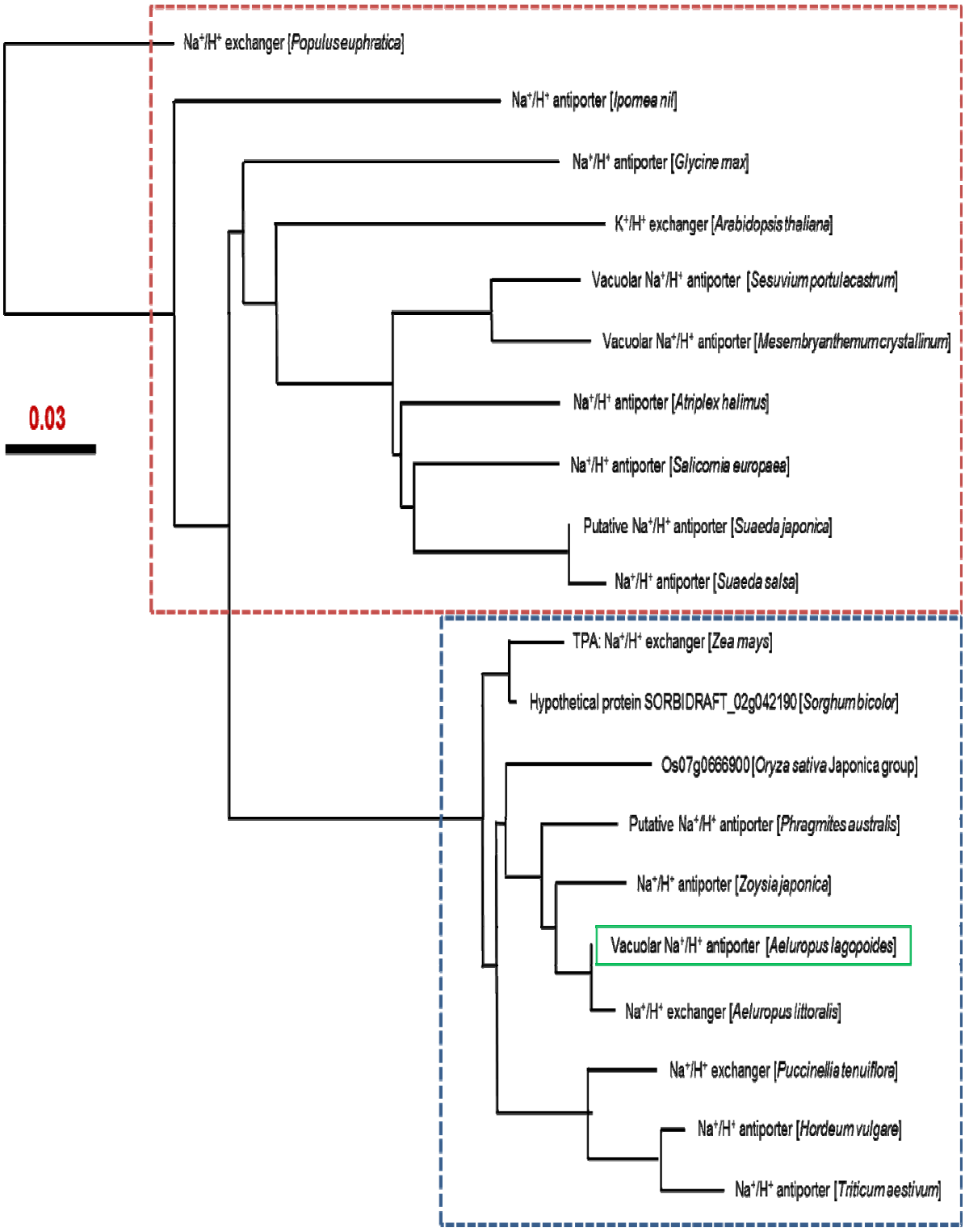


Fig. 2. The cluster analysis of AlaNHX with closely related sequences of different plant species present on NCBI. The protein sequences were aligned by NCBI software. The accession numbers for the protein sequences in the decreasing order of homology with *Aeluropus lagopoides* (AlaNHX: ACZ97405.1), were as follows: *Aeluropus littoralis* (AAV80466.1), *Zoysia japonica* (ABY19311.2), *Phragmites australis* (BAD95562.1), *Oryza sativa* (BAA83337.1), *Puccinellia tenuiflora* (BAK23261.1), *Hordeum vulgare* (BAC56698.1), *Triticum aestivum* (AAK76738.2), *Salicornia europaea* (AAN08157.1), *Glycine max* (AAY43006.1), *Sesuvium portulacastrum* (AFD97541.1), *Suaeda japonica* (BAE95195.1), *Mesembryanthemum crystallinum* (CAN99589.1), *Suaeda salsa* (AAK53432.1), *Ipomoea nil* (BAD91200.1) and *Populus euphratica* (ACU01855.1). Blue and red doted boxes represent monocots and dicots species groups, respectively.

Table 2. The PROSITE patterns for AlaNHX: positions of important sites.

Site name	Site position in protein	Site sequence	Randomized probability
N-glycosylation	051 – 054 294 – 297 369 – 372	NESI NVTE NLTK	$p<0.005$
Protein kinase C phosphorylation	070 – 072 251 – 253 298 – 300 302 – 304 371 – 373 380 – 382 460 – 462 490 – 492	TTK TDR SSR TTK TKK TWR SPK SLR	$p<0.01$
Casein kinase II phosphorylation	017 – 020 158 – 161 251 – 254 475 – 478 530 – 533	STSD SATD TDRE SDLE SPTE	$p<0.01$
N-myristoylation	013 – 018 060 – 065 118 – 123 121 – 126 154 – 159 230 – 235 234 – 239 284 – 289 391 – 396	GVLSST GLCTGV GAVGTM GTMISF GAIFSA GVFAGL GLLSAY GIVMSH GLMRGA	$p<0.01$
ATP/GTP-binding site motif A	335 – 342	ASDSPGKS	$p<0.00001$
Leucine zipper	257 – 278	LMMLMAYLSYMLAELSDLSGIL	$p<0.00001$

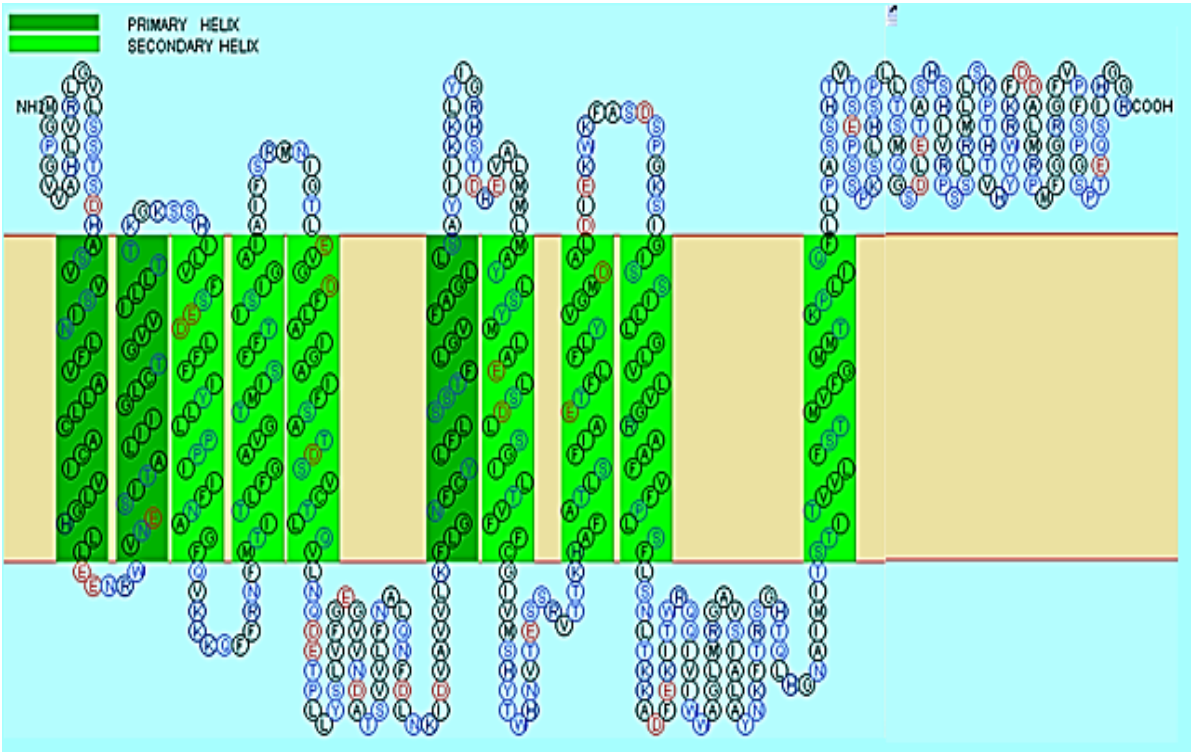


Fig. 3. Hydrophobicity plot of alaNHX. The hydrophobicity values were calculated by the program SOSUI.

Table 3. The proportion of different amino acids in the structure of AlaNHX protein.

Code	Amino acid	Quantity in protein	% Contribution
A	Alanine	36	6.67%
C	Cysteine	6	1.11%
D	Aspartic acid	17	3.15%
E	Glutamic acid	16	2.96%
F	Phenylalanine	42	7.78%
G	Glycine	39	7.22%
H	Histidine	17	3.15%
I	Isoleucine	40	7.41%
K	Lysine	21	3.89%
L	Leucine	69	12.78%
M	Methionine	19	3.52%
N	Asparagine	16	2.96%
P	Proline	17	3.15%
Q	Glutamine	11	2.04%
R	Arginine	17	3.15%
S	Serine	55	10.19%
T	Threonine	39	7.22%
V	Valine	44	8.15%
W	Tryptophan	7	1.30%
Y	Tyrosine	12	2.22%
	Total	**540**	

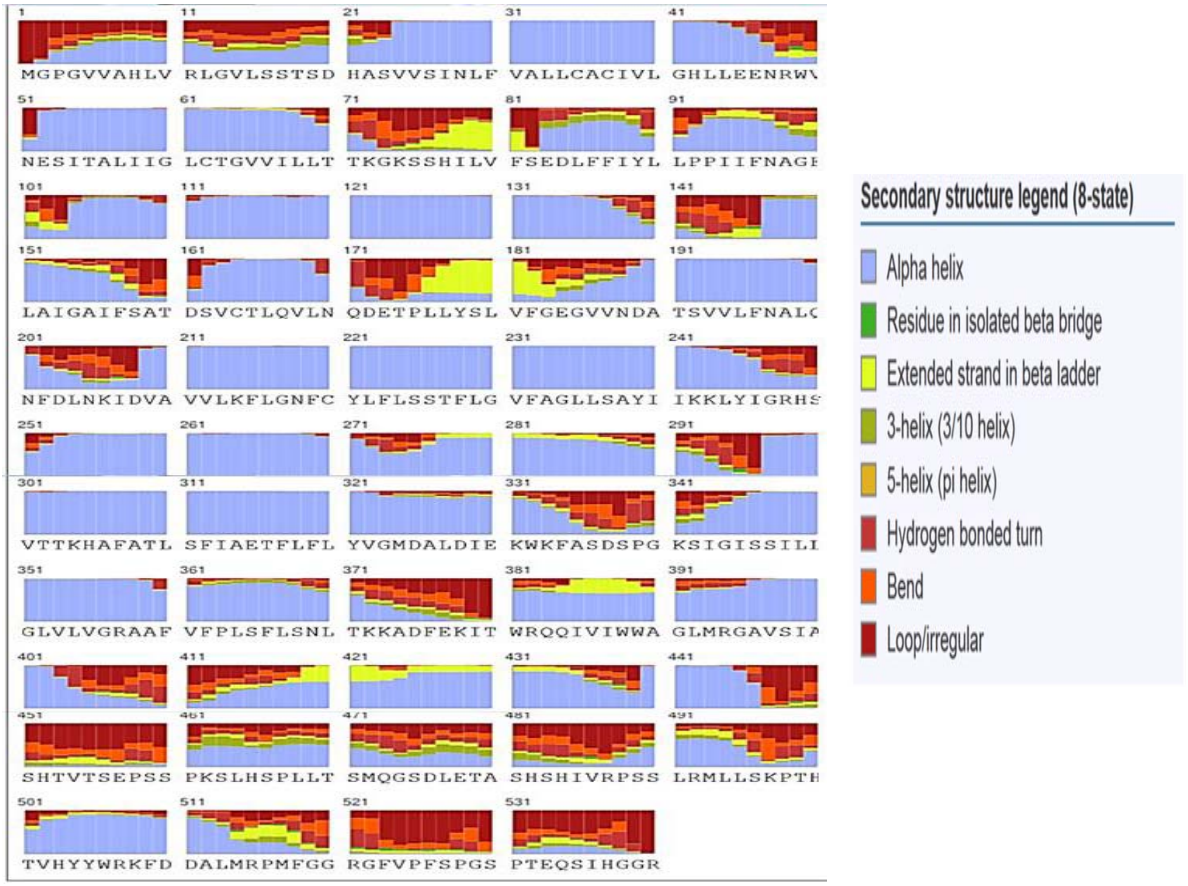


Fig. 4. Proportion of secondary structures in AlaNHX protein predicted by online RaptorX services.

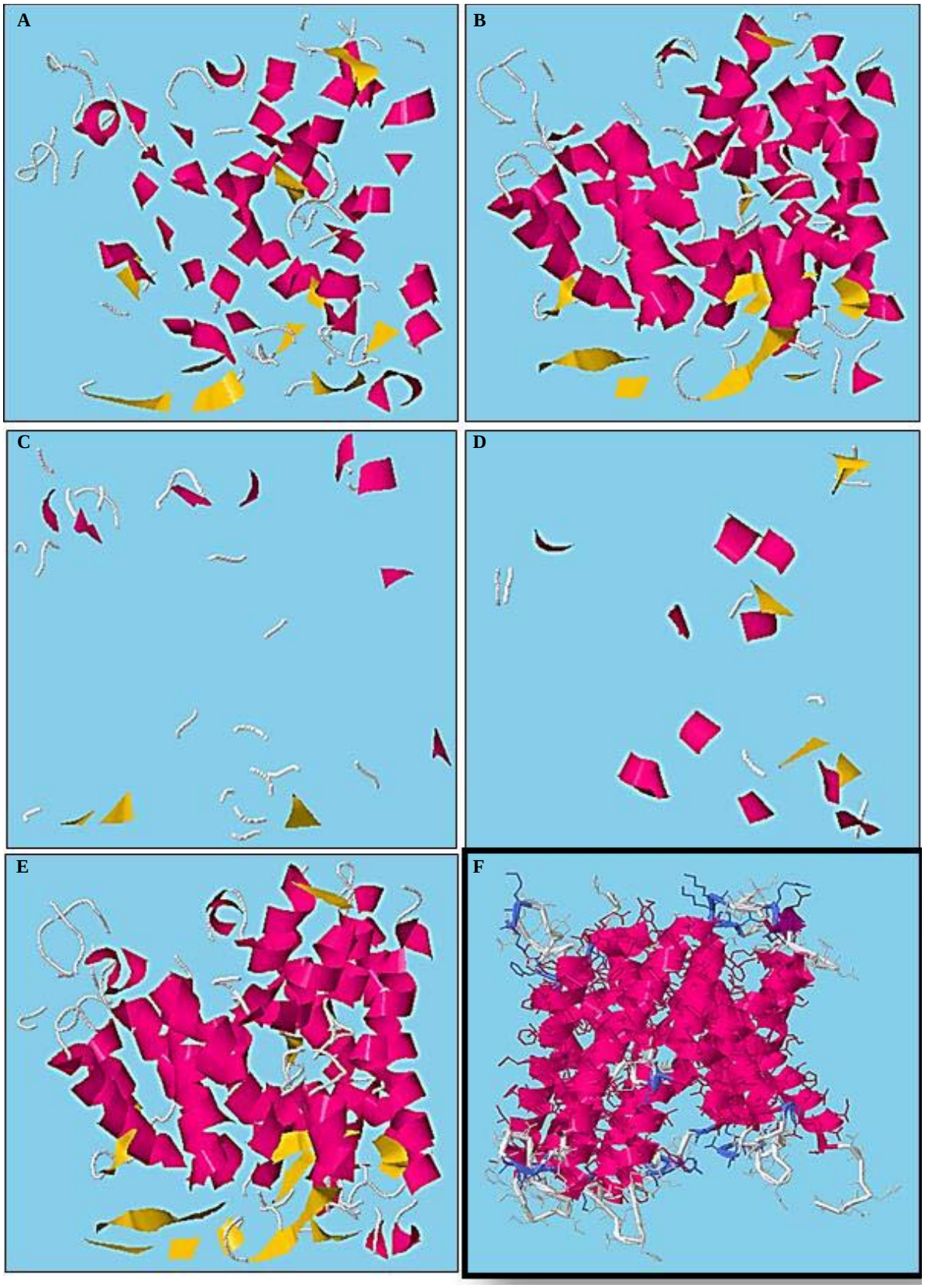


Fig. 5. Profiling of amino acids in AlaNHX. A; polar amino acids, B; non-polar amino acids, C; basic amino acids, D; acidic amino acids, E; neutral amino acids and F; predicted 3D shape of protein through online RaptorX services.

Conclusion

The bioinformatics of Na^+/H^+ antiporter channel protein of *A. lagopoides* was performed by using isolated *AlaNHX* sequence information and a theoretical 3D model was constructed with the help of homology modeling. The possible presence of various regulatory elements along with long carboxylic tail in AlaNHX protein might be the reason of its efficient function in halophytic grass. In addition, e the specific amino acid contribution in the makeup of protein structure could improve the half-life under stress condition. The predicted protein sequence and structure could help in better understanding about the function of this protein under salinity stress. This information could further be used for improving the efficiency of Na^+/H^+ antiporter in various conventional crop species through modern molecular techniques.

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