In-silico analysis of a vacuolar Na^+/H^+ antiporter gene from *Triticum aestivum* cv. Kharchia Local.

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.**ABSTRACT**

Kharchia Local wheat variety known for its tolerance to salinity, an Indian salt tolerant land race. Previously we have reported transcriptome sequencing of wheat under salt stress with large number differential expressed genes. qRT-PCR analysis showed up-regulation in response to salinity of many known and unknown genes. In this report, we cloned and analyzed bioinformatically a gene encoding protein vacuolar Na^+/H^+ antiporter (NHX1). The *NHX* gene is 1,641bp long, encoding 546 amino acids protein with estimated molecular mass of 59.7kDa and pI 8.13. The *NHX1* shows high amino acid similarity with other NHX gene of Poaceae family and belongs to Class I type NHXs. The hydropathy plot shows NHX has 11 strong transmembrane helices. The study throws light into the structural features of *NHX1*, a potential candidate for developing salinity tolerant crop plants.

Keywords: *In-silico* analysis, salt stress, vacuolar Na^+/H^+ antiporter gene

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INTRODUCTION

Abiotic stresses approximately decreases 70% of crop productivity (Acquaah 2007). Plant response to these stresses is complex and involves changes at the transcriptomic, proteomic, cellular and physiological levels. Stress tolerance in plants is a synchronized action of plethora of genes, which once activated can trigger components of different pathways (Tuteja, 2007). Salinity stress is one of the major stresses among various abiotic stresses. Growth and productivity of crops worldwide is affected by salinity stress (Gao *et al.,* 2007). Over 800 million hectares of land is affected worldwide by salinity stress (FAO 2008, http:www.fao.org/ag/agl/agll/spush/).

Of the several mineral nutrients required for growth and development of plants, Na^+ is not considered an essential mineral nutrient, however, its excess adversely affects the growth of the plant and is known to delay flowering as well as cause yield loss (Gill 1979, Hasegawa *et al*. 2000, Zhu 2001, Chinnusamy *et al*. 2006). Saline soils usually contain very high concentration of Na^+ which disrupts absorption of K^+ and other mineral (Zhu 2001). There are mainly three known mechanisms to prevent accumulation of Na^+ in the cytosol of a plant cell: restriction of Na^+ influx, activation of Na^+ efflux and compartmentalization of Na^+ into the vacuole (Niu *et al*., 1995; Blumwald *et al*., 2000; Zhu 2001, Goyal *et al*., 2013). Na^+ homeostasis is maintained either by sequestration of excess Na^+ into the vacuoles by means of vacuolar Na^+/H^+ antiporters (*NHX1*) or by eliminating it through *SOS1* (plasma membrane Na^+/H^+ antiporter) (Shi *et al.,* 2000; 2003). The transport of Na^+ inside vacuoles is a strategy to withstand salt stress. For sequestering excess Na^+ into the vacuole, vacuolar antiporter utilizes the proton motive force generated by pyrophosphatases and vacuolar ATPases, thereby decreasing the lethal effects of Na^+ inside the cytosol (Blumwald *et al.,* 2000; Niu *et al.,* 1995; Munns and Tester, 2008).

We previously reported transcriptome analysis and studied the global gene expression of the salt-tolerant wheat variety under salt stress (Goyal *et al*., 2016). The results showed up-regulation of *NHX1* gene under salt stress. For this study, we have cloned and performed *in-silico* analysis of the vacuolar Na^+/H^+ antiporter gene from salt tolerant bread wheat cultivar Kharchia Local.

MATERIAL AND METHODS

Plant Material

Kharchia Local seeds (*T. aestivum*, 2n=42, AABBDD) were taken from Central Soil Salinity Research Institute (CSSRI), Karnal, India. The present investigations were undertaken in hydroponics system with Kharchia local (tolerant to salinity). Seedlings of Kharchia Local were raised from seeds in hydroponics containing Hoagland solution under light/ dark cycle of 16/8 h at 25 ºC, at National Phytotron Facility (IARI Campus, New Delhi). The seven days old seedlings growing in hydroponics system were imposed with 250mM salt (NaCl) stress for 24 h. The root samples were taken and processed to total RNA isolation.

RNA Isolation and Reverse Transcription (RT)

Total RNA was isolated from salt treated sample using RaFlex™ Total RNA Isolation Kit (GeNei™, Bangalore, India) as per the manufacturer's protocol. The quality of the total RNA was checked by running the samples on 1.2% Formaldehyde Agarose Gel electrophoresis under denaturing conditions. The cDNA was prepared from 2µg of RNA using Superscript® III First Strand Synthesis Kit (Invitrogen) as per the manufacturer's protocol.

Isolation of *vacuolar Na^+/H^+ antiporter* gene

NHX1 full-length cDNA sequences obtained from the NCBI database with accession number AY040245.1. The PCR was performed using cDNA as template using Platinum® Pfx DNA Polymerase (Invitrogen, USA), 10pM each of NHXF (5′ ATGGGCTGCGTGTTGTCATC 3′) and NHXR (5′ TTATTTGCTGATTCCACTGT 3′) primers. PCR condition were 94°C 3min, 1 cycle; 94°C, 1min; 58°C, 30sec and 72°C, 2min 30 cycles and last 72°C, 10min, 1 cycle. The PCR product was purified and cloned into pGEM®T – Easy Vector (Promega, USA) for sequencing.

***In-silico* analysis**

The NCBI database was used as a source of protein and nucleotide sequences. TMpred software was used for the prediction of transmembrane domains and Clustwal W for sequence alignment. Secondary structure prediction was carried out by PSIPRED protein structure prediction tool. Phosphorylation motifs were predicted by Expasy ScanProsite (http://www.expasy.ch/tools/). Hydropathy plot at http://www.virginia.edu, using a window of size 19 was used to find TM domain and also by the method described by Kyte and Doolittle (1982). MEGA 3.1 software was used for phylogenetic analysis. The phylogenetic tree was generated based on minimal evolution criterion using the neighbor-joining method with 1000 times of bootstrap.

RESULTS

Soil salinity is among one of the abiotic stresses which affect growth and productivity of crop plants throughout the world. Moreover, the problem of salinity is increasing day by day which is rendering more areas of arable lands unproductive. To overcome this, there are two primary lines of action; one being the recovery of salt-affected soils by chemical amendments and other is to develop salt tolerant plant varieties which can grow on salinity affected soils (Epstein, 1980; Shannon and Grieve, 1999; Ashraf and McNeilly, 2004). Out of these, the latter approach seems to be economically feasible and more effective and requires us to understand the basis of salt tolerance.

We have previously performed transcriptome sequencing from *T. aestivum* cv. Kharchia Local and found that *NHX1* gene was up-regulated both in RNA-seq and qRT-PCR analysis (Goyal *et al.,* 2016). The *NHX1* gene was cloned and submitted to GenBank viz. accession number KT273926. Full length *NHX1* gene was obtained using gene specific primer by PCR using cDNA as template encompassing the ORF of 1,641bp (Fig. 1). The DNA from the excised band was eluted, purified and sequenced. The obtained sequence of the amplicon was subjected to NCBI nucleotide BLAST search. The sequence showed homology to known *NHX1* sequences available in the database. This confirmed that the amplified fragment from wheat is *NHX* gene.

The full length cDNA of Na^+/H^+ antiporter gene from Kharchia Local named *NHX* (accession number: KT273926) has theoretical pI and Mw of 8.13 and

59.7 kD, respectively (Fig. 2). BlastP and CDD (Conserved Domain Database) analysis suggested the presence of a conserved NhaP (Na^+/H^+ antiporter) domain containing a N-terminal transmembrane region and a C-terminal cytoplasmic tail along the peptide chain of *NHX*.

The BLAST hits were manually screened for the sequence alignment of *NHX* with other NHX antiporters. The comparison of Na^+/H^+ antiporters across plant species revealed a high level of conservation in protein structure (Fig. 3). The membrane-spanning regions are well conserved in eukaryotic Na^+/H^+ antiporters. Within these regions, *NHX* shares high degree of similarity with other vacuolar Na^+/H^+ antiporters such as *ZmNHX1*, *SbNHX1*, *Aegilops tauschii NHX1* and *HvNHX1*. The phylogenetic analysis showed that NHX belongs to the vacuolar Na^+/H^+ antiporter proteins and is more closely related to *TaNHX1* (Fig. 4).

The membrane-spanning regions and their orientation were predicted by the method of TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) (Fig. 5A, Table 1). The hydropathy plot shows NHX has 11 strong transmembrane helices (Fig. 5A), indicating its potential helix regions. There were 11 inside to outside helices and 12 outside to inside helices. The protein sequence 85-FFIYLLPPII-94 in NHX protein is highly conserved. This sequence was identified as an amiloride binding site in mammals which inhibits eukaryotic Na^+/H^+ antiporter. It was predicted in 3rd domain and cation binding sites were predicted in 5th and 6th domain. This property confirmed that *NHX* is a vacuolar Na^+/H^+ antiporter. Two dimensional secondary structure of NHX protein sequence predicted using TMRPres2D (Fig. 5B). The 3D structure of the protein predicted using Swiss model is shown in Fig. 5C.

The analysis of NHX showed three potential N-glycosylation sites, eleven N-myristoylation sites, five Protein kinase C phosphorylation sites, one cAMP- and cGMP-dependent protein kinase phosphorylation site and eleven Casein kinase II phosphorylation sites (Table 2). The secondary structure was predicted by PSIPRED Protein Structure Prediction Server (http://www.combio.dundee.ac.uk/) and it revealed a structure of 22 coils, 17 alpha-helices and 7 beta-strands (Fig. 4.6).

DISCUSSION

The highest sequence homology among NHXs takes place at N-terminal which forms the membrane pore, while C-terminal domains are different. In mammals, NHX comprises amiloride sequence which inhibits Na^+/H^+ exchange (Counillon and Pouyssegur 2000). Hence, NHX proteins can be identified by the presence of consensus sequence coding for ameloride (sodium) binding site (FFXXLLPPI, where X may be any amino acid). The vacuolar NHX members are divided into two classes i.e. Class-I and Class-II which share only 20-25% identity (Pardo *et al.,* 2006). Recently, genes encoding vacuolar Na^+/H^+ antiporters have been isolated from several plant species, including *Arabidopsis thaliana* (Apse et al., 1999; Gaxiola et al., 1999), *Oryza sativa* (Fukuda et al., 1999), *Atriplex gmelini* (Hamada et al., 2001), *Mesembryanthemum crystallium* (Chauhan et al., 2000), *Suaeda salsa* (Ma et al., 2004), *Leptochloa fusca* (Rauf et al., 2014), *Nitraria sibirica* (Wang et al., 2016), tomato (Zhang and Blumwald 2001), *Brassica napus* (Zhang et al., 2001), *Triticum*

aestivum (Xue et al., 2004) and *Brassica juncea* (Rajagopal et al., 2007).

The cDNA of vacuolar *Na^+/H^+ antiporter* gene from Kharchia Local named *NHX* (accession number: KT273926) is 1,641 bp, encoding a polypeptide of 546 amino acid residues with an estimated molecular mass of 59.7 kDa and isoelectric point of 8.13. Amino acids blast (BLAST P) showed the presence of a conserved *NhaP* (Na^+/H^+ antiporter) domain. Topological analysis and structure-function studies have so far only been performed with the *AtNHX1* protein. Eleven strong transmembrane domains were predicted using TMpred analysis in *NHX* which is in accordance with the results in other plants like *Salicornia europaea*, *S. brachiata*, *Suaeda japonica* and *Mesembryanthemum crystallinum*. Whereas, *Atriplex gmelini, Nitraria sibirica, Leptochloa fusca, S. bigelovii, Chenopodium glaucum* and *Kalidium foliate* have twelve transmembrane domains. The dissimilarity in the number of transmembrane domain is interesting, thereby suggesting that these regions may be important for antiporter function. It is reported that glycosylation plays an important role in biosynthetic processing of transporter proteins in yeast. *ScNHX1* isolated from yeast is reported to be a glycoprotein (Wells and Rao, 2001). The secondary structure was predicted using PSIPRED protein structure prediction server, which revealed that a total of 22 coils, 17 alpha-helices and 7 beta-strands were present in *NHX*. To investigate the molecular evolution and phylogenetic relationships among Na^+/H^+ antiporters in plants, Na^+/H^+ antiporter protein sequences from both halophytes and glycophytes were aligned and phylogenetic tree was constructed. In the present study, we observed three putative glycosylation sites along with eleven myristoylation sites. There were seventeen protein kinase phosphorylation sites, involving, casein kinase II (11 sites) and protein kinase C (5 sites) and one cGMP/cAMP dependent protein kinase phosphorylation. The protein sequence of 85-FFIYLLPPII-94 in *NHX* is extremely conserved. This region was identified as the binding site of amiloride in mammals, which inhibits the activity of eukaryotic Na^+/H^+ antiporter. These features confirm that the *NHX* isolated from Kharchia Local is a vacuolar type *Na^+/H^+ antiporter*. Kharchia *NHX* gets clustered within the Class-I type of NHX proteins, while it was showing distance from *OsNHX1*, *SbNHX1* and *ZmNHX1*. This indicates that *NHX* gene belongs to the family of plant vacuolar protein which functions as Na^+/H^+ antiporter.

In conclusion, it can be explained that Kharchia Local has efficient mechanisms to sequester Na^+ into vacuoles. Kharchia Local is a salt tolerant bread wheat land race and can be grown in high salt and also accumulate salt in the root tissue therefore, it is reasonable to isolate antiporter gene from this plant and validate it by *in-silico* analysis. *NHX* gene isolated in this study belonged to Na^+/H^+ antiporter Class I gene family which is located in vacuole. In future, *NHX* gene can be a potential candidate gene for enhancing abiotic stress tolerance, however more experiments are required for defining its role in adaptive response.

Tables Legends:

Table 1: The amino acid sequence of *NHX* is a Membrane bound Protein which has 11 strong transmembrane helices.

No.	N terminal	Transmembrane region	C terminal	Length
1	23	IVAINIFIALLCGCIVFGHLLEG	45	23
2	57	VLGLITGGVILICTKGVNSRILI	79	23
3	84	IFFIYLLPPIIFNAGFQV	101	18
4	114	ILFGAAGTLISFVIITFGAMGLF	136	23
5	218	FLYLFFTSTVLGVAAGLLSAYII	240	23
6	267	LSMLLDLSGILTVFFCGIVMSHY	289	23
7	304	HTFATLSFIAEIFLFLYV	321	18
8	342	IALSAVILGLVMVGRAAFVFPLS	364	23
9	384	VIIWWAGLMRGAVSIALAYN	403	20
10	415	VNAVMITSTIIVVLFSTMVFGLL	437	23
11	509	RPMFGGRGFVPFVPG	523	15

Table 2: The PROSITE patterns for NHX: positions of important sites

Position	Prediction site/patterns	Position	Prediction site/patterns
2 - 7	MYRISTYL *N-myristoylation site*	18 – 21	CK2_PHOSPHO_SITE *Casein kinase II phosphorylation site*
14 - 19		137 – 140	
59 - 64		157 – 160	
117 - 122		250 – 253	
120 - 125		370 – 373	
153 - 158		452 – 455	
229 - 234		460 – 463	
233 – 238		472 – 475	
283 – 288		524 – 527	
390 – 395		538 – 541	
534 – 539		540 – 543	
50 - 53	ASN_GLYCOSYLATION *N-glycosylation site*	371 – 374	CAMP_PHOSPHO_SITE *cAMP- and cGMP-dependent protein kinase phosphorylation site*
293 - 296			
368 - 371			
250 - 252	PKC_PHOSPHO_SITE *Protein kinase C phosphorylation site*		
297 - 299			
301 - 303			
337 - 339			
370 - 372			

Figures Legends:

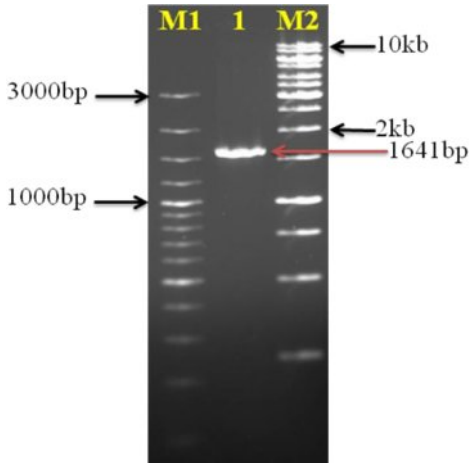

Figure 1: Amplification of full length *NHX* gene. M1: 100bp plus DNA ladder; Lane 1: Amplified PCR product; M2: 1kb DNA ladder

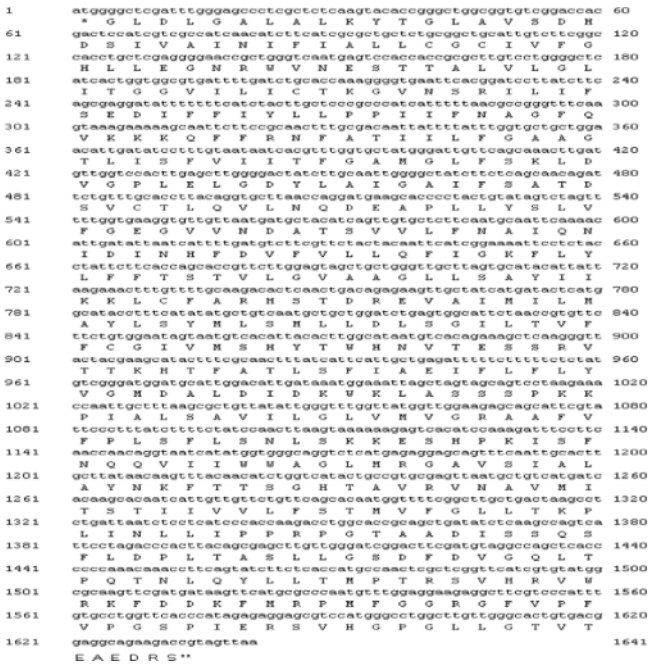

Figure 2: Nucleotide and deduced amino acid sequence of the *NHX* (GenBank accession number, KT273926). Amino acid sequence is represented by a single-letter code under each codon. Putative start and stop codons are indicated by * and **, respectively.

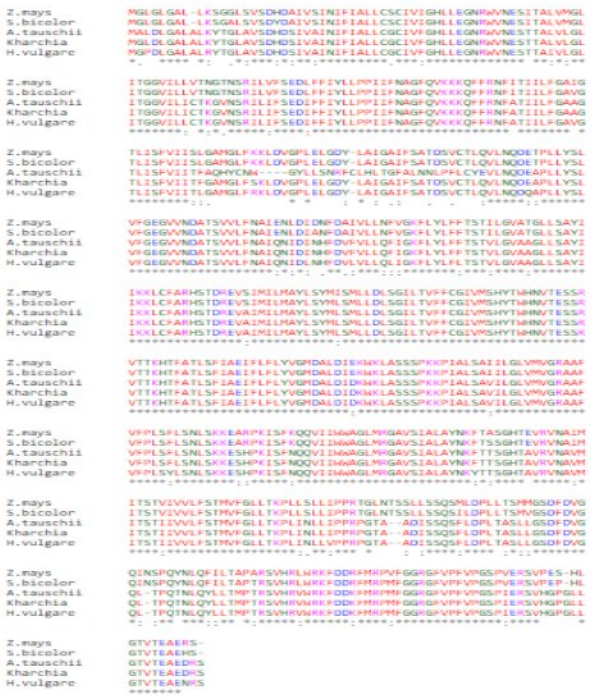

Figure 3: Sequence alignment (ClustalW2; http://www.ebi.ac.uk/) of the NHX deduced amino acids with the NHX sequences from other plants: gb| KXG21297.1| *Sorghum bicolor*, gb|AAO91943.2| *Hordeum vulgare*, gb| NP_001169551.1 |*Zea mays*, gb|EMT25578.1| *Aegilops tauschii*; where, gap are represented as dashes; asterisks, colons and dots indicate identical amino acid residues, conserved substitutions, and semi-conserved substitutions, respectively, in all sequences used in the alignment.

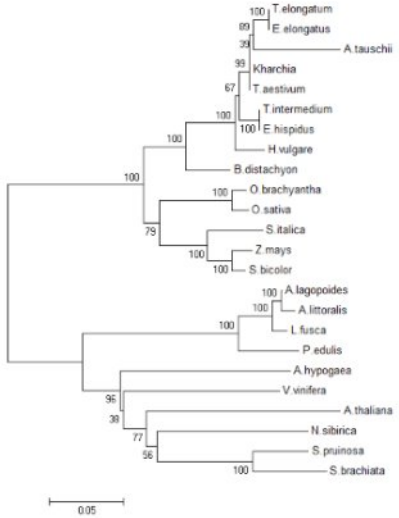

Figure 4: Phylogenetic tree of Na^+/H^+ antiporter gene from different plants.

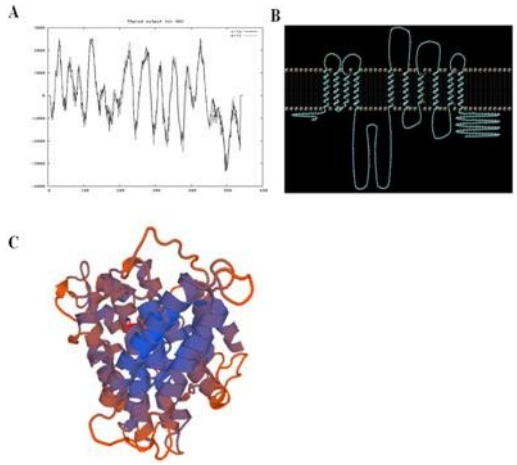

Figure 5: Structural analysis of *NHX*: (A), the hydrophobicity values were calculated by the program TMpred available at http://www.ch.embnet.org/software/TMPRED-form.html; (B) Proposed topological model of NHX. Coiled portion indicates the 11 transmembrane domains. Third domain contain amiloride binding site while cation binding domains is present in 5th and 6th domain; (C) 3-dimensional model of NHX protein.

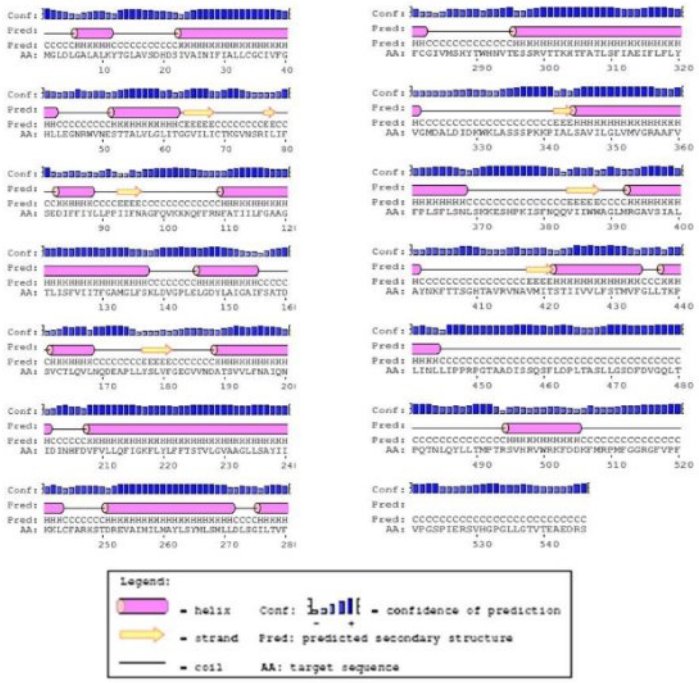

Figure 6: Predicted secondary structure for Na^+/H^+ antiporter from *T. aestivum*

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