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Identification, structural characterization and phylogenetic analysis of Na +/H + antiporter (*NHX*) genes across the fava bean's (*Vicia faba* L.) genome

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Abstract

Plant Na +/H + antiporter (*NHX*) genes enhance salt tolerance by sequestering Na+ into vacuoles or effluxing it across the plasma membrane. This study aimed to identify and characterize *NHX* genes in fava bean. Seven *VfNHX* genes were explored and named as *VfNHX*1-*VfNHX*7 on the basis of their order in the phylogenetic tree. The protein length, isoelectric points and GRAVY of all 7 proteins ranged from 529-1155, 5.2-8.9 and 0.142-0.637, respectively. The vacuole was predicted as a major residence for all 7 *NHX* proteins. Amiloride was conserved in the third motif of all members except *VfNHX*7. Exons' number in *VfNHX* genes ranged from 14-23. Segmental duplication contributed 28.5% to the *VfNHX* gene family expansion. Ka/Ks ratio of paralogs revealed that they were all under purifying selection. Salt-associated cis-acting elements, including GT1-motif, TGACG-motif, ABRE, G-box, MBS, and TGA, were detected in *VfNHX* promoters. This study provides a base for functional validation of *VfNHX* genes and their contribution towards salt tolerance.

Keywords: Fava bean, *NHX*, salt stress, genome-wide analysis

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Introduction

Salt stress is one of the major abiotic stresses that affects plant growth and development (Deinlein et al., 2014). Excessive salt concentration has drastically reduced the area under cultivation of important crops, squeezing the existing farmland and irrigated land by 6% and 20%, respectively (Munns & Tester, 2008). Under salt stress, higher accumulation of Na+ and Cl ions in the chloroplasts decreases the chlorophyll content, thus inhibiting photosynthesis (Chutipaijit et al., 2011). Poor seed germination, growth and flowering are some of the early symptoms of salinity (Chandna et al., 2013). The plant tissues become toxic due to elevated Na+ concentrations for a prolonged period, affecting intracellular K^+ homeostasis (Shabala & Cuin, 2008). A critical Na^+/K^+ ratio in the cells is mandatory for the efficient functioning of cytosolic enzymes (Mahajan et al., 2008).

The Na^+/H^+ antiporters (*NHX*) are proteins embedded in the vacuolar membrane that exchange sodium (Na^+) or sometimes potassium ions (K^+) for protons (H^+) across cellular membranes (Brett et al., 2005). The genes encoding these transport proteins constitute a family known as the *NHX* family that plays a key role in maintaining ion homeostasis, pH regulation and conferring resistance to salt stress in plants (Van Zelm et al., 2020). Two H^+ pumps, including vacuolar H^+-ATPases (V-ATPase) and H^+-pyrophosphatases (H^+-PPase), create a proton gradient across the tonoplast by actively pumping H^+ ions from the cytosol into the vacuole at the expense of energy derived from ATP or pyrophosphate (PPi) (Aharon et al., 2003). This H^+ gradient across membranes is essential to power secondary transporters like *NHX*. Under salt stress, *NHX* exchanges cytosolic Na^+ with vacuolar H^+ across the tonoplast. This helps sequester excess Na^+ into the vacuole, protecting the cytoplasm from Na^+ toxicity and contributing to salt tolerance in plants (Bassil et al., 2011). Potassium (K^+) is a vital macronutrient for plants and is involved in enzyme activation, osmoregulation, and turgor pressure maintenance (Yamaguchi et al., 2003). H^+/K^+ exchangers (particularly in vacuoles) allow K^+ to be sequestered into vacuoles during surplus and released when needed, keeping cytosolic K^+ levels optimal. Eight *NHX* genes were first identified across the *Arabidopsis* genome with 3 subgroups (Brett et al., 2005).

NHX genes are associated with many biochemical processes like coping with salt stress, regulating cell division, vesicle transportation across the membrane and maintaining a relatively constant pH. Irregular cell division in Arabidopsis has been witnessed upon inactivating *AtNHX5* and *AtNHX6,* which has led to poor root and embryo development (Dragwidge et al., 2019). Similarly, *OsNHX1,* 2, 3 and 5 have exhibited elevated expression upon exposure to salt and abscisic acid stress (Fukuda et al., 2011). In other studies, rye grass modified with the rice *OsNHX1* gene was able to withstand the detrimental effects of salt stress. Likewise, genetically modified B. napus plants grew well under high salt concentration (200 mM salt. Genetically manipulated rice with the *AgNHX1* gene from *Atriplex gmelinii* exhibited better growth compared to the wild type at elevated salt stress. Overexpression of At*NHX*1 and Ss*NHX*1 has significantly contributed to salt tolerance of tomato (Manik et al., 2015) and *M. sativa* (Wu et al., 2019a)

Fava bean is an important leguminous crop that belongs to the family Fabaceae.

This crop is grown on 2.6 million hectares with an annual production of 5.4 million tonnes (FAO, 2022). Fava bean is a nutritionally rich crop with high content of protein (27–40%) and carbohydrate (50–60%) (Kumar et al., 2015). It is also an excellent source of L-DOPA, a precursor used in treating Parkinson's disease (Singh et al., 2013). Additionally, fava bean contributes to sustainable agriculture by increasing crop yields through their ability to fix nitrogen (Barton et al., 2014). Fava beans can tolerate a considerable level (up to ~5.5–6 dS/m) of saline water (Katerji et al., 2005). Comparative studies have revealed a higher salinity threshold for fava bean compared to chickpea and lentil (Arslan, 2016). Fava bean has exhibited a better salt tolerance at the germination stage among the legume crops (El-Kholy et al., 2021). Similarly, different varieties of fava bean have shown a variable level of salt tolerance upon exposure to salt stress ranging from 25–100 mM NaCl (Abdel Latef et al., 2014). Keeping in view the salt-tolerant nature of fava bean, the present study was conducted with the assumption that fava bean is a potential repository of *NHX* genes.

Material and Methods

Identification of *NHX* genes across the faba bean (*Vicia faba*) genome

At*NHX*1, 5, 7 and 8 sequences of *Arabidopsis thaliana* were isolated from Phytozome v.13 (https://phytozome-next.jgi.doe.gov) and blasted against the fava bean proteome available in Phytozome v.13 for extracting *VfNHX* transcripts. Short sequences, truncated sequences/hits were removed.

Physico-chemical characterization of *VfNHX*s proteins

Protein length and CDS of *VfNHX*s were taken from phytozome. Similarly, other features of *VfNHX* proteins, including PI, molecular weight, and GRAVY, were obtained from Expasy Protparam (https://web.expasy.org/protparam)

Conserved domain and interspecific phylogeny of *VfNHX* Proteins

Two files, including renamed protein and hitdata files, mandatory for finding conserved domains, were obtained from TBtool.v1.09854 (Chen et al., 2018) and conserved domain database (Marchler-Bauer et al., 2015), respectively. The files were subjected to TBtool.v1.09854 for generating domain architecture (Chen et al., 2018). *VfNHX* sequences were inserted in MEGA 12 (Kumar et al., 2016) for exploring phylogeny among *Vicia faba, Arabidopsis thaliana, Glycine max*, *Medicago truncatula, Oryza sativa and Solanum lycopersicum* based on maximum likelihood (ML, 1,000 bootstrap replicates).

Conserved motif and structure of *VfNHX* genes

Conserved motifs were visualized in *VfNHX* proteins using Multiple Em for Motif Elicitation (http://memesuite.org). CDS and genomic sequences of *VfNHX* genes were extracted from Pytozome and subjected to Gene Structure Display Server 2.0 (http://gsds.gao-lab.org/) for visualizing the gene structure.

Mapping, duplication and synteny analyses of *VfNHX* genes

The data, like chromosome number, position of *VfNHX* genes and chromosome length, were inserted in PhenoGram Plot for mapping *VfNHX* genes on chromosomes (http://visualization.ritchielab.psu.edu/phenogram s/plot). The values of non-synonymous (Ka) and synonymous (Ks) substitutions, of all duplicated gene pairs, were obtained from TBTool (Chen et al., 2018). For synteny analysis, Fasta and GFF3 files of V. faba, A. thaliana, M. truncatula, G. max and S. lycopersicum plants were downloaded from Phytozome

v.13 databases and analyzed through the step MCScanX function in the TBtools program (Wang et al., 2012). Results were visualized via the Dual Synteny Plot for the MCScanX function in TBtools software (Chen et al., 2020).

Promoter Region Analysis

A sequence of 1500bp upstream was taken from the Phytozome of each gene. PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/) was used for exploring *cis*-regulatory elements in *VfNHX* genes (Lescot et al., 2002).

Results

Genome-wide identification of *NHX* genes in fava bean and their physicochemical characterization

Seven full-length genes were left after removal of truncated sequences/genes and were designated as *VfNHX*1-*VfNHX*7. A highly conserved Na^+/H^+ exchanger domain was found in all *VfNHX* proteins (Figure 1). *VfNHX*7 was recorded as the largest gene with 3468 bp CDS, 1145 amino acids protein length (PL) and 128.206 kDa protein molecular weight (PMW), while *VfNHX*3 was found as the smallest one with only 1590 bp CDS, 529 PI and 58.236 kDa PMW (Table 1). Aliphatic index of all *VfNHX* ranged from 99 to 117, showing high thermal stability. Phylogenetic tree revealed vacoule as the major predicted site for *NHX* proteins (5), followed by 1 each in plasma membrane and endosomal region (Figure 1). Isoelectric points and GRAVY of *VfNHX* were found in the range of 5.2–8.9 and 0.142–0.637, respectively (Table 1)

Figure 1: (Annexure A) The yellow rectangular boxes represent the Na^+/H^+ exchanger domain.

Table 1: (Annexure B) Physico-chemical characterization of VfNHX family

Inter-specific phylogeny of *NHX* Proteins

Forty-six *NHX* proteins across different species, including fava bean (*Vicia faba*), *Arabidopsis thaliana, Glycine max*, *Medicago truncatula, Oryza sativa and Solanum lycopersicum*, were clustered into 3 groups (*NHX*1, *NHX*2 and *NHX*3) based on the predicted location of NHX protein in organelles. Vacuole-localized proteins belong to the *NHX*1 group, endosomal-localized proteins belong to the *NHX*2 group, and Plasma membrane-localized proteins belong to the *NHX*3 group. The *NHX*1 group comprises 30 genes, while *NHX*2 and *NHX*3 consist of 9 genes and 7 genes, respectively. Along with genes from other plants, 5, 1 and 1 genes from fava bean were recorded in *NHX*1, *NHX*2 and *NHX*3 groups, respectively (Figure 2).

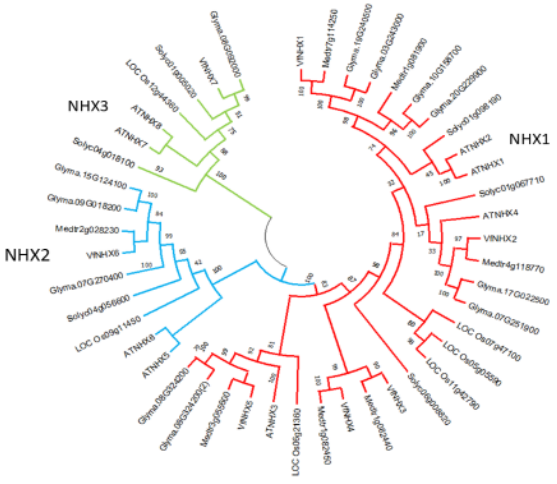

Figure 2: Comparative analysis of NHX proteins across different plant species. The phylogenetic tree was constructed through MEGA-12 using the Maximum Likelihood (ML) method. Different groups (NHX1, 2 and 3) are highlighted with different colours.

Chromosomal mapping, duplications and Ka/Ks ratio of *NHX* genes

Seven genes were mapped on 6 chromosomes. Two *VfNHX* genes (maximum) were placed on chromosome number 4, while only 1 *VfNHX* (minimum) was found on the rest of the chromosome. Segmental duplication was witnessed in all

the paralogs (2) (Figure 3). The detection of a less than 1 Ka/Ks ratio for both paralogs revealed its evolution under purifying selection (Table 2).

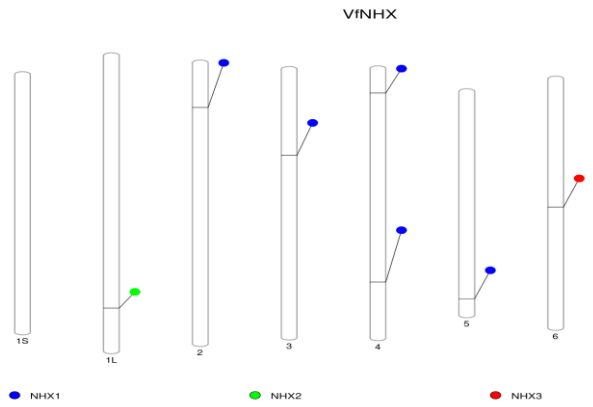

Figure 3: Chromosomal locations and duplication patterns of VfNHX genes across the fava bean genome.

The red circle represents the members of the NHX1 group, the green of the NHX2 and the blue of the NHX3 group. The chromosome number is mentioned in each bar. Each line connects to two genes that represent paralogs.

Table 2: The Ka/Ks ratio for paralog pairs

Seq_1	Seq_2	Ka	Ks	Ka_Ks
VfNHX1	VfNHX2	0.203768	1.415166	0.143989
VfNHX3	VfNHX4	0.114173	0.410067	0.278426

Intra-specific phylogenetic tree, Gene structures and motifs

Based on phylogenetic analysis, *VfNHX* were clustered into 3 groups named as *NHX*1, *NHX*2 and *NHX*3. *NHX*1 consist of *VfNHX*1-*VfNHX*5, *NHX*2 has *VfNHX*6 and *NHX*3 has *VfNHX*7. The phylogenetic tree of fava bean revealed that *VfNHX*1/*VfNHX*2 and *VfNHX*3/*VfNHX*4 are closely related to each other (Figure 4a). The *VfNHX* gene structures are arranged to match their evolutionary tree. *NHX*1 group members detected with 13 to 14 exons, *NHX*2 and *NHX*3 have 20 and 22 exons, respectively. The number of introns in all 7 *VfNHX* genes ranged from 12 to 21 (Figure 4b). Conserved motifs in *VfNHX* proteins have been arranged in line with their evolutionary tree. The number of amino acids in motifs of *VfNHX* proteins ranged from 6–50. Motif 1, 2, 3, 4, 7, and 9 were marked as the longest ones with 50 amino acids, while the minimum amino acids was reported in motif 12. *NHX*1 group detected with a range of 10–11 motifs, and 5 motifs were detected in each member of *NHX*2 (motif1, 3, 11, 13 and 15) and *NHX*3 (motif11, 12, 13, 14, and 15) groups (Figure 4c). Highly conserved amiloride binding site [FFIYLLPPI] was found in all *VfNHX* proteins except *VfNHX*7 (Figure 4d).

Figure 4: (Annexure C) (**a**) Intra-specific phylogenetic analyses, (**b**) Gene structure analysis, (**c**) conserved motifs, (d) Amiloride binding site [FFIYLLPPI].

Synteny analysis

In synteny between V. faba and A. thaliana, *VfNHX*1, *VfNHX*5, and *VfNHX*7 revealed collinear regions at chromosomes 2, 5, and 6 in V. faba and chromosomes 1, 2, 3 and 5 in A. thaliana (Figure 5a). Similarly, *VfNHX*1, *VfNHX*2, *VfNHX*5, *VfNHX*6 and *VfNHX*7 exhibited collinear regions on chromosomes 1, 2, 4, 5 and 6 in V. fava and on chromosome 3, 7, 8, 9, 10, 15, 17, 19 and 20 in the genome of G. max. *VfNHX*1 revealed 4 copies on chromosomes 3, 10, 19, and 20 in G. max (Figure 5b). Likewise, *VfNHX*1, *VfNHX*2, *VfNHX*5 and *VfNHX*6 showed collinear regions at chromosomes 2, 4, 5 and 6 in *fava bean* and chromosome 1, 2, 3, 4 and 7 in M. truncatula. Two copies of *VfNHX*1 were found in *M. truncatula*, one on chromosome 1 and the other on chromosome 7 (Figure 5c). The synteny between fava bean and *S. lycopersicum* presented that *VfNHX*1, *VfNHX*2, *VfNHX*5 and *VfNHX*7 have collinear regions on chromosomes 2, 4, 5, and 6 in *fava bean* and on chromosomes 1 and 10 in the genome of *S. lycopersicum* (Figure 5d).

Figure 5: (Annexure E) Collinearity analysis of fava bean with (**A**) A. thaliana, (**B**) G. max, (**C**) M. truncatula and (**D**) S. lycopersicum.

In synteny, the upper (orange) and lower (green) boxes/lines represent the chromosome number of both species. Red lines connect NHX genes in both species.

Promoter Analysis

Promoter sequences of *VfNHX* genes were examined for cis-regulatory elements using PlantCARE software. One hundred thirty-four (134) cis-acting regulatory elements were explored in the 1500bp upstream region of 7 *VfNHX* genes (Figure 6). Cis-regulatory elements were dominated by (a) light-responsive elements (67), followed by (b) hormone-responsive elements (44), (c) environmental stress (15), and (d) developmental elements (5). The light-responsive cis-elements include G-box, Box 4, GT1-motif, TCCC-motif, and TCT-motif. The ABRE (ABA response elements) constituted a major part of hormone-responsive elements present in *VfNHX*1, *VfNHX*2, *VfNHX*3 and *VfNHX*5. Similarly, ARE and LTR were detected as the prominent environmental stress-related elements, while GCN4_motif element was witnessed as the main developmental-related element. Various cis-acting elements in the promoter region of each fava bean gene (*VfNHX*) have been shown in 6e. Salt-associated cis-acting elements, including GT1-motif, TGACG-motif, ABRE, G-box, MBS, and TGA, were detected in *VfNHX* promoters.

Figure (Annexure F)

Figure 6: **(Annexure G)** Cis-regulatory elements in the 1500 bp upstream promoter region of VfNHX genes. Predicted cis-acting elements responsive to (a) Light, (b) Hormone, (c) Environmental stresses, and (d) Development, while various colored boxes in (**e**) represent different types of cis-acting elements in the promoter of each VfNHX gene.

Discussions

The identification of 7 *VfNHX* genes and detection of highly conserved Na^+/H^+ exchanger domain, in the present study, is in line with previous studies of S. bicolor (Kumari et al., 2018).

Similarly, greater than 0 GRAVY, as recorded for all *VfNHX*, exhibited a hydrophobic nature of these proteins. These results strengthened earlier reports of Bhattacharya et al (2018). A higher range of aliphatic index (99 –117) revealed that a large portion of *VfNHX* proteins is covered by hydrophobic side chains, contributing to their stability to heat stress. The range of exons (13–22) in *VfNHX* genes was similar to those reported in Sl*NHX* genes (Cavusoglu et al., 2023) and reported by Shen et al (2023) in Cmo*NHX*.

The number of motifs in VfNHX proteins ranged from 5 to 11. The presence of a highly conserved amiloride binding site [FFIYLLPPI] in 6 members of the *VfNHX* family (except *VfNHX*7) is a characteristic feature of membrane-bound *NHX* transporter in plants (Wu et al., 2011). The amiloride impairs the function of the Na^+/H^+ antiporter, resulting in the accumulation of Na^+ in the cytoplasm. This condition is beneficial only when there is a scarcity of sodium in the soil. The *VfNHX*7 lacking amiloride binding reduces the cytotoxicity by sequestering Na+ into vacuoles. Such similarity in motif configuration points toward a resemblance in the functions of *VfNHX* proteins. *Medicago truncatula* and tomato have shown such similarity in motif configuration (Sandhu et al., 2018; Cavusoglu et al., 2023).

The location of *NHX* genes inside plant cells helps plants withstand the detrimental effects of stress. The researchers can enhance the resilience of

crops against stress by relocating these genes. For example, in *Arabidopsis thaliana*, the *NHX*1 gene is found in the tonoplast. It helps move salt (Na^+) ions into the vacuole for safe storage, which supports plant growth and helps the plant survive under stressful conditions like high salt levels. *VfNHX1, 2, 3, 4 and 5 were* predicted to localize in the vacuole. (Apse et al., 1999; Zhang et al., 2012). In fava bean, *VfNHX7* was found in the plasma membrane, which revealed its possible involvement in maintaining pH and salt levels at the cell surface. They likely help swap sodium and hydrogen ions, which can affect important cell functions like controlling cell size, keeping pH balanced, and sending signals inside the cell (Wang et al., 2015).

The phylogeny of *VfNHX* proteins revealed a closest evolutionary relationship with *M. truncatula*, followed by G. max, sharing a common ancestry with fava bean. Closest evolutionary relationship exhibited on account of higher sequential homology in protein sequences of both the crops.

Segmental duplication was witnessed in *VfNHX*1/*VfNHX*2 and *VfNHX*3/*VfNHX*4 paralogs, which contributed significantly (28.5%) to the expansion of the *VfNHX* gene family. The ratio of synonymous and non-synonymous substitution (Ka/Ks) reveals the force causing gene modification during the course of evolution. Less than 1, equal to 1 and greater than 1Ka/Ks values represent purifying, neutral and positive selections, respectively (Wang et al., 2011). Less than 1 Ka/Ks of *VfNHX* paralogs (Ka/Ks < 1) exhibits purifying selection as an evolutionary force for gene modification.

Collinearity analysis compared *NHX* genes in fava bean with those in *Arabidopsis*, *Medicago truncatula*, soybean, and tomato. It showed that some gene regions are conserved. The strongest match was detected between fava bean and soybean, followed by *M. truncatula*, Arabidopsis and tomato. The collinear chromosomal segments are believed to have the highest degree of conserved gene order and orientation pointing towards their common ancestral chromosome. The genes in collinear regions are less likely to have undergone structural rearrangements or losses, indicating their functional conservation. The strongest match between fava bean and soybean predicts the most likely ancestral relationship (true orthologs) and conserved functional collinearity in their genomes. These results are in line with earlier results witnessed in chickpea (Parveen et al., 2023).

Cis elements act as main players in regulating transcription, controlling response to growth hormones and stresses (Ding et al., 2018). Different cis-acting elements adopt different modes for turning genes on or off in plants. Notably, the ABRE element interacts with plant hormone ABA (abscisic acid) (Wang and Huang, 2019). Similarly, various elements, including G-box, AT-rich, GT1-motif, and I-box, are stimulated by light (Gilmartin et al., 1992). Büyük et al (2016) have reported the role of GT1 and TGACG motifs in coping with salt stress. The involvement of ABRE, G-box, MBS, and TGA-elements was also highlighted during salt stress. These elements play a role in helping plants respond and adapt to salty conditions (Saeediazar et al., 2014). ABRE, TGACG-motif, and TGA-element are linked to hormone responses, while GT1-motif and G-box respond to light, and MBS is related to environmental stress. The detection of these elements in the promoter region of *VfNHX* genes strengthened earlier reports of Parveen et al (2023) and

Cavusoglu et al (2023) in chickpea and tomato, respectively. We predict that *VfNHX1* and *VfNHX5*, due to their promoter elements and vacuolar localization, are the primary candidates for mediating salt tolerance in fava bean.

Conclusions

This study was designed to reveal the *VfNHX* that highly responds to salt stress. The physico-chemical parameters were similar to those reported for the *NHX* gene family in other plants. Amiloride motifs were conserved in all *VfNHX* genes except *VfNHX*7. Segmental duplications contributed 28.5% to the *VfNHX* gene family expansion. Paralogs were under purifying selection. Promoter region of *VfNHX* genes enriched with cis-acting elements including GT1-motif, TGACG-motif, ABRE, G-box, MBS, and TGA-element. The putative salt-responsive genes, as explored in the current study based on their promoter analysis, could be the potential elements for enhancing the tolerance of fava bean against salt stress. After functional validation. In fact, these findings identify prime candidate genes (*VfNHX1-6*) for further functional characterization via overexpression to confirm their role in salt tolerance."

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Competing Interest

The authors have no relevant financial or non-financial interests to disclose.

Author contributions

ZS and AU designed the research. AU extracted the data under the supervision of ZS and SUR. ZS and AU prepared and finalized the draft. ZK helped in analysis and manuscript writing, while IS assisted in figure setting. All the authors approved for submission of the manuscript.

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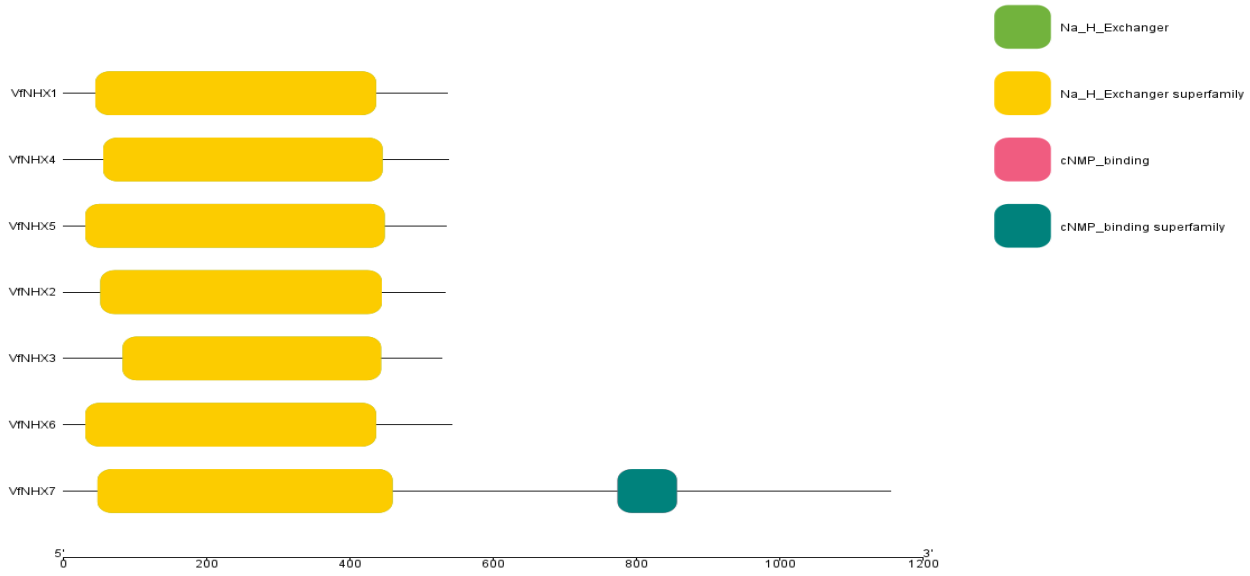
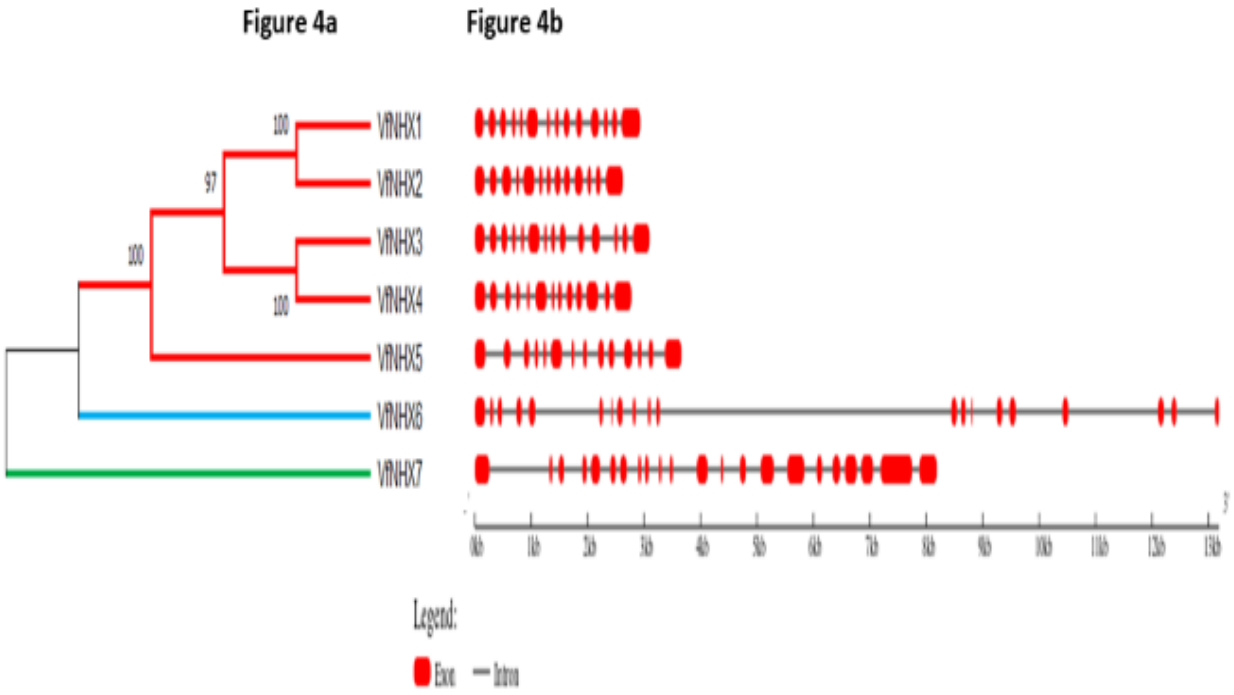
Annexure A



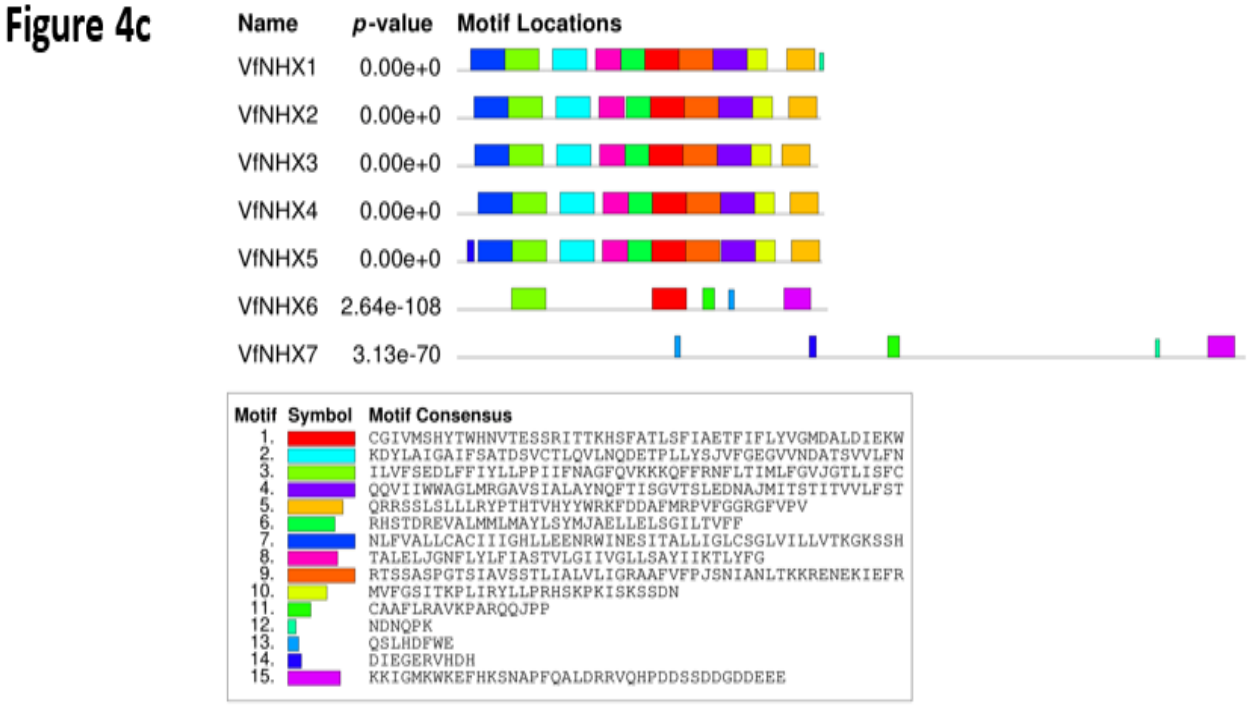
Anneure B

Transcript ID	Gene names	chromosome	start	end	strand	Protein length	Molecular Weight (kDa)	CDS	Pi	aliphatic index	GRAVY
Vf5g183120	VfNHX1	5	1266655386	1266658320	reverse	537	59805.28	1614	8.1	109.96	0.508
Vf4g189280	VfNHX2	4	1303652501	1303655128	forward	533	59232.92	1602	8.9	116.14	0.592
Vf3g085760	VfNHX3	3	526695302	526698400	reverse	529	58236.16	1590	7.6	114.06	0.601
Vf4g216880	VfNHX4	4	148781408	1487816861	forward	538	60164.54	1617	5.5	117.21	0.637
Vf2g045640	VfNHX5	2	273805254	273808917	reverse	535	59872.69	1608	8	112.77	0.582
Vf1g442360	VfNHX6	1	1542547448	1542560636	forward	543	59390.42	1632	5.2	99.48	0.443
Vf6g087320	VfNHX7	6	782740555	782748744	reverse	1155	128206.11	3468	6.1	105.26	0.142

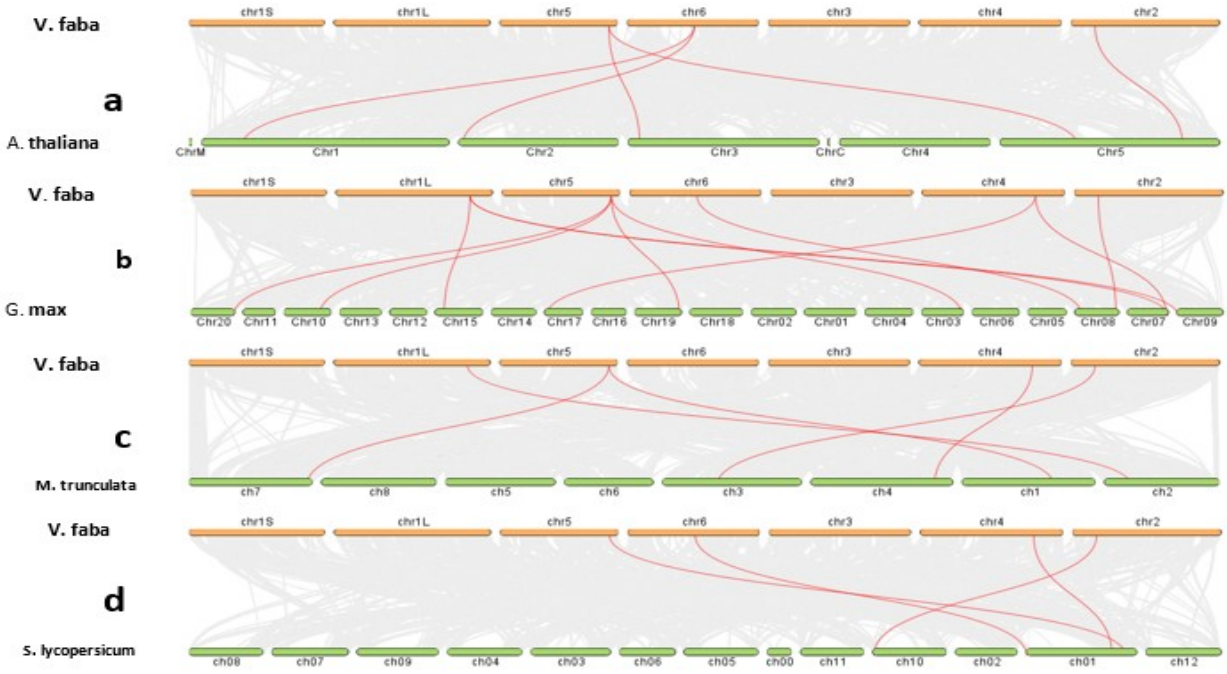
Annexure C



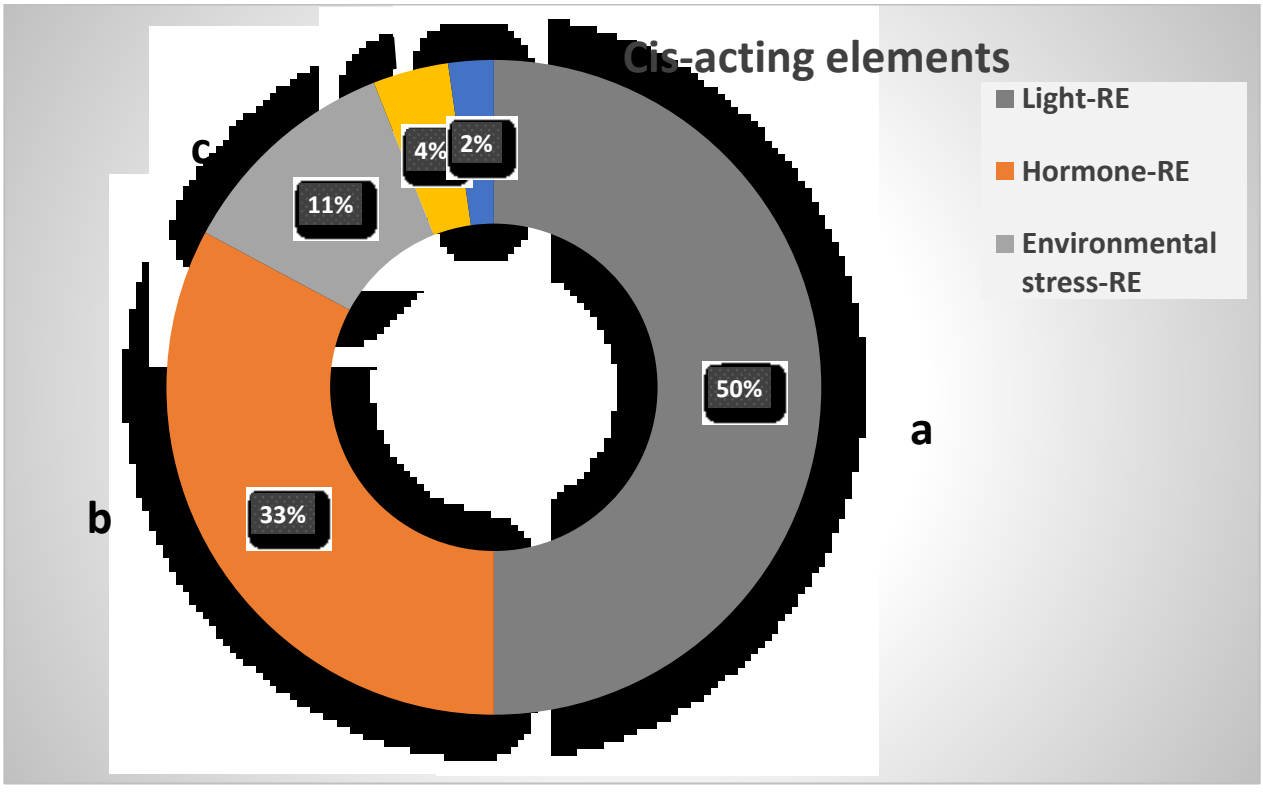
Annexure D



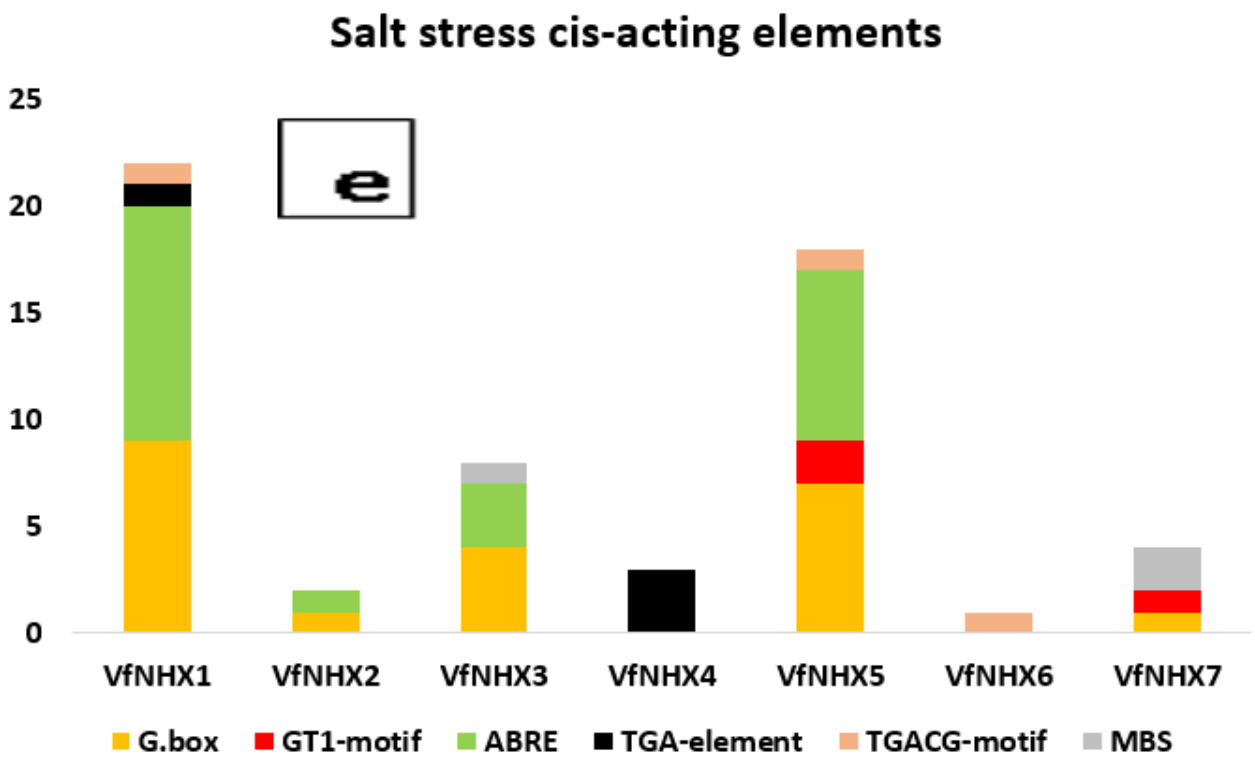
Annexure E



Annexure F



Annexure G


Salt stress cis-acting elements
e
25
20
15
10
5
0
VfNHX1
VfNHX2
VfNHX3
VfNHX4
VfNHX5
VfNHX6
VfNHX7
G.box
GT1-motif
ABRE
TGA-element
TGACG-motif
MBS