

Systematic identification, evolution and expression analyses of *Brachypodium distachyon* NHX gene family in response to salinity

Hatem Boubakri

hatem.boubakri@cbbc.rnrt.tn

Center of Biotechnogy of Borj Cédria

Siwar Messaoudi

Center of Biotechnogy of Borj Cédria

Mhemmed Gandour

Center of Biotechnogy of Borj Cédria

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Abstract

Salinity stress is a major abiotic factor affecting plant cultivation and productivity worldwide. *Brachypodium distachyon* emerged as a model for understanding stress adaptation mechanisms in grasses. Here, Sodium/Hydrogen antiporter gene (NHX) family in *B. distachyon* (BdNHX) was systemically identified, characterized and their expression pattern was assessed in response to salinity by Real Time q-PCR. Overall, 8 NHX genes were identified in the *B. distachyon* genome (*BdNHX1-8*). Phylogenetic, motifs and gene structure analyses allowed the classification of BdNHX genes into 3 groups as a function of their subcellular localization: Vacuole (Vac-Class), endosomal (Endo-Class) and plasma membrane (PM-Class). Protein-protein interactions (PPIs) network determination indicated that several BdNHX proteins interacts with CIPK24, CBL4 and KEA4, implying their involvement in CBL-CIPK pathway. Expression studies using Real Time quantitative-PCR (RT q-PCR) revealed the upregulation of specific BdNHX genes under salinity in both leaves and roots, suggesting their involvement in salinity tolerance mechanisms in *B. distachyon*. Collectively, these results provided valuable targets of *B. distachyon* NHX members for genetic engineering to improve grasses resilience to salinity.

Introduction

Plants are sessile organisms facing various abiotic stresses like drought, salinity, and extreme temperatures. Salinity seriously limited plant growth, development and productivity in many regions of the world. Salinity affects plant growth mainly through osmotic stress and ionic stresses (He et al. 2018). In fact, high salt concentrations reduced water uptake and nutrient absorption, hampering plant growth (He et al. 2018). Regarding ion toxicity, the Accumulation of salt ions disrupts metabolic processes, accelerates aging, and triggers oxidative stress (He et al. 2018). To overcome salt-induced ionic stress, plants might maintain their cell ion homeostasis. This ion homeostasis is crucial for salt tolerance as it prevents the toxic effects of excessive Na^+ while maintaining essential K^+ levels required for plant growth and function (He et al. 2018). Sodium/Hydrogen antiporter (NHX) genes play a crucial role in helping plants cope with salt stress through adjusting ion equilibrium. These proteins are part of the cation-proton antiporter (CPA) superfamily and function by exchanging sodium (Na^+) or potassium (K^+) ions for protons (H^+), utilizing the proton electrochemical gradient generated by H^+ -ATPase and H^+ -PPase (Santhoshi et al. 2025). NHX proteins are located in cell and organelle membranes and distinguished by the presence of a distinct “ Na^+/H^+ exchanger” domain that is required for the transportation of Na^+ ions in exchange for H^+ under saline conditions (Dong et al. 2024). In addition, some NHX proteins harbor a potential “amiloride-binding site” domain (FFIYLLPPI), allowing the binding of amiloride with a high affinity as a competitive inhibitor for Na^+ ions. NHX transporters also contain 10 to 12 transmembrane helix domains (TMs) (Dong et al. 2024). According to their subcellular location, NHX proteins were classified into three groups: vacuolar (Vac-Class), plasma membrane (PM-Class), and endosomal (Endo-Class) (Dong et al. 2024). The vacuolar class of NHX (Vac-Class) transporters are involved in the vacuolar compartmentalization of Na^+ ions accumulated in the cell cytoplasm, preventing their toxicity inside the cell (Sharma et al. 2022). Vac-Class NHXs are known to possess an “amiloride-binding site” domain in the TM3 in their N-terminus region. Na^+/H^+ antiporters belonging to PM class play a crucial role in the maintenance of Na^+ homeostasis by transporting Na^+ from the cytoplasm to the extracellular cell space (Dong et al. 2024). *Arabidopsis thaliana* contains 8 NHX genes, including 2 genes (*AtNHX7* and *AtNHX8*) belonging to the PM-Class, 2 genes (*AtNHX5* and *AtNHX6*) belonging to the Endo-Class, and 4 genes (*AtNHX1-4*) belonging to the Vac-Class (Bassil et al. 2011). The overexpression of *RtNHX1*, a Na^+/H^+ antiporter from *Reaumuria trigyna*, in transgenic *Arabidopsis thaliana* enhanced salinity tolerance by maintaining a favorable ion balance. Specifically, it helped accumulate more K^+ and less Na^+ compared to wild-type (WT) plants (Li et al. 2027). The overexpression of *RtNHX1* also enhanced osmotic regulation and antioxidant defenses, which helped protect plants from oxidative damage caused by salt stress (Li et al. 2027). Additionally, the ectopic expression of *Zygophyllum xanthoxylum* Na^+/H^+ transporter gene (*ZxNHX*), together with *ZxVP1-1*, a H^+ -PPase gene in the legume model, *Medicago sativa*, conferred tolerance to both salinity and drought stresses (Bao et al. 2015). Therefore, incorporating NHX genes into breeding programs could lead to the development of

salt-tolerant crop varieties. However, in the model grass model plant that is largely used for functional genomics research, *Brachypodium distachyon*, the Na⁺/H⁺ antiporter (BdNHX) family is not yet characterized. Thus, the present study aims to identify the BdNHX protein family in *B. distachyon* (line Bd21), elucidate their organization, classification and evolution and analyze their expression profiles in response to salinity by using RT-q PCR.

Material and methods

Identification and characterization of the NHX proteins in *Brachypodium distachyon*

The NHX protein sequences of Arabidopsis and rice were downloaded from The Arabidopsis Information Resource (TAIR, <https://www.Arabidopsis.org/>) and Rice Genome Annotation Project (RGAP, <http://rice.plantbiology.msu.edu/>), respectively and used as queries for searching *Brachypodium distachyon* NHX protein sequences (BdNHX) by performing a BLASTP search against *Brachypodium distachyon* proteome (*Brachypodium_distachyon_v3.0*, strain Bd21) available at the NCBI database (<https://www.ncbi.nlm.nih.gov/>) with E-value cutoff set as $1e^{-10}$. The presence of the domain "Na_H_Exchange" domain characterizing 'NHX' proteins was confirmed using the SMART program (<http://smart.embl-heidelberg.de/>). BdNHX isoforms were named according to their degree of homology to the primary sequences of the model plant *A. thaliana*. WoLF-PSORT (<http://www.genscript.com/wolf-psort.html>), Plant-mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>) and CELLO v.2.5 (<http://cello.life.nctu.edu.tw/>) programs were used to predict the subcellular localization of the different NHXs proteins. The ExPASy Compute pI/Mw tool (https://web.expasy.org/compute_pi/) was used to determine the molecular weight (Mw), length and the theoretical isoelectric points (pI) of each protein. Similar results were obtained from these programs. The multiple alignment of BdNHX proteins was generated using ClustalW, and the conserved regions were highlighted using GenDoc software. Finally, the Protter program (<http://wlab.ethz.ch/protter/start/>) was used to predict transmembrane helices (TMHs) in BdNHX protein sequences.

Phylogenetic analysis of NHX proteins in plants

NHX protein sequences from de *Populus trichocarpa*, *Solanum tuberosum*, *Oryza sativa*, *Sorghum bicolor*, *Cucurbita maxima*, *Solanum lycopersicum*, *Vitis vinifera*, *Eutrema halophilum*, *Spinacia oleracea*, *Arabidopsis thaliana* and *B. distachyon* were aligned with the aid of ClustalW program. Then, the obtained multiple sequence alignment was used to generate the phylogenetic tree using MEGA11 software (<https://www.megasoftware.net/history.php>). The tree was constructed based on the Neighbor-Joining (NJ) method, with 1000 bootstrap replicates (Kumar et al. 2016). The Itols online tool was used to visualize the obtained tree (<https://itol.embl.de/>).

Gene structure and motif composition

To identify conserved motifs, the MEME Suite program (<http://meme-suite.org/ouils/meme>) was used with default parameters. Parameter settings influencing the result of query execution were defined as follows: maximum number of motifs 10 and optimal motif width of 6 to 50. The intron/exon distribution pattern of *NHX* genes was determined by using the GSDS2.0 Software (<http://gsds.cbi.pku.edu.cn/>) by aligning the coding sequence (CDS) with their corresponding genomic DNA sequences.

Promoter region Cisacting regulatory elements (CAREs) analysis

The 2,000 bp promoter regions of the *NHX* genes of *Brachypodium distachyon* were extracted from the Phytozome V13 database in order to analyze their cis-acting regulatory elements (CAREs). The PlantCARE website tool

(http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html) was used to search for cis-acting regulatory elements (CAREs).

Chromosomal location and syntenic analysis of BdNHX gene family

Physical positions on chromosomes of *BdNHX* genes were drawn using the Phenogram software (<https://ritchielab.org/software/phenogram-downloads>). Regarding the comparative syntenic analysis, sequence similarity among NHX members of *B. distachyon*, *A. thaliana*, and *Oryza sativa* was visualized by the Circoletto tool (<https://bat.infospire.org/circoletto/>).

Tertiary structural prediction of BdNHX proteins

The three-dimensional (3-D) structure of BdNHX proteins was predicted using the “Phyre²” program (<http://www.sbg.bio.ic.ac.uk/phyre2/html/flibview.cgi?pdb=c6z3y>). To identify the closest structure of each BdNHX protein, the program performs an alignment of existing primary sequences in the Protein Data Bank (PDB) with the query sequence. The PDB file obtained by Phyre² was submitted to the “Ezmol” program (<https://www.sbg.bio.ic.ac.uk/ezmol/>) for the visualization of the 3-D structures.

Analysis of protein–protein interactions network for BdNHX proteins

The molecular interaction network of **BdNHX** proteins was determined using STRING v11.0 database (<https://string-db.org>). The proteins showing the highest bit scores, based on the similarity search against the Arabidopsis proteome at STRING v11.0 database, were considered for the analysis.

Plant culture conditions and salt stress application

The study was carried out on seedlings from caryopses of *Brachypodium distachyon* L. (line Bd-21). Bd-21 line was provided by Dr David Garvin (USDA-ARS, Plant Science Research Unit and Department of Agronomy and Plant Genetics, University of Minnesota, St Paul, MN, USA). First, Bd-21 seeds were disinfected in 70% bleach for 1 min and then rinsed 5 times before being allowed to germinate in Petri dishes containing a double layer of filter paper soaked in distilled water at room temperature and in the dark. After 7 days, the seedlings obtained were then transferred to the hydroponic system in 5-litre plastic pots, each containing 12 seedlings. These pots contained a diluted (1/4) nutrient solution (Hewitt et al. 1966), under continuous aeration using an air pump. After 3 weeks of growing, plantlets were divided into 2 batches. A portion of the plants were used as controls (untreated plants) and the second was used for the salt treatment with sodium chloride (150 mM NaCl). Leaves and roots were then harvested at 0 (control), 3, 6 and 24 h after salt stress application and immediately frozen in liquid nitrogen and then stored at -80°C.

RNA extraction and cDNA synthesis

Total RNA was isolated from 100 mg leaf or root tissue of *B. distachyon* using the ‘RNeasy plant mini kit’ (Qiagen). RNA concentration was measured using NanoDrop 1000 (Thermo Fisher Scientific). To get an idea of the quality of the RNA, we determined for each sample, the A260/A280 and A260/A230 ratios, which should be greater than or equal to 2 for A260/A280 and close to 2 for A260/A230. The genomic DNA was removed by DNase RNase-Free (Qiagen) treatment for 15 min followed by a purification step on column using the RNeasy Plant Mini Kit (Qiagen). Reverse transcription (cDNA synthesis) was performed on 500 ng mRNA as a template using the M-MuLV First Strand cDNA Synthesis kit (Biomatik) according to the manufacturer’s instructions. cDNAs were then diluted at 1:10 for gene expression assays.

RT-qPCR assays

The expression of *BdNHX* genes in response to salinity was monitored by using real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) as previously described by Boubakri et al. (2021). The primer sequences of *BdNHX* genes (**Table S1**) were designed by the Primer-BLAST tool at the NCBI (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). RT-qPCR was performed using qPCR SYBR Green 1 Master Mix' (Biomatic) in 96-well plates in a final volume of 20 µl sealed with an optical film. The reaction mixture contains 10 µl of SYBR Green 1 Master Mix, 5 µl diluted cDNA, and 300 nM primers. All reactions were analyzed using by a thermal cycler (Applied Biosystems 7300 RealTime PCR System) where PCR conditions were 1 min at 95°C followed by 60 cycles, each consisting of a step of denaturation (10 s at 95°C) and a step of hybridization and annealing (1 min at 60°C). The ubiquitin gene (*BdUbg*) was used as an internal reference gene to normalize gene expression values. The specificity of the primers was checked by analyzing the melting curves obtained after each reaction between 65°C and 95°C. At the end of the amplification phase, DNA temperature was gradually increased from 55 to 95°C in steps of 0.5°C, and the fluorescence was recorded at each temperature step. If a single peak is obtained, the specificity of the amplification is validated. Normalized expression values of *BdNHX* genes were determined based on the Ct (cycle threshold) calculation method (Livak and Schmittgen, 2001).

Statistical analysis

Expression data were analyzed by ANOVA using the Statistica 7.1 Software (StatSoft, Tulsa, OK, USA). The significance of the differences between mean expression values was determined with Tukey's HSD test ($P \leq 0.05$).

Results

Identification and characterization of BdNHX proteins

In total, 8 BdNHX proteins were identified from the *Brachypodium distachyon* proteome using the NHX protein sequences of the model plant *A. thaliana* as queries. BdNHX proteins were renamed according to the order of their corresponding id (Fig. 1A). As shown in Table 1, BdNHX proteins are localized in vacuoles (Vac), plasma membrane (PM) and endosomes (Endo). In terms of molecular characteristics, BdNHX isoforms have a size between 450 aa (BdNHX5) and 1128 aa (BdNHX7), an isoelectric point between 4.99 (BdNHX5) and 8.70 (BdNHX2) and a molecular weight between 51.24 (BdNHX5) and 125 (BdNHX7) KDa. In total, 8 BdNHX proteins were typical transmembrane transporters. The number of transmembrane helical domains (TMHs) in NHX proteins varies from 11 to 13 (Fig. 1B). BdNHX1 and BdNHX7 contains 13 transmembrane helices. BdNHX2 and BdNHX3 contain 12 TMHs. BdNHX4 and BdNHX6 contain 11 TMHs. Finally, BdNHX5 and BdNHX8 contains 10 and 9 TMHs, respectively. All BdNHX proteins harbor the conserved 'amiloride binding site' (FFYLFL) domain, characteristic of the superfamily of monovalent cation/proton antiporters (CPA1) (Fig. 1A). A 'signal' peptide in both BdNHX6 and BdNHX8 was detected (Fig. 1B). Signal peptides are N-terminal pre-sequences responsible for targeting proteins to the endo-membrane system and subsequent subcellular or extracellular compartments. In addition, several sites containing the 'N-glyco-motif' motif were observed in all BdNHX proteins (Fig. 1B).

Table 1

Molecular characteristics of NHX proteins in *Brachypodium distachyon*. Vac: vacuole; End: endosome; PM: plasma membrane; Id: identifier.

Gene Name	NCBI id	Phytozome13 Id	Length	Ip	MW	TMHs	Localization
BdNHX1	XP_003562486.1	Bradi1g18360.1.p	543	8.47	59.64	13	Vac
BdNHX2	XP_003577286.2	Bradi4g11500.1.p	555	8.70	60.64	12	Vac
BdNHX3	XP_003568941.1	Bradi2g36000.1.p	545	8.19	59.66	10	Vac
BdNHX4	XP_003560776.1	Bradi1g42737.1.p	528	8.38	58.13	11	Vac
BdNHX5	XP_024319099.1	Bradi4g17381.1.p	460	4.99	51.24	8	End
BdNHX6	XP_003577939.1	Bradi4g27334.1.p	534	5.09	58.35	9	End
BdNHX7	XP_003576505.1	Bradi4g00290.1.p	1128	7.41	125.0	13	PM
BdNHX8	XP_003576666	Bradi4g33420.4.p	530	5.22	58.70	9	End

Phylogenetic relationships between *B. distachyon* NHX proteins and those in other plants

The phylogenetic tree (Fig. 2) was built using NHX protein families from 11 plant species. It demonstrated the phylogenetic relationships between NHX proteins from *B. distachyon* (Bd) and others from *Oryza sativa* (Os); *Sorghum bicolor* (Sb); *Populus trichocarpa* (Pt); *Solanum tuberosum* (St); *Cucurbita maxima* (Cm); *Solanum lycopersicum* (Sl); *Vitis vinifera* (Vv); *Eutrema halophilum* (Eh); *Spinacia oleracea* (So) and *Arabidopsis thaliana* (At). According to their subcellular location, the plant NHX family was clustered into three classes which are: Vac, Endo and PM (Fig. 1). Vac-class NHX proteins are the most abundant in plants. According to the phylogenetic analysis, *B. distachyon* NHX proteins were divided into only two classes (Vac and Endo). NHX proteins of the Vac-class included BdNHX1, BdNHX2, BdNHX3, BdNHX4 and BdNHX7. The Endo-class is formed by 3 members (BdNHX5, BdNHX6 and BdNHX8).

Analysis of the conserved motifs of BdNHX proteins

To further explore the structural diversity of BdNHX proteins, motif composition in the proteins was determined using the MEME program which identified a total of 10 putative motifs (Fig. 2). Generally, BdNHX members of the same class shared similar motif compositions. The conservative distribution of motifs in the BdNHXs proteins further supported the classification observed in the phylogenetic tree. The 'Na⁺/H⁺ Exchanger' domain, present in motif 2, was detected in all BdNHX proteins (Table 2). Additionally, the amiloride-binding site (FFIYLLPPI), which is a typical feature of NHX proteins, was found in motif 3 of all BdNHX proteins. These differences in the composition of the conserved motifs suggest a functional divergence for the different isoforms of the BdNHX family.

Table 2
Motifs detected in BdNHX protein family and their function according to Pfam database

Motif	Motif Consensus	Sites	Width	Function
Motif 1	TDREVALMVLMAVLSYMLAELLGLSGILTVFFCGIVMSHYTWHNVTESSR	7	50	Na ₂ H ₂ Exchanger
Motif 2	LYSLVFGEGVVNDATSVVLFNAIQNFDLN	8	29	Na ₂ H ₂ Exchanger
Motif 3	EDLFFIYLLPPIIFNAGFQVKKKQFFRN	6	29	amiloride binding domain (FFYLFL)
Motif 4	VTTKHFFATLSFJAETFJFYVGMD	7	25	-
Motif 5	VDCLAFGAJFSATDPVCTLQVLQZDETP	7	28	Na ₂ H ₂ Exchanger
Motif 6	KRPPHKISPRQQVVIWWAGL	7	20	-
Motif 7	CGCIVFGHLLZENRWLNESHTALIIGLTT	7	29	-
Motif 8	MRGAVSIALAYNKFTKSGHTQLPVNAIMITSTIIVVLFSTMVFGLLTKPL	4	50	Na ₂ H ₂ Exchanger
Motif 9	RMLLTKPTRSVHYWWRKFDDAFMRPVFGGRGFV	4	33	-
Motif 10	GGSTGTMLEALQVVGDSNRYRHPYEENFDGNNAAYEEGTSTPSKFQLKLR	3	50	-

Conserved motifs and BdNHX gene organization

To better identify the structural characteristics of *BdNHX* genes, the exon/intron organizations of these genes were analyzed and compared (Fig. 3A). Members of the same group have relatively similar genetic structures (number of exons/introns and length of exons/introns). The results (Fig. 3A) showed that *BdNHX1*, *BdNHX2*, *BdNHX3*, *BdNHX4*, and *BdNHX7* contain, respectively, 14, 13, 14, 14 and 3 exons, while the other 3 genes (*BdNHX5*, *BdNHX6* and *BdNHX8*) have 22, 19 and 19 exons, respectively. However, this exon/intron organization varied depending on the group, suggesting that intra-genetic rearrangements (insertion/deletion of exons/introns) have contributed to the evolution of the *NHX* family in *B. distachyon*. In general, this gene structure analysis also consolidated the classification obtained by phylogenetic analysis.

Figure 3B, showing a phylogenetic tree and the conserved motifs of the BdNHXs protein family. The conservative distribution of the detected motifs in BdNHXs proteins is consistent with the classification observed in the phylogenetic tree. Consequently, BdNHXs of the same class have similar motif compositions. For motifs 1 to 9, they are mainly present in class I NHXs. However, only motif 2 is present in the BdNHX7 protein. Interestingly, a binding domain for the diuretic compound amiloride (FFI/LY/FLLPPI) was identified in BdNHXs proteins (Table 2). These differences in conserved motif composition suggest a functional divergence for the different isoforms of the BdNHX family.

Chromosomal distribution, synteny and evolution of NHX genes in *B. distachyon*

To examine the distribution of the *BdNHX* genes, chromosome mapping was performed using the Phenogram software (<https://visualization.ritchielab.org/phenograms/plot>). As shown in Fig. 4A, *NHX* genes in *B. distachyon* are distributed

over 3 chromosomes. Two genes, *BdNHX1* and *BdNHX4*, are located on chromosome 1, while *BdNHX2*, *BdNHX5*, *BdNHX6*, *BdNHX7* and *BdNHX8* are located on chromosome 4. The *BdNHX3* gene is located on chromosome 2. The synteny analyses between NHX proteins from *B. distachyon* and those from monocotyledon (*O. sativa*) and dicotyledon plants (*A. thaliana*) were performed via the Circoletto tool (tools.bat.infospire.org/circoletto/) to better understand the origin of *BdNHX* genes. Obviously, almost all *BdNHX* genes exhibited synteny relationships with the majority of *NHX* genes from both *A. thaliana* (Fig. 4B) and *O. sativa* plant species (Fig. 4C). The higher number of syntenic blocks between *NHX* genes from *B. distachyon* and *A. thaliana* plants implied their existence before the monocot-dicot divergence.

Tertiary structure prediction of BdNHX proteins

The 3-D structure of *B. distachyon* NHX proteins was predicted using the Phyre² program (Fig. 5). The protein 'c6z3yB' in the 'PDB' database corresponding to an NHX-type antiporter (sodium/hydrogen exchanger) was used as a template by the Phyre² program for modelling the 3-D structure of *BdNHX* proteins. The side chain residues constituting the amiloride diuretic binding domain (FFI/LY/FLLPPI) are shown in green (Fig. 5). The position of the amino acids forming the amiloride binding domain (FFI/LY/FLLPPI) varied among the different NHX proteins, suggesting a functional divergence within the *B. distachyon* NHX family.

BdNHX gene promoter analysis for Cis-acting regulatory elements (CAREs)

To identify Cis-acting regulatory elements (CAREs), we submitted upstream 1500 bp sequences in the promoter region of *BdNHX* genes to PlantCARE database. The results (Fig. 6A) showed that 20 CAREs involved in the stress response were found, including LTR, O₂-site, ABRE, G-Box, CAAT-box, TGA-element, GARE motif, CGTCA-motif, GATA-motif, MBS, MRE, Sp1, P-box, TATC-box, Case 4, TCA-element. All members of the *BdNHX* genes contain MeJA-sensitive cis-acting regulatory elements, such as the CGTCA motif and the TGACG motif. Except *BdNHX7*, the remaining *BdNHX* members contained the ABA-responsive element (ABRE). In total, 40% of *BdNHX* genes contain auxin response-related regulatory elements such as the AuxRR-core and TGA-element motifs. The promoters of *BdNHX2* and *BdNHX3* genes showed the highest number of stress-related CAREs, while *BdNHX4* promoter region contained the lowest number (Fig. 6B). Overall, these data indicated a potential role for *BdNHX* genes in development and stress response.

Analysis of BdNHX gene expression under salt stress

The expression profiles of *BdNHX* genes in response to salinity were determined by RTq-PCR in both leaves and roots of *B. distachyon* plants grown under 150 mM NaCl for 0-, 3-, 6- and 24-hours post-treatment (hpt). In response to salt stress, certain *BdNHX* genes (*BdNHX1*, *BdNHX2*, *BdNHX3*, *BdNHX6* and *BdNHX7*) were upregulated in leaf tissues, particularly at 24 hpt (Fig. 7A). However, *BdNHX4*, *BdNHX5* and *BdNHX8* genes were not induced by salt stress in the leaves at different time intervals tested. In roots, specific *BdNHXs* members (*BdNHX1*, *BdNHX2*, *BdNHX3*, *BdNHX4* and *BdNHX7*) were upregulated in response to salinity mainly at 24 hpt (Fig. 7B). The two genes *BdNHX1* and *BdNHX4* showed the highest expression levels in response to salt in root tissues, while *BdNHX5* and *BdNHX8* members were not affected by salt in roots at the different time points tested. The results of the RT q-PCR analysis indicated that the expression patterns of *BdNHX4* were tissue-specific under NaCl treatment (Fig. 7) as it was highly induced in roots (at 6 and 24 hpt) and unaffected in leaves, suggesting differential functional roles.

Protein interaction network analysis

To further explore the function and metabolic pathways related to *B. distachyon* NHX proteins, the protein-protein interaction (PPI) network was constructed with the online tool String (Fig. 8A). The deduced PPI network involved almost all *BdNHX* proteins which shared the same interactive proteins (Fig. 8B), including CIPK24 (CBL-interacting serine/threonine-protein kinase 24), CBL4 (Calcineurin B-like protein 4) and KEA4 (K⁺ efflux antiporter 4). CIPK24 is involved in the regulatory pathway for the control of intracellular Na⁺ and K⁺ homeostasis and salt tolerance. CIPK

activated the vacuolar H^+/Ca^{2+} antiporter (CAX1) and operated in synergy with CBL4/SOS3 to activate the plasma membrane Na^+/H^+ antiporter SOS1. CIPK also interacts with CBL proteins. Binding of a CBL protein to the regulatory “NAF domain” of CIPK protein led to the activation of the kinase in a calcium-dependent manner. CIPK phosphorylates CBL1, CBL4 and CBL10. For example, BdNHX2 (Fig. 8A) interacts with CBL4 which corresponds to a regulatory subunit of serine/threonine phosphatase 2b, acting as a calcium sensor involved in the signaling pathway during abiotic stress response. It contributed to the regulation of stress-related transcription factors (TFs) such as CBF/DREB. The interaction of the CBL1 protein with NHX2 protein would enable vacuolar compartmentalization of Na^+ ions, which is necessary for detoxification of cytoplasmic Na^+ . Additionally, certain BdNHX proteins were found to interact with HKT1 (Sodium transporter HKT1), CHX18 (Cation/ H^+) antiporter 18, GORK (Potassium channel GORK) and SKOR (Potassium channel SKOR) proteins (Fig. 8B). HKT1 could play a significant role in Na^+ recirculation to roots from shoots. This interaction controls intracellular homeostasis of Na^+ and K^+ ions and consequently salt tolerance. CHX18 belongs to the monovalent cation/proton antiporter 2 (CPA2) transporter family and may operate as a cation/ H^+ antiporter. The potassium channel SKOR is highly selective outward-rectifying potassium channel. It is involved in potassium release into the xylem sap toward the shoots. Overall, all BdNHX proteins operated together with other ones to ensure salinity stress response.

Discussion

NHX antiporters belong to the CPA1 family and play an important role in plant cells. Under salt or osmotic stress, they help to absorb K^+ ions, control turgor and regulate pH (Li et al. 2017). NHX proteins also have a protective function through vacuolar compartmentalization of K^+ and in some cases Na^+ , preventing toxic Na^+/K^+ ratios in the cytosol (Li et al. 2017). In our present work, 8 *NHX* genes were identified in the grass model system *B. distachyon* (BdNHX1-8). Our results also showed that the number of *NHX* genes in *B. distachyon* is lower than that previously reported in other grasses. Differences in the number of *NHX* genes in plants were mainly attributed to the duplication and loss of genes during evolution (Wu et al. 2019). In Arabidopsis, 8 *ATNHX* genes were identified and subdivided into three classes according to their subcellular location, including 2 PM-class genes, 2 Endo-class genes and 4 Vac-class genes (Li et al. 2017; Kumari et al. 2018). In our case, 8 *BdNHX* genes were identified and classified into 2 classes (5 Vac-class genes and the 3 Endo-class). The number of *NHX* genes belonging to the Vac-class is the highest, indicating that Vac-class NHX proteins may have a very important role in plant cells (Li et al. 2017; Kumari et al. 2018).

Both gene structure and motif composition of BdNHX family are relatively conserved within the same class. This further consolidated the classification obtained in phylogenetic study. The number of exons for the three NHX classes (PM-, Endo- and Vac-classes) in poplar is 23, 22 and 14, respectively (Tian et al. 2017), which is significantly higher than that of the *NHX* genes in *B. distachyon*. In sugar beet, the highest number of exons was found for the *BvNHX4* gene (Endo-class) with 26 exons, followed by the *BvNHX5* gene (PM-class) with 23 exons (Wu et al. 2019). These findings indicated a diversity in exons/introns organization across species, suggesting the contribution of intragenic rearrangements such as insertion/deletion in the evolution of *NHX* gene family.

Previous studies have shown that most N-terminal domains of NHX proteins contain 9–12 transmembrane domains (TMs), which are highly conserved (Wu et al. 2019). The ‘amiloride- binding site’ present in NHX antiporters is responsible for Na^+ ion transport (Wu et al. 2004). Amiloride is a competitive inhibitor of NHX mediating Na^+ transport. In our work, 7 NHX proteins were found to contain this binding site in *B. distachyon*. This site was also observed in the *Arabidopsis* AtNHX1-4 proteins (Li et al. 2017) and the *Zanthoxylum* ZxNHX1 protein (Wu et al. 2011). This indicates that Vac-class NHX antiporters are highly conserved during evolution.

In this work, we also determined the CAREs present in *BdNHX* gene promoters. The results showed that a proportion of the detected cis-acting elements are linked to hormones response (IAA, GA, SA, ABA and MeJA). Different *NHX* genes were previously reported to be regulated by hormones such as ABA and MeJA (Fu et al. 2020). Therefore, BdNHX

proteins might be responsive to a variety of hormones and stresses. Other stress-related CAREs (CAAT-box, W-BOX and TATA-box) were also identified in the promoter regions of *BdNHX* genes. The promoter of *BdNHX1* gene contained the highest number of abiotic stress-related CAREs. W-Box cis-element was previously reported to be linked to salt stress response (Xu et al. 2018). These results indicated that *BdNHX* antiporters may participate in plant development through various hormonal pathways, abiotic stress response, and other physiological processes.

The PPI network showed that almost all *BdNHX* proteins shared the same interactive proteins including CIPK24, CBL4, HKT1, CHX18, GORK, SKOR proteins and SOS3. All these proteins play crucial roles in salinity stress response as shown in the schematic representation in Fig. 8. The overexpression of *AtAVP1* enhanced salinity tolerance in transgenic *Arabidopsis* lines (Gaxiola et al. 2001). *AtHKT1* was found to play an important role in salinity tolerance mechanisms by extracting Na^+ from the xylem of different tissues and organs (Romero-Aranda et al. 2020). Additionally, SOS2/CIPK24, a protein kinase, was reported to reduce adverse effects of salinity stress in *A. thaliana* (Wilkins et al. 2016). Kumari et al. (2018) have described calcineurin B-like proteins (CBL4)/Salt Over Sensitive (SOS3), a Ca^{2+} sensor, was able to escort SOS2 and SOS1(NHX1) to promote Na^+ efflux from the cells. The calcium binds to CBL4 causing dimerization of this protein and enhancing the activity of CIPK24 (SOS2) serine/threonine protein kinase (Fig. 8). The resulting CBL4/CIPK24 (SOS3/SOS2) complex activated the SOS1(NHX1 antiporter) by phosphorylation to promote its exchanging activity. However, CIPK24 together with SCABP (SOS3 like protein) are involved in the activation of the V-ATPase (Fig. 8). The overexpression of the gene encoding H^+ -ATPase has resulted in an enhanced salinity tolerance in transgenic *Arabidopsis* lines (Bao et al. 2008). Moreover, AKT1 was reported to reduce salt damage by regulating the amount of K^+ (Manik et al. 2015). Under salt stress conditions, AKT1 transporter was found to play an important role in K^+ uptake, maintenance and balance. In fact, many plants were described as able to overcome salinity stress by maintaining high K^+/Na^+ ratios (Isayenkov and Maathuis, 2019).

In *Arabidopsis*, some vacuolar Na^+/H^+ exchangers including *AtNHX1*, *AtNHX2* and *AtNHX5* were induced by salt stress (Yokoi et al. 2002). To gain an idea on the physiological role of *B. distachyon* *NHX* genes in response to environmental stresses, their expression profiles were assessed by RT q-PCR under salinity (150 mM NaCl) treatment. Salt stress induced the expression of targeted *BdNHX* genes, particularly within 24 hpt in both leaves (*BdNHX1*, *BdNHX2*, *BdNHX3*, *BdNHX6* and *BdNHX7*) and roots (*BdNHX1*, *BdNHX2*, *BdNHX3*, *BdNHX4* and *BdNHX7*), suggesting a potential role for these antiporters in the regulation of salinity tolerance mechanisms in *B. distachyon*. The over-expression of *AtNHX1* allowed transgenic plants to tolerate high Na^+ concentrations, exceeding 200 mM (Apse et al. 2003). *AtNHX1* is expressed in all plant tissues and found on the tonoplast and membranes of the internal organelles (Apse et al. 2003). Thus, the upregulated *BdNHX* genes under salt stress might perform different functions in *B. distachyon* leaves and roots. It can be suggested that *BdNHX* proteins might alleviate the harmful effects of Na^+ by vacuolar compartmentalization of the excessive leaf Na^+ cations, while restricting its accumulation in root tissues. Overall, our study provided findings valuable in subsequent functional studies on targeted *BdNHX* members to better understand their physiological role and to improve salinity tolerance in grasses using the recently emerged genome-editing tools.

Conclusion

NHX antiporters are among the most-induced genes in response to salt stress. These exchangers sequester Na^+ in the vacuole and regulate intracellular pH, ion homeostasis and osmoregulation. In addition, they were shown to be capable of transporting monovalent cations other than Na^+ , particularly K^+ . In this work, we identified *NHX* genes in the grass model plant *B. distachyon* and analyzed their expression profiles in response to salt stress to identify salt stress-responsive members. Additionally, using systematic bioinformatics analyses, we explored gene structure, motif composition, evolution and CAREs of the *BdNHX* family. Using RT q-PCR analysis, we obtained fundamental results that

are useful in subsequent functional studies on specific *BdNHX* members to gain more insights into their physiological role and to improve the tolerance of grasses to salinity stress in future engineering programs.

Declarations

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Figures

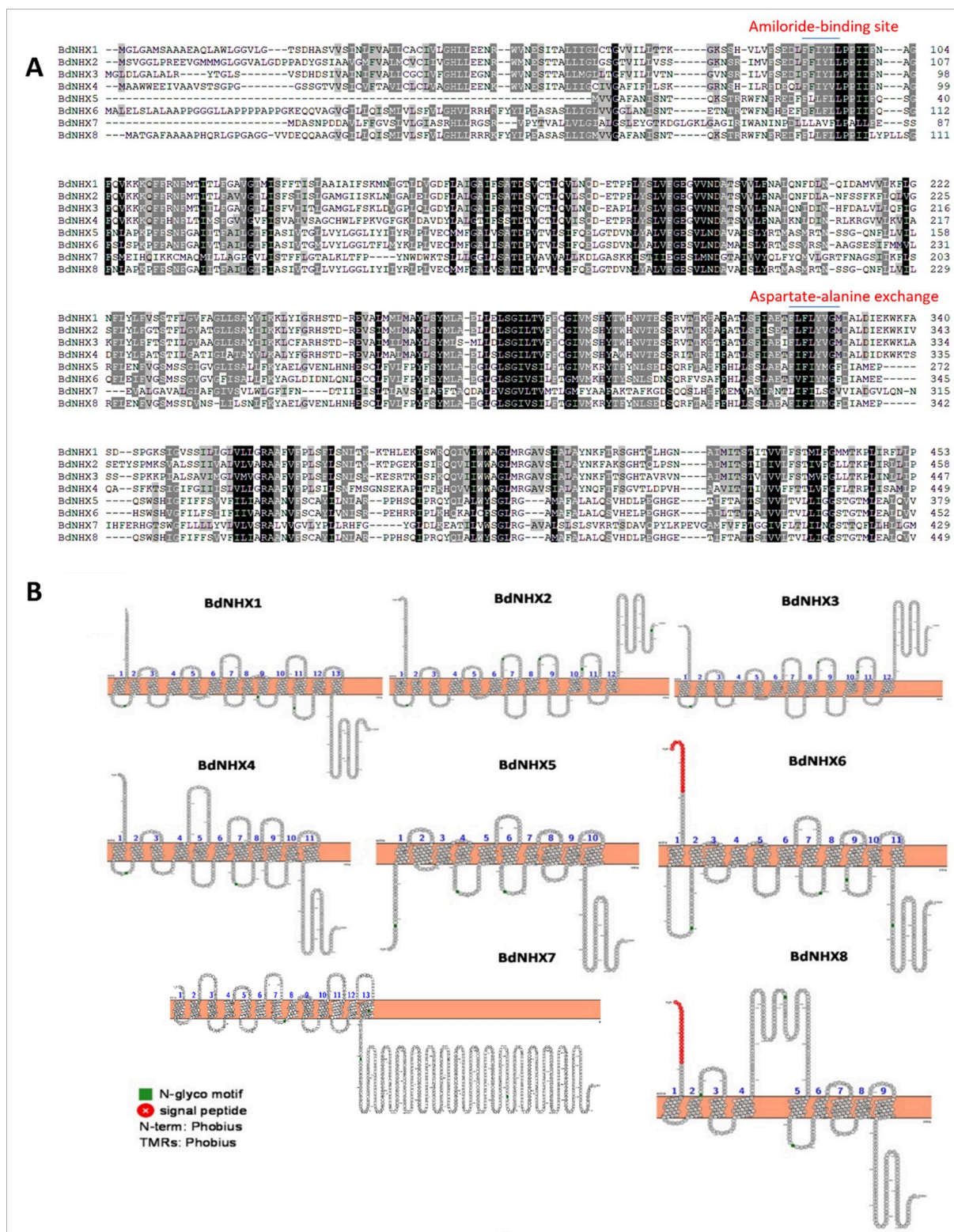


Figure 1

A: Multiple sequence alignment of BdNHX proteins. **B:** Illustration of TMHs in BdNHX proteins

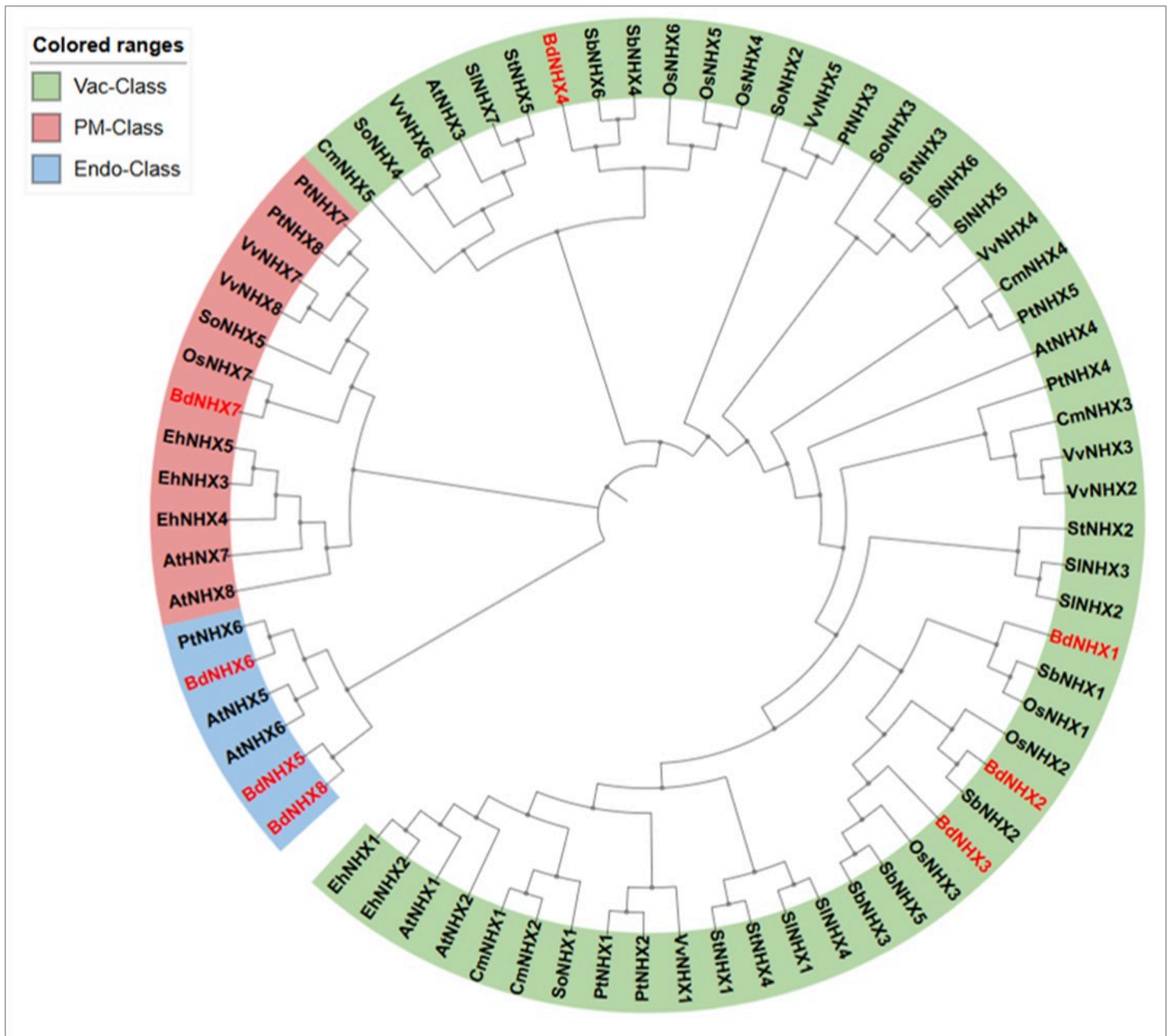


Figure 2

Phylogenetic tree of the NHX proteins from *B. distachyon* (Bd) and others from *Oryza sativa* (Os); *Sorghum bicolor* (Sb); *Populus trichocarpa* (Pt); *Solanum tuberosum* (St); *Cucurbita maxima* (Cm); *Solanum lycopersicum* (Sl); *Vitis vinifera* (Vv); *Eutrema halophilum* (Eh); *Spinacia oleracea* (So) and *Arabidopsis thaliana* (At). The tree was determined by the Neighbor-Joining method (NJ) with 1000 bootstrap replicates using MEGA11. According to the clustering of the NHX proteins, NHX proteins were clustered into three different classes including Vac-, Endo-, and PM-classes. Different label colors indicated different classes.

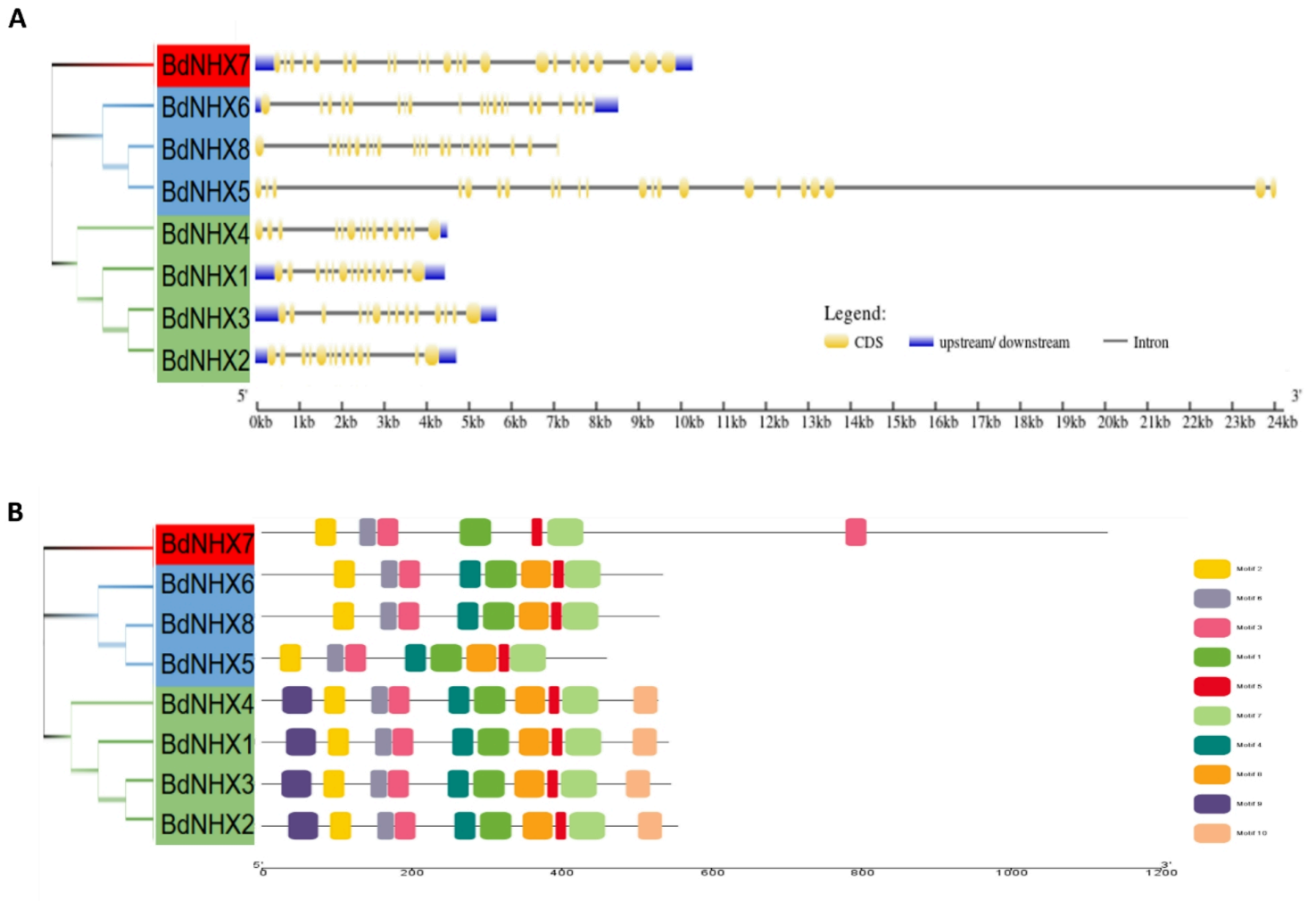


Figure 3

A: Gene structure of the *BdNHX* gene family. **B:** Conserved motifs. Different colored boxes represented different conserved motifs.

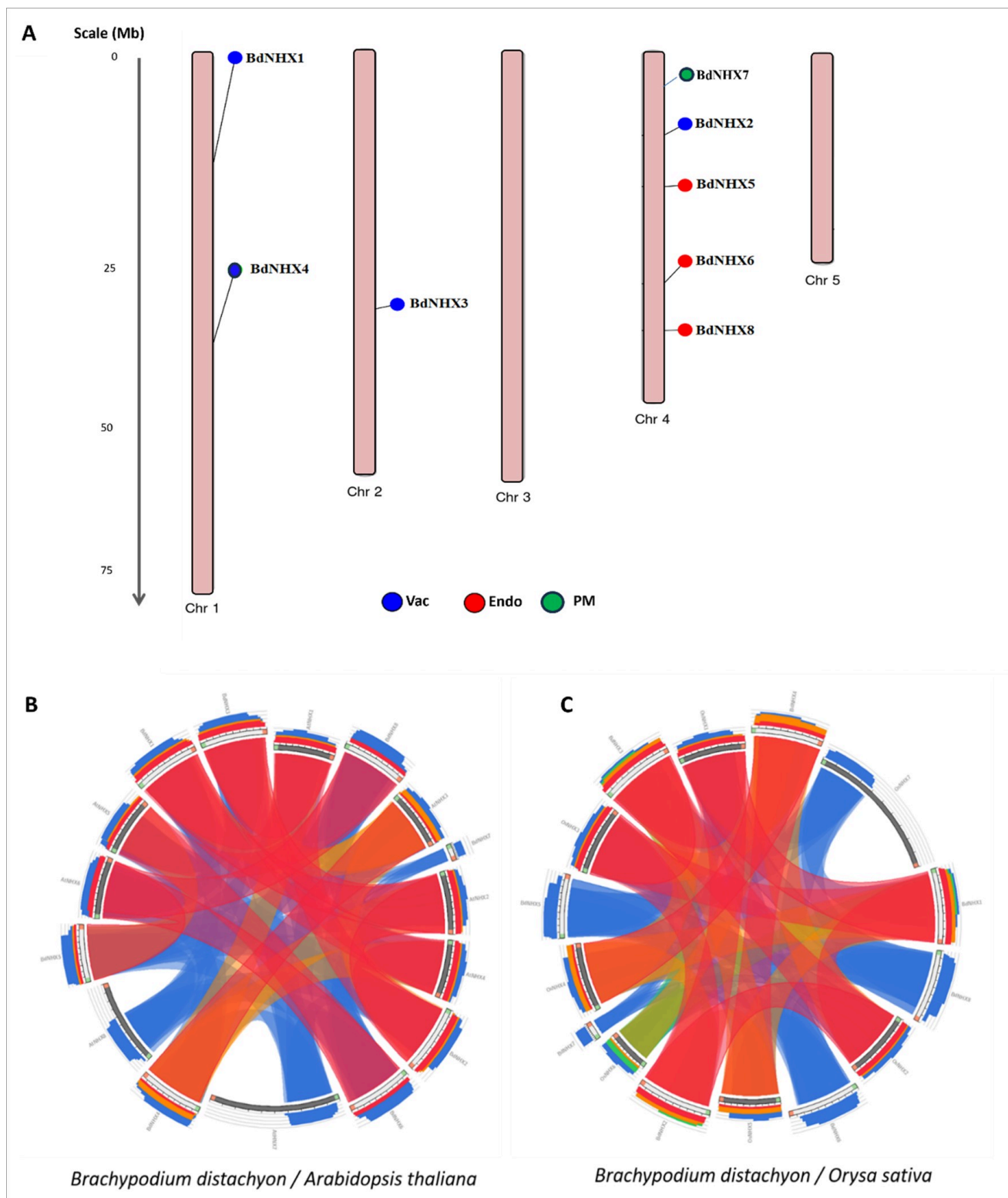


Figure 4

A: Chromosomal localization of the *BdNHX* gene family. The chromosome numbers are indicated at the end of each vertical bar. The gene name on the right side of each chromosome corresponds to the locations of each *NHX* gene. The scale is in mega-bases (Mb). **B:** Synteny relationship between the *NHX* members of *B. distachyon* and those of *A. thaliana*. **C:** Synteny relationship between the *NHX* members of *B. distachyon* and those of *O. sativa*.

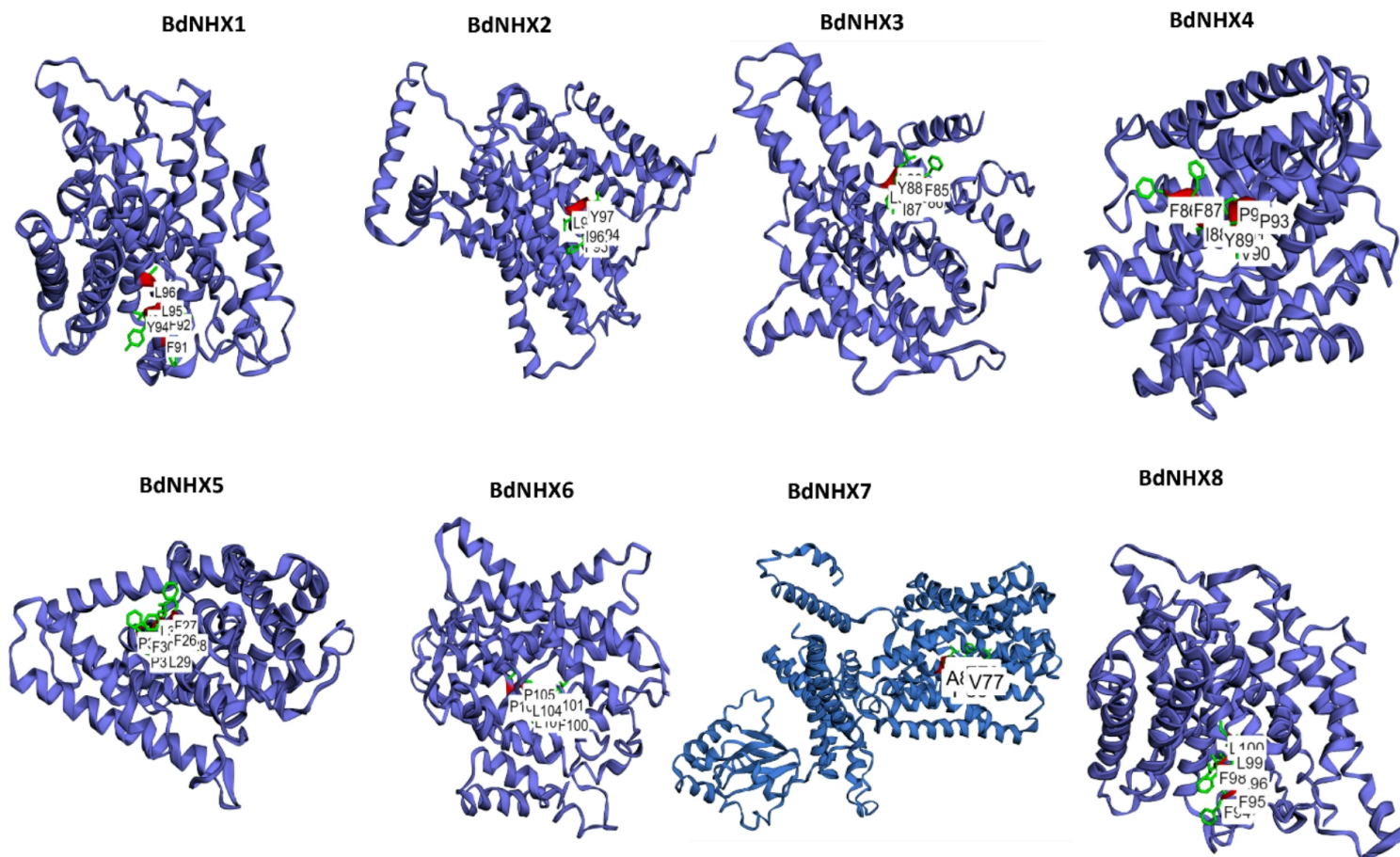


Figure 5

Tertiary structure of BdNHX proteins. The models of proteins were obtained by the online Phyre² program. The protein 'c6z3yB' in the 'PDB' database corresponding to an NHX-type antiport (sodium/hydrogen exchanger) was used as a template by the Phyre² program to predict the models of *B. distachyon* NHX proteins. Amino acids forming the side chain of the amiloride-binding domain (FFI/LY/FLLPPI) are marked in green.

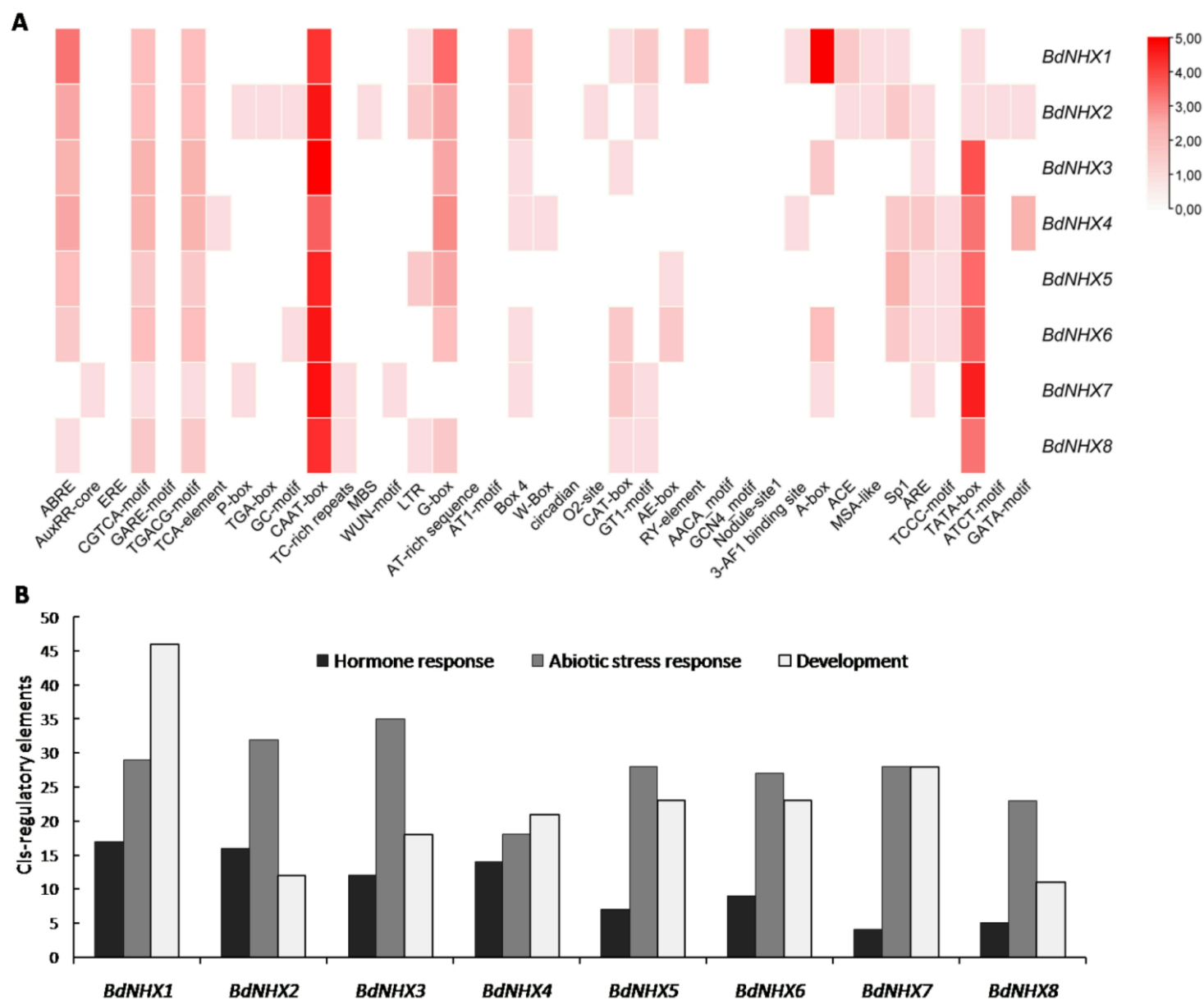


Figure 6

CAREs detection in *B. distachyon* *NHX* promoter regions. A: The cis-acting regulatory elements (CAREs) were detected on the online tool PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/search_CARE.html). B: Predicted functions of the different CAREs present in promoter regions of *B. distachyon* *NHX* genes.

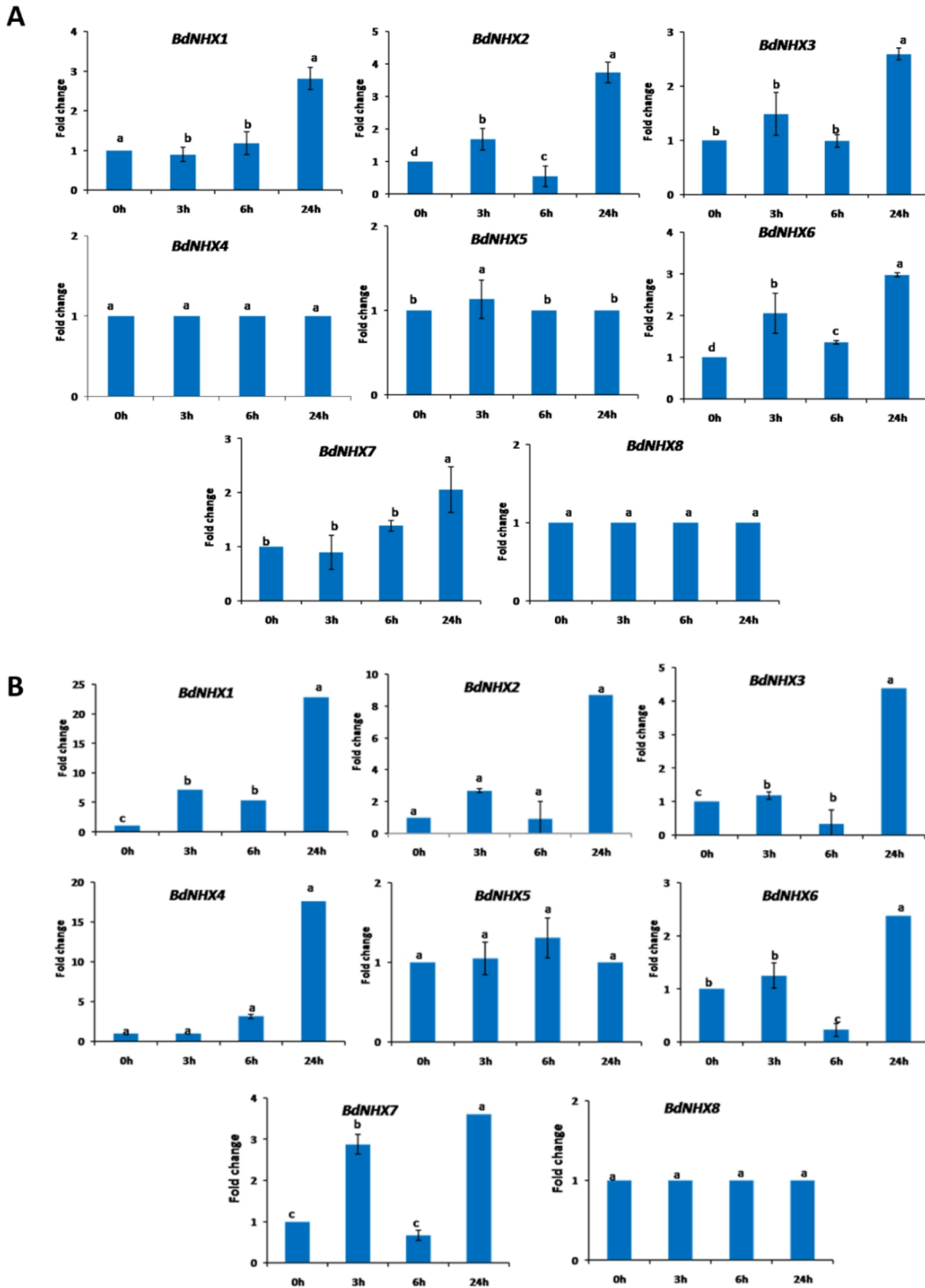


Figure 7

Expression changes of 8 *NHX* genes in *B. distachyon* in response to salt stress. Plant leaves (**A**) and roots (**B**) were harvested within 0, 3, 6 and 24 h after exposure to salinity stress for RNA extraction and RT-qPCR analysis. The $2^{-\Delta\Delta Ct}$ method was used to calculate relative gene expression. The default expression value for each gene was 1 at 0 h before treatment (control). The *BdUbq* gene was used as an internal control. Expression data are mean values of three replicates.

CBL-interacting serine/threonine-protein kinase 24 (CIPK24, SOS2). The CIBL4/CIPK24 complex activates NHX (SOS1) and inhibits Na⁺ uptake by HKT2. CIPK24 together with SCaBP (SOS3 like protein) are involved in the activation of the V-ATPase. CIPK24 participates in the activation of vacuolar transporters such as CAX and NHX.