

Sweet cherry genome-wide Identification of the NHX gene family and functional dissection of PavNHX37 a key salt tolerance gene

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Abstract

Background

Soil salinization severely limits sweet cherry (*Prunus avium* L.) growth and productivity, yet the NHX (Na⁺/H⁺ antiporter) gene family, which is central to salt tolerance, remains uncharacterized in this species.

Results

Here, we identified 37 *PavNHX* genes unevenly distributed across eight chromosomes. All *PavNHX* proteins were predicted to localize to the plasma membrane except *PavNHX21* (vacuolar), and their secondary structures were dominated by α -helices and random coils. Phylogenetic analysis divided the family into three groups, with Group I containing both sweet cherry and *Arabidopsis* homologs, indicating evolutionary conservation. Expression profiling revealed diverse tissue-specific patterns, and qRT-PCR under salt stress identified *PavNHX37* as the most significantly upregulated gene. Subcellular localization confirmed *PavNHX37* at the plasma membrane. Stable overexpression of *PavNHX37* in *Nicotiana benthamiana* dramatically improved salt tolerance: transgenic lines exhibited superior growth, higher proline accumulation, elevated SOD/POD/CAT activities, and reduced MDA content compared to wild-type plants under 150 mM NaCl.

Conclusion

This study provides the first genome-wide characterization of the NHX gene family in sweet cherry and demonstrates that *PavNHX37* functions as a key positive regulator of salt tolerance. These findings establish a theoretical foundation and genetic resources for improving salt tolerance in sweet cherry.

1. Introduction

Sweet cherry (*Prunus avium* L.) is a commercially important fruit tree of the Rosaceae family. It is widely cultivated due to the rich nutrition and economic value of its fruits. However, sweet cherry exhibits moderate sensitivity to salinity, and soil salinization has become a major factor limiting the expansion of cultivation and the stability of yield, particularly in arid and semiarid regions [1]. Soil salinization is a key abiotic stress factor restricting global agricultural productivity, affecting an estimated 1 billion hectares of cultivated land worldwide [2]. High concentrations of salt in the soil disrupt cellular ion homeostasis, induce osmotic stress, and trigger excessive accumulation of reactive oxygen species (ROS), ultimately inhibiting plant growth, reducing yield, and even leading to plant death [3–4].

The Na⁺/H⁺ exchanger gene family plays a critical role in maintaining cellular ion homeostasis under salt stress by mediating the transmembrane transport of Na⁺ and H⁺ [5]. NHX proteins are divided into two subgroups: those localized to the plasma membrane (PM) and those localized to the endosomal or vacuolar membrane. These two sub-groups reduce Na⁺ toxicity in the cytoplasm and maintain K⁺/Na⁺ balance by either extruding Na⁺ out of the cell or sequestering Na⁺ into the vacuole, respectively [6]. Overexpression of *AtNHX1* enhances salt tolerance in *Arabidopsis thaliana* [7–8], while *OsNHX1* and *OsNHX2* contribute to salt adaptation in rice by regulating Na⁺ sequestration into the vacuole [9]. In woody plants, *MdNHX* from apple (*Malus domestica* L.) and *PpNHX2* from peach (*Prunus persica* L.) have been shown to improve salt tolerance when heterologously expressed in tobacco [10–11]. High salt concentrations disrupt cellular ion homeostasis, induce osmotic stress, and trigger excessive accumulation of reactive oxygen species (ROS) such as superoxide anion (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (-OH) [12]. These ROS cause oxidative damage to lipids, proteins, and nucleic acids, leading to membrane lipid peroxidation, metabolic disorders, and ultimately growth inhibition or even plant death [13]. In response, plants activate their antioxidant defense system to alleviate ROS induced damage, in which superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) play key roles: SOD catalyzes the dismutation of O₂⁻ into H₂O₂ and O₂, while POD and CAT further scavenge H₂O₂ to prevent oxidative injury [14]. Malondialdehyde (MDA), a product of lipid peroxidation, is widely used as a key indicator of membrane damage under stress [15]. Moreover, under salt stress, osmotic regulatory substances such as proline rapidly accumulate in plant cells, lowering the cellular osmotic potential to facilitate water uptake and protecting enzymes and membrane structures [16].

With the publication of the sweet cherry genome sequence [17], genome wide identification and functional analysis of gene families have become feasible. To date, several stress responsive gene families, such as WRKY and AP2/ERF, have been identified in sweet cherry; however, the NHX family, which plays a central role in ion homeostasis regulation, has not yet been reported [18–19]. Given the conserved function of NHX genes in ion transport and the unique salt response mechanism of sweet cherry, a comprehensive analysis of the NHX family is necessary to fill this research gap. Through genome wide homology screening and domain validation, a total of 37 NHX family members were identified in sweet cherry. Their molecular characteristics, chromosomal distribution, phylogenetic relationships, gene structures, cis acting elements, and expression patterns in different tissues and under salt stress were systematically analyzed. Furthermore, the subcellular localization of the salt responsive gene *PavNHX37* was verified, and its function in regulating the antioxidant system was analyzed in stably overexpressing transgenic tobacco plants. This study reveals the molecular mechanism of salt tolerance in sweet cherry and provides genetic resources for the genetic improvement of salt tolerant varieties.

2. Materials and Methods

2.1. Plant Materials

Plant materials used in this study were in vitro plantlets of sweet cherry cultivar 'Tieton' (*Prunus avium* L.), preserved in the Laboratory of Fruit Tree Physiology and Molecular Biology, Tianshui Normal University. The cultivar was originally obtained and formally identified from the university's Fruit Tree Germplasm Resource Bank. No wild plant materials were sampled. All experimental procedures involving plants were performed in accordance with institutional guidelines and relevant national regulations of China.

Sweet cherry plantlets were subcultured on MS medium containing 1.0 mg/L 6-BA, 0.1 mg/L IBA, 30 g/L sucrose and 7 g/L agar (pH 5.8). Using the paper bridge method, plantlets were exposed to NaCl solutions at concentrations of 0, 50, 100 and 150 mmol/L for 24 h. Leaves were collected with three biological replicates, snap-frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction and reverse transcription.

Nicotiana benthamiana seeds were conserved in the same laboratory. Plants were grown in a controlled environment with a 3:1 (v/v) mixture of nutrient soil and vermiculite, maintained at $25 \pm 2^{\circ}\text{C}$ under a 16 h light/8 h dark photoperiod. Four-week-old seedlings with consistent and vigorous growth were selected for subcellular localization analysis.

For genetic transformation, tobacco seeds were surface-sterilized: rinsed with sterile water for 1 min, immersed in 75% ethanol for 40 s, treated with 5% sodium hypochlorite for 10 s, and washed 4–5 times with sterile water. Sterilized seeds were sown on MS medium supplemented with 30 g/L sucrose and 7 g/L agar (pH 5.8). Seedlings with two cotyledons were used for subsequent transformation experiments.

2.2. Sweet Cherry NHX Gene Family Identification and Physicochemical Property Analysis

The accession numbers of NHX genes were obtained from the Arabidopsis genome database (<https://www.arabidopsis.org>), and the coding sequence of each gene was retrieved from NCBI. The sweet cherry genome annotation file was downloaded from the NCBI genome database (<https://www.ncbi.nlm.nih.gov/datasets/genome/>). The CDS sequences of the sweet cherry genome were converted into protein sequences and simplified using TBtools- v2.382. Homology alignment was performed between the protein sequences of Arabidopsis and sweet cherry, followed by a secondary alignment of the preliminarily screened protein sequences using the BLAST module of NCBI. The gene IDs of the retained sequences were recorded, and their amino acid sequences were extracted. Functional domain screening was conducted using NCBI-CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Candidate genes lacking the specific domains of NHX genes were excluded. Through this screening, a total of 37 NHX gene family members in sweet cherry were identified. Based on the selected NHX amino acid sequences, their basic information, including amino acid number, molecular weight, isoelectric point, and hydrophilicity, was obtained using ExPASy (<https://web.expasy.org/protparam/>).

2.3. Sweet Cherry NHX Gene Family Chromosomal Localization Subcellular Localization and Protein Secondary Structure

The chromosomal positions of sweet cherry NHX genes were predicted and visualized using TBtools- v2.382. Subcellular localization of the NHX gene family was predicted using WoLF PSORT (<https://www.genscript.com/wolf-psort.html>). Protein secondary structure analysis of the NHX gene family was performed using NPSA (https://npsa.lyon.inserm.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html).

2.4. Sweet Cherry NHX Gene Family Phylogenetic Tree Construction and Synteny Analysis

A phylogenetic tree was constructed using the One Step Build a ML Tree function in TBtools- with the neighbor-joining (NJ) method. The tree was then beautified using the ChiPlot tool (<https://www.chiplot.online/#BioPlot>).

Genome and annotation files for synteny analysis were downloaded from the NCBI genome database (<https://www.ncbi.nlm.nih.gov/datasets/genome/>) for grape (*Vitis vinifera* L.), Arabidopsis thaliana, apple (*Malus domestica* L.), woodland strawberry (*Fragaria vesca*), and peach (*Prunus persica* L.). Synteny analysis of sweet cherry NHX genes was performed and visualized using the synteny tool in TBtools-.

2.5. Sweet Cherry NHX Gene Family Gene Structure Motif and Cis-Acting Element Analysis

Gene structure prediction was performed using TBtools- software. Conserved motif analysis was carried out using the MEME website (<http://meme-suite.org/>) with the number of motifs set to 10. Conserved protein domains were analyzed using the NCBI-CDD website (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). The promoter sequences (2000bp upstream of the start codon) of NHX gene family members were extracted. Cis-acting element screening was completed using the PlantCARE website (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>), and visualization was performed using TBtools- software.

2.6. Sweet Cherry NHX Gene Family Codon Usage Bias Analysis and Protein-Protein Interaction Network Analysis

Codon usage bias analysis was performed using R software (version 4.3.3) with the following bioinformatics and statistical packages: seqinr (v4.2-25) for biological sequence manipulation, Biostrings (v2.68-1) for codon parsing of CDS sequences, and ggplot2 (v3.4-4) for visualization.

The protein sequences of the sweet cherry NHX family were used to construct a protein-protein interaction network via the STRING database (<https://string-db.org/>).

2.7. Sweet Cherry NHX Gene Family Tissue-Specific Expression Profile Analysis

Using published sweet cherry tissue expression data [17] the corresponding gene accession numbers of NHX protein members were retrieved. The obtained data were organized using Excel 2007 and subsequently visualized using the ChiPlot tool.

2.8. Sweet Cherry NHX Gene Family qRT-PCR Analysis

Primers were synthesized by Shanghai Bioengineering Technology Co., Ltd. (Table 1). The PCR reaction system (total 20μL) consisted of 7μL ddH₂O, 1μL cDNA, 2μL forward and reverse primers, and 10μL 2× SYBR TaKaRa Premix Ex Taq II. The PCR amplification conditions were 35 cycles as follows: 95°C for 30s, 95°C for 10s, 58°C for 30s, and 72°C for 20s. Three biological replicates and three technical replicates were performed to ensure the reliability of the results. Relative expression levels were calculated using the 2^{-ΔΔCt} method.

Table 1
Primers used in the experiment.

Use	Names of primers	Primer of sequence (5'-3')
Gene-amplification	pCAMBIA2300-PavNHX37-F	GAGAACACGGGGGACGAGCTCATGGACATTGGTTTCCTGGAGA
	pCAMBIA2300-PavNHX37-R	CACCATGGTGTGCGACTCTAGACTCTACTATCTGCTGCTCCCCAA
Plant identification	35s-F	GACGCACAATCCCACTATCC
qRT-PCR	PavNHX03-F	GCCGTCCAGACTGACTTGAA
	PavNHX03-R	GTGTCACCCACGCAATTTC
	PavNHX05-F	GATCCCTACGCTGCTCCTTC
	PavNHX05-R	GGAGACATTGGACTCGGTGG
	PavNHX08-F	GTGTCCTTGTTGGTTTCGGC
	PavNHX08-R	GAGAAGTTTCAGGTCCGCCA
	PavNHX09-F	TCCTGGCGGAGAATTTGCAT
	PavNHX09-R	TCGAACGTCATGCACCTCAA
	PavNHX17-F	GGTGATGGCCTTGAGGGTAC
	PavNHX17-R	TGTGAATGACGCTGTGCTCT
	PavNHX19-F	TCTCGGATCTGCGTTGCTTT
	PavNHX19-R	GTGGTCGCAAAACTGGCAT
	PavNHX22-F	AGCTCAACCAAAAACGACGC
	PavNHX22-R	CCGCCATAGTCTTCTCGTC
	PavNHX25-F	GGTTGCCACCATGATCCAGA
	PavNHX25-R	GATGTTGGTGGCTGTTGCTG
	PavNHX29-F	GCCCTGGTCATAGGCATCTC
	PavNHX29-R	TTCCATCGTGACCGTTCGTT
	PavNHX30-F	CCTTGCTCAGTCGGTGTTCT
	PavNHX30-R	CCTCCCAAAAACAGCACAGC
	PavNHX36-F	TGGAAGCCAGAACCTAGCG
	PavNHX36-R	AAGCGCAACAAAAGCCTCAG
	PavNHX37-F	ACTCAGGCAGCATCATGTCC
	PavNHX37-R	CGGCCATATCCCACTTTGGA
	pavEF1-a1-F	TGAGAGGCTGACTGTGCTGTTC
	pavEF1-a1-R	GGAGTAGTGGCATCCATCTTGTT

2.9. PavNHX37 Overexpression Vector Construction and Nicotiana benthamiana Subcellular Localization

The overexpression vector pCAMBIA2300-PavNHX37-GFP was constructed. Primers were designed with restriction sites (Sacl and XbaI) (Table 1). The construct was then transformed into Agrobacterium tumefaciens strain GV3101. After overnight shaking, the bacterial suspension was stored at -80°C.

The *Agrobacterium* cells harboring the fusion vector were collected by centrifugation after overnight shaking, washed twice with sterile water, and centrifuged again, and the supernatant was discarded. The bacterial pellet was resuspended in resuspension buffer (10mM 2-ethanesulfonic acid, MES pH 5.8, 10mM MgCl₂, 200μM Acetosyringone, AS) to an OD₆₀₀ of 0.6–0.8, followed by shaking at 180rpm at 28°C in darkness for 2–3h. The resuspended bacterial suspension was injected into tobacco leaves that had been water-deprived for two days using a needleless syringe. After dark culture for 24h, the plants were transferred to normal light conditions for an additional 24h. Subcellular localization of the pCambia2300-*PavNHX37*-GFP fusion protein was observed under a laser confocal scanning microscope (LSM710, Carl Zeiss, Germany).

2.10. *Nicotiana benthamiana* Stable Genetic Transformation and Identification

Healthy and sterile *Nicotiana benthamiana* test-tube plantlets were selected, and their leaves were cut into 5mm × 5mm pieces. These leaf pieces were placed on pre-culture medium and cultured in darkness for 2–3 days. The pre-cultured leaf pieces were immersed in an *Agrobacterium* solution containing the overexpression vector pCambia230-*PavNHX37*-GFP. After removing excess bacterial solution, the leaf pieces were transferred to co-culture medium and cultured in darkness for another 3 days. Following co-cultivation, the leaf pieces were transferred to selection medium and cultured under light conditions, with subculture to fresh medium every 1–2 weeks. Once new shoots regenerated, they were transferred to rooting medium. After roots had developed, leaf samples were collected to test the positivity of transgenic plants.

After obtaining the overexpressing tobacco plants, leaves were randomly selected from different plants and individually labeled. Genomic DNA was extracted from the leaves using the TIANGEN Plant Genomic DNA Extraction Kit (Beijing, China). The extracted DNA was used as a template for PCR amplification with gene-specific primers. The amplification products were detected by agarose gel electrophoresis to complete the molecular positive identification of the overexpressing tobacco plants.

2.11. Overexpressing *PavNHX37* Tobacco Enzyme Activity Assay

Tobacco leaf tissue samples from wild-type (WT) and overexpressing (OE) lines treated for 72 h under different conditions were collected. The samples were flash-frozen in liquid nitrogen and ground into powder. Then 0.1g of powder was weighed and pre-cooled extraction buffer (50mmol/L Tris(hydroxymethyl)aminomethane, Tris-HCl pH 8.0, 1mmol/L Ethylenediaminetetraacetic acid, EDTA, 1mmol/L Phenylmethylsulfonyl fluoride, PMSF, 1% Polyvinylpyrrolidone, PVP) was added. After homogenization on ice, the mixture was centrifuged at 12 000r/min for 20min at 4°C. The supernatant was collected as the crude enzyme extract. The activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) as well as the contents of malondialdehyde (MDA) and proline (Pro) were determined using commercial assay kits purchased from Suzhou Grace Biotechnology Co., Ltd.

2.12. Data Processing and Statistical Analysis

All experimental data were statistically analyzed using SPSS 23.0 software. One-way analysis of variance (ANOVA) was performed followed by an ANOVA test ($P < 0.05$). Graphs were generated using Origin Pro 2024 software. All measured data were derived from three independent biological replicates.

3. Result

3.1. Sweet Cherry *pavNHX* Gene Family Identification and Secondary Structure Analysis

A total of 37 NHX family genes were identified in sweet cherry through homology analysis and were named *PavNHX01*–*PavNHX37*. These genes were distributed on eight chromosomes of sweet cherry. Among them, ten genes were located on chromosomes 2 and 6, followed by five genes on chromosomes 1 and 4, three genes on chromosome 5, two genes on chromosome 8, and only one gene on chromosomes 3 and 7 (Fig. 1). The number of amino acids encoded by NHX members ranged from 150 to 1223, with substantial variation even among genes within the same subfamily. The molecular weights of the proteins ranged from 15,971.08 to 132,067.04 Da, the theoretical isoelectric points (pI) ranged from 4.6 to 9.14, and the instability indices ranged from 24.71 to 49.37. The hydrophilicity values ranged from 0.060 to 1.127, with higher values indicating stronger hydrophilicity. Subcellular localization prediction showed that all genes were localized to the plasma membrane except *PavNHX21*, which was localized to the vacuole. The atomic composition of *PavNHX36* could not be calculated due to ambiguous bases in the analyzed sequence (Table 2).

The secondary structure of sweet cherry NHX family proteins was dominated by α-helices (42.00%–68.68%) and random coils (19.95%–39.27%), followed by β-turns (1.18%–4.55%) and extended strands (8.67%–16.64%) (Table 3).

Table 2
physical and chemical properties of *PavNHX* gene family.

gen ID	gen name	Location of Chromosome	Amino acid	Molecular weight/ KD	PI	Instability index:	Aliphatic index:	hydropathicity	Formula	Sub localization
FUN_001618-T1	<i>PavNHX01</i>	chr1	551	60730.92	8.19	45.79	107.35	0.499	C ₂₇₉₀ H ₄₃₄₈ N ₆₉₆ O ₇₆₈ S ₂₅	Plasma membrane
FUN_003199-T1	<i>PavNHX02</i>	chr1	816	88278.88	8.9	37.5	109.31	0.41	C ₄₀₀₈ H ₆₄₄₉ N ₁₀₄₁ O ₁₁₁₅ S ₃₈	Plasma membrane
FUN_004934-T1	<i>PavNHX03</i>	chr1	1148	127491.96	5.94	39.62	102.97	0.096	C ₅₇₈₅ H ₉₀₈₃ N ₁₄₉₉ O ₁₆₆₉ S ₃₆	Plasma membrane
FUN_005739-T2	<i>PavNHX04</i>	chr1	255	27578.24	4.6	29.27	102.43	0.089	C ₁₂₂₈ H ₁₉₅₇ N ₃₁₇ O ₃₉₁ S ₅	Plasma membrane
FUN_005739-T1	<i>PavNHX05</i>	chr1	591	63803.82	5.78	29.29	122.52	0.581	C ₂₉₀₇ H ₄₆₇₅ N ₇₃₁ O ₈₂₇ S ₂₂	Plasma membrane
FUN_008475-T1	<i>PavNHX06</i>	chr2	765	84271.72	7.26	38.9	112.09	0.399	C ₃₈₆₃ H ₆₁₁₅ N ₉₇₃ O ₁₀₇₀ S ₃₀	Plasma membrane
FUN_008684-T1	<i>PavNHX07</i>	chr2	765	84271.72	7.26	38.9	112.09	0.399	C ₃₈₆₃ H ₆₁₁₅ N ₉₇₃ O ₁₀₇₀ S ₃₀	Plasma membrane
FUN_008760-T1	<i>PavNHX08</i>	chr2	841	92082.37	6.21	38.34	102.69	0.179	C ₄₀₉₉ H ₆₅₅₆ N ₁₀₉₂ O ₁₂₂₁ S ₄₄	Plasma membrane
FUN_011354-T1	<i>PavNHX09</i>	chr2	912	100683.18	5.99	34.46	98.12	0.084	C ₄₄₉₆ H ₇₁₃₃ N ₁₁₉₅ O ₁₃₂₆ S ₄₈	Plasma membrane
FUN_012393-T1	<i>PavNHX10</i>	chr2	813	90061.57	9.13	40.34	109.91	0.264	C ₄₁₀₀ H ₆₅₄₈ N ₁₀₇₂ O ₁₁₃₄ S ₃₃	Plasma membrane
FUN_012394-T1	<i>PavNHX11</i>	chr2	1223	132067.04	5.13	41.37	104.88	0.06	C ₅₈₅₄ H ₉₄₅₂ N ₁₅₉₆ O ₁₈₀₁ S ₃₃	Plasma membrane
FUN_012399-T1	<i>PavNHX12</i>	chr2	685	75828.07	5.95	37.33	111.53	0.416	C ₃₄₂₆ H ₅₄₆₁ N ₈₈₁ O ₉₆₂ S ₄₅	Plasma membrane
FUN_012409-T1	<i>PavNHX13</i>	chr2	292	32247.32	8.78	32.53	124.28	0.623	C ₁₅₀₂ H ₂₃₇₅ N ₃₆₇ O ₃₉₃ S ₁₂	Plasma membrane
FUN_012716-T2	<i>PavNHX14</i>	chr2	150	15971.08	7.67	24.71	126.67	1.127	C ₇₄₃ H ₁₁₆₄ N ₁₇₆ O ₁₉₃ S ₁₀	Plasma membrane
FUN_012716-T1	<i>PavNHX15</i>	chr2	816	90019.67	6.71	44.19	117.34	0.39	C ₄₀₆₄ H ₆₅₄₆ N ₁₀₇₆ O ₁₁₃₉ S ₄₁	Plasma membrane
FUN_017854-T1	<i>PavNHX16</i>	chr3	572	61910.63	5.64	31.76	117.55	0.622	C ₂₈₃₂ H ₄₄₉₈ N ₆₉₈ O ₇₉₇ S ₂₆	Plasma membrane
FUN_031503-T1	<i>PavNHX17</i>	chr4	540	59315.46	5.53	40.03	99.69	0.427	C ₂₇₂₅ H ₄₁₅₃ N ₆₇₅ O ₇₆₁ S ₂₄	Plasma membrane
FUN_031504-T1	<i>PavNHX18</i>	chr4	824	90950.62	7.32	45.92	109.3	0.331	C ₄₁₁₁ H ₆₅₆₈ N ₁₀₇₂ O ₁₁₅₄ S ₄₆	Plasma membrane
FUN_032382-T1	<i>PavNHX19</i>	chr4	540	59315.46	5.53	40.03	99.69	0.427	C ₂₇₂₅ H ₄₁₅₃ N ₆₇₅ O ₇₆₁ S ₂₄	Plasma membrane
FUN_035179-T1	<i>PavNHX20</i>	chr4	551	61702.79	7.21	37.22	112.96	0.58	C ₂₈₅₃ H ₄₄₄₆ N ₇₀₂ O ₇₅₈ S ₃₁	Plasma membrane
FUN_035478-T1	<i>PavNHX21</i>	chr4	525	58253.25	7.18	32.95	115.89	0.601	C ₂₇₀₂ H ₄₁₉₅ N ₆₆₅ O ₇₂₆ S ₂₀	Vacuole
FUN_024568-T1	<i>PavNHX22</i>	chr5	856	92668.69	5.5	35.91	108.9	0.355	C ₄₁₆₁ H ₆₆₄₇ N ₁₁₀₅ O ₁₂₀₀ S ₄₁	Plasma membrane
FUN_025222-T1	<i>PavNHX23</i>	chr5	790	87368.57	6.23	37.18	109.59	0.331	C ₃₉₇₉ H ₆₂₅₇ N ₁₀₂₅ O ₁₁₁₈ S ₃₂	Plasma membrane
FUN_025223-T1	<i>PavNHX24</i>	chr5	800	86656.83	8.79	40.99	114.7	0.415	C ₃₉₁₅ H ₆₃₃₇ N ₁₀₄₅ O ₁₀₉₃ S ₃₅	Plasma membrane
FUN_018802-T1	<i>PavNHX25</i>	chr6	808	87403.31	8.75	36.16	108.13	0.351	C ₃₉₃₂ H ₆₃₄₃ N ₁₀₅₇ O ₁₁₁₀ S ₃₈	Plasma membrane
FUN_018803-T1	<i>PavNHX26</i>	chr6	758	83582.65	6.19	40.16	107.57	0.365	C ₃₇₉₅ H ₅₉₉₉ N ₉₆₃ O ₁₀₇₂ S ₄₁	Plasma membrane
FUN_018804-T1	<i>PavNHX27</i>	chr6	754	83357.53	8.06	37.76	105.46	0.343	C ₃₇₉₀ H ₅₉₇₂ N ₉₈₄ O ₁₀₄₅ S ₄₁	Plasma membrane

gen ID	gen name	Location of Chromosome	Amino acid	Molecular weight/ KD	PI	Instability index:	Aliphatic index:	hydropathicity	Formula	Sub localization
FUN_018805-T1	<i>PavNHX28</i>	chr6	759	83622.04	7.15	49.37	111.73	0.389	C ₃₈₀₄ H ₆₀₄₈ N ₉₆₆ O ₁₀₆₆ S ₃₉	Plasma membrane
FUN_018806-T1	<i>PavNHX29</i>	chr6	696	77297.88	7.87	48.48	105.42	0.312	C ₃₄₆₅ H ₅₅₅₃ N ₉₀₇ O ₉₈₀ S ₅₃	Plasma membrane
FUN_018816-T1	<i>PavNHX30</i>	chr6	757	83456.67	6.37	45.41	114.32	0.39	C ₃₇₉₄ H ₆₀₃₇ N ₉₆₇ O ₁₀₆₉ S ₃₆	Plasma membrane
FUN_021849-T1	<i>PavNHX31</i>	chr6	858	94339.05	6.13	35.5	110.83	0.371	C ₄₃₂₀ H ₆₇₉₁ N ₁₁₀₅ O ₁₁₉₄ S ₃₂	Plasma membrane
FUN_021851-T1	<i>PavNHX32</i>	chr6	848	91916.5	9.14	39.94	107.84	0.294	C ₄₁₅₉ H ₆₆₉₅ N ₁₁₁₇ O ₁₁₆₃ S ₃₀	Plasma membrane
FUN_022777-T1	<i>PavNHX33</i>	chr6	782	84719.33	8.73	43.09	114.46	0.38	C ₃₈₂₂ H ₆₁₉₂ N ₁₀₃₀ O ₁₀₇₂ S ₃₁	Plasma membrane
FUN_022826-T1	<i>PavNHX34</i>	chr6	543	59396.33	6.51	36.53	107.96	0.57	C ₂₇₄₃ H ₄₂₆₉ N ₆₆₅ O ₇₆₀ S ₂₁	Plasma membrane
FUN_039269-T1	<i>PavNHX35</i>	chr7	539	59876.29	8.83	34.46	113.91	0.545	C ₂₇₇₀ H ₄₃₃₀ N ₆₉₀ O ₇₄₄ S ₂₁	Plasma membrane
FUN_029070-T1	<i>PavNHX36</i>	chr8	616	66511.05	5.72	35.9	116.93	0.574	-	Plasma membrane
FUN_031269-T1	<i>PavNHX37</i>	chr8	785	85778.87	7.58	35.07	118.78	0.523	C ₃₉₅₂ H ₆₂₉₀ N ₉₉₄ O ₁₀₈₀ S ₂₄	Plasma membrane

Table 2
Secondary structure analysis of *PavNHX* protein.

Gen name	Alpha helix	Beta turn	Extended strand	Random coil
PavNHX01	48.82	3.09	13.07	35.03
PavNHX02	45.1	3.68	14.58	36.64
PavNHX03	55.66	3.22	10.19	30.92
PavNHX04	57.25	1.18	11.37	30.2
PavNHX05	59.73	2.03	10.15	28.09
PavNHX06	46.54	4.18	15.42	33.86
PavNHX07	44	4.04	14.98	36.98
PavNHX08	42	4.17	15.57	38.27
PavNHX09	68.68	2.7	8.67	19.95
PavNHX10	44.16	3.94	15.13	36.78
PavNHX11	46.04	3.9	16.1	33.96
PavNHX12	45.84	4.38	16.64	33.14
PavNHX13	43.75	3.43	15.44	37.38
PavNHX14	62.67	3.33	12.67	21.33
PavNHX15	60.31	1.92	11.19	26.57
PavNHX16	44.3	3.88	14.81	37.01
PavNHX17	51.11	3.52	14.07	31.3
PavNHX18	51.11	3.52	14.07	31.3
PavNHX19	44.75	4.12	15.62	35.5
PavNHX20	46.86	3.81	14.76	34.56
PavNHX21	44.28	3.39	14.6	37.73
PavNHX22	45.44	3.42	14.68	36.46
PavNHX23	45.62	3.5	15.5	35.38
PavNHX24	44.31	3.71	15.22	36.76
PavNHX25	44.69	3.98	16.45	34.88
PavNHX26	44.33	4.09	16.09	35.49
PavNHX27	44.01	4.35	16.07	35.57
PavNHX28	44.12	4.49	16.38	35.01
PavNHX29	45.69	4.02	16.24	34.05
PavNHX30	42.54	4.55	14.57	38.34
PavNHX31	45.91	3.71	15.6	34.78
PavNHX32	43.04	3.77	13.92	39.27
PavNHX33	49.36	3.5	14	33.15
PavNHX34	49.35	3.53	13.54	33.58
PavNHX35	59.58	2.44	12.18	25.81
PavNHX36	55.54	3.44	11.97	29.04
PavNHX37	47.91	3.27	13.43	35.39

3.2. Sweet Cherry *pavNHX* Gene Family Evolutionary Analysis

To further understand the evolutionary characteristics of the NHX gene family, a phylogenetic tree was constructed using NHX family members from sweet cherry and *Arabidopsis thaliana* (Fig. 2). The family members were divided into three groups. Group I contained eight sweet cherry genes and eight *Arabidopsis* genes. Group II and Group III consisted entirely of sweet cherry NHX family members, with six and twenty-three members respectively. Among

the three groups, only Group I contained Arabidopsis NHX family members, indicating that NHX gene family members are highly conserved within the groups.

3.3. Sweet Cherry *pavNHX* Gene Family Protein Structure and Motif Analysis

The conserved structural domains of the sweet cherry NHX gene family were analyzed online using the MEME software, and the family structures were compared and localized. Among the members, *PavNHX03*, *PavNHX04*, *PavNHX18*, *PavNHX17*, and *PavNHX33* contained only one motif. *PavNHX22*, *PavNHX20*, *PavNHX08*, *PavNHX07*, *PavNHX21*, *PavNHX32*, *PavNHX31*, *PavNHX02*, *PavNHX24*, *PavNHX23*, *PavNHX30*, *PavNHX10*, *PavNHX12*, *PavNHX13*, *PavNHX16*, *PavNHX06*, *PavNHX26*, *PavNHX28*, and *PavNHX27* all shared the same motifs, indicating a high degree of conservation and suggesting that their functions are largely similar. Furthermore, *PavNHX37*, *PavNHX01*, and *PavNHX35* contained only three motifs, while *PavNHX34* contained only two motifs (Fig. 3a).

Protein structure analysis of the sweet cherry NHX gene family revealed that these genes contain 0 to 22 introns. *PavNHX25* contained no intron, while the longest intron was found in *PavNHX12*. Additionally, *PavNHX05*, *PavNHX35*, *PavNHX15*, *PavNHX14*, *PavNHX17*, *PavNHX33*, *PavNHX34*, *PavNHX01*, *PavNHX09*, *PavNHX26*, *PavNHX32*, *PavNHX24*, and *PavNHX23* possessed exons, whereas the remaining genes lacked exons. Notable differences in exon length and position were observed, which may contribute to functional divergence among these genes (Fig. 3b).

3.4. Sweet Cherry *pavNHX* Gene Family Promoter Cis-Acting Element Prediction

Among the 37 predicted cis-acting promoter elements of the sweet cherry NHX gene family, 32 response elements were identified. All NHX gene family members contained the following elements: a cis-acting regulatory element involved in MeJA responsiveness, a cis-acting element involved in salicylic acid responsiveness, a cis-acting element involved in abscisic acid responsiveness, a MYB binding site involved in drought inducibility, a cis-acting element involved in auxin responsiveness, a cis-acting regulatory element root specific, an auxin-responsive element, a MYB-like binding site in flavonoid biosynthetic genes regulation, and a cis-acting regulatory element related to meristem specific activation. Different members contained different response elements. In addition to the elements listed above, *PavNHX01*, *PavNHX11*, and *PavNHX27* contained a cis-acting regulatory element involved in zein metabolism regulation. *PavNHX04* and *PavNHX05* lacked the cis-acting element involved in low-temperature responsiveness. *PavNHX23* and *PavNHX12* contained the fewest response elements, only 12 elements, whereas *PavNHX01* contained the most with 23 elements (Fig. 4).

3.5. Sweet Cherry *pavNHX* Gene Family Tissue-Specific Expression Analysis

To elucidate the functions of *PavNHX* genes in sweet cherry growth and development, the expression patterns of 37 *PavNHX* genes across 15 tissues and fruit developmental stages were analyzed. After $\text{Log}_2(\text{FPKM} + 1)$ normalization, a heatmap was generated (Fig. 5). The results showed that the *PavNHX* gene family exhibited significant tissue-specific and stage-specific expression characteristics. All members could be clearly divided into two major expression clusters. The first cluster was a high-expression cluster, containing genes such as *PavNHX24*, *PavNHX23*, *PavNHX34*, and *PavNHX18*. These genes maintained consistently high expression levels in vegetative organs including annual shoots, young leaves, and mature leaves. Among them, *PavNHX24* and *PavNHX34* showed the highest expression levels and stable expression in most tissues. The second cluster was a low-expression cluster, containing genes such as *PavNHX22*, *PavNHX30*, *PavNHX29*, and *PavNHX06*. These genes exhibited extremely low expression in most vegetative organs but displayed distinct targeted expression characteristics: *PavNHX29* was significantly upregulated at stem stages 2–3; *PavNHX30* and *PavNHX22* showed elevated expression in middle-to-late fruit development stages; *PavNHX06* and *PavNHX08* accumulated a small amount of expression in floral organs. In addition, some genes such as *PavNHX04* and *PavNHX16* maintained low expression levels across all 15 tissues and stages without obvious tissue preference.

3.6. Sweet Cherry *pavNHX* Gene Family Synteny Analysis

Intraspecific synteny analysis of the sweet cherry NHX gene family revealed three pairs of duplicated genes located on chromosomes 1, 7, 6, and 2. However, no linked genes were found on chromosomes 3, 4, 5, and 8 (Fig. 6). To further elucidate the phylogenetic mechanism of the sweet cherry NHX gene family as well as the relationship between paralogy and orthology, synteny analysis was performed between sweet cherry and five representative species. The results showed that sweet cherry shared 16 syntenic gene pairs with grape berry, 17 with Arabidopsis thaliana, 12 with apple, 22 with woodland strawberry, and 30 with peach (Fig. 7). Some NHX genes were found to be associated with more than two syntenic gene pairs, while others had no syntenic counterpart. These results indicate that syntenic relationships exist both within the sweet cherry NHX gene family and between sweet cherry and other species, and that some family genes are closely linked but not entirely orthologous.

3.7. Sweet Cherry NHX Gene Family Codon Usage Bias Analysis

The relative synonymous codon usage (RSCU) of the 37 sweet cherry NHX genes was analyzed (Fig. 8). $\text{RSCU} > 1$ indicates preferred codons. $\text{RSCU} < 1$ indicates non-preferred codons. $\text{RSCU} = 1$ indicates no usage bias. For leucine (Leu) and serine (Ser) which have two codon families each they were analyzed separately as Leu1/Leu2 and Ser1/Ser2. All 22 amino acids showed varying degrees of synonymous codon usage bias. The total RSCU values varied among amino acids. Leu1 had the highest total RSCU value significantly higher than Leu2. The total RSCU values of alanine (Ala) arginine (Arg) glycine (Gly) proline (Pro) threonine (Thr) and valine (Val) were all close to 4. The total RSCU values of asparagine (Asn) aspartic acid (Asp) cysteine (Cys) glutamine (Gln) glutamic acid (Glu) histidine (His) isoleucine (Ile) lysine (Lys) methionine (Met) phenylalanine (Phe) threonine (Thr) tryptophan (Trp) and tyrosine (Tyr) were all close to 2. In addition, the rare codons showed specific RSCU values. For example, the RSCU value of Trp's UGG was approximately 2. Overall, the preferred codons of the 37 sweet cherry NHX genes all ended with U or C while non-preferred codons tended to end with A or G. This indicates that the synonymous codon usage of these genes exhibits a significant bias toward U or C endings.

3.8. Sweet Cherry NHX Gene Family Protein-Protein Interaction Network Analysis

The interaction network analysis tool was used to predict the interaction relationships among sweet cherry PavNHX proteins (Fig. 9). The PavNHX gene family members formed a complete interaction network. Thirty members showed interaction connections while seven members were isolated nodes indicating extensive interactions among family members. PavNHX35, PavNHX22 and PavNHX06 were the core nodes in the network. These nodes had the largest size and the darkest color with interaction strength close to the highest threshold value of 0.900. They also exhibited dense connections with many surrounding genes making them key hubs in the regulatory network. PavNHX19, PavNHX36 and PavNHX14 showed intermediate node sizes and interaction strengths. In contrast PavNHX12, PavNHX07 and PavNHX27 had the smallest node sizes and the lightest colors with relatively low interaction strength. These genes had only single connections with a few genes and were located at the periphery of the interaction network.

3.9. Sweet Cherry NHX Gene Family Member Expression Analysis

To further analyze the expression patterns of gene members twelve genes were selected based on the gene family phylogenetic tree for qRT-PCR analysis. The qRT-PCR results showed that the expression levels of six sweet cherry NHX gene family members decreased with increasing salt concentration. Compared with the control *PavNHX03*, *PavNHX08* and *PavNHX17* showed significantly reduced expression under high salt stress among which *pavNHX17* decreased by 10-fold. The relative expression levels of *PavNHX19* and *PavNHX36* did not change significantly under the salt stress. In addition, the relative expression trends of *PavNHX09* and *PavNHX25* first decreased and then increased. Furthermore, we found that the relative expression of *PavNHX37* increased significantly reaching 8.9-fold that of the control (Fig. 10).

3.10. *PavNHX37* Sequence Analysis and Subcellular Localization

The *PavNHX37* gene is located on sweet cherry chromosome 8 at positions 207796 to 211408. Its full length is 1991 bp. The CDS coding region is 1656 bp in length encoding 551 amino acids (Fig. 11a). A conserved Na⁺/H⁺ exchanger domain is present at amino acid positions 34 to 455. The fusion expression vector pCambia2300-*PavNHX37*-GFP was used (Fig. 11b). The constructed fusion expression vector and the pCambia2300-GFP negative control were injected into tobacco leaves via *Agrobacterium*. The results showed that the negative control was expressed in both the nucleus and the plasma membrane whereas *PavNHX37* was localized to the plasma membrane. This result is consistent with the subcellular localization prediction (Fig. 11c).

3.11. *PavNHX37* Overexpressing Tobacco Functional Verification

Positive transgenic lines were screened by genomic PCR using gene-specific primers. As shown in Fig. 12a a specific band of approximately 1500 bp was successfully amplified in most candidate overexpression lines. This band was consistent with the positive control (P0) and the expected size of the target gene fragment. In contrast wild-type (WT) plants showed no such specific band. Four lines OE-8 OE-13 OE-14 and OE-17 failed to amplify the target gene fragment and were therefore excluded from subsequent experiments.

The expression levels of the target gene in wild-type and overexpressing lines under different NaCl treatments were detected by qRT-PCR (Fig. 12c). Under normal control conditions (0 mM NaCl) the basal expression level of the target gene was very low in wild-type plants. All tested overexpression lines showed significantly elevated gene expression levels approximately 40-fold higher than that of the wild-type ($P < 0.05$). This confirmed that the exogenous gene had been successfully overexpressed.

Wild-type plants and the selected overexpression lines were treated with 0 50 100 and 150 mM NaCl solutions. Under non-stress conditions (0 mM NaCl) no significant differences in growth status or morphological characteristics were observed between wild-type and overexpression lines (Fig. 12b). This indicated that overexpression of the target gene did not affect normal plant growth and development. With increasing NaCl concentration wild-type plants showed obvious growth inhibition leaf yellowing and wilting. In contrast the overexpression lines exhibited significantly enhanced salt tolerance. Under 50 mM NaCl treatment the overexpression lines showed greater growth vigor larger leaves and higher biomass compared to wild-type plants. Under moderate (100 mM NaCl) and severe (150 mM NaCl) salt stress the growth advantage of the OE lines became more pronounced. Notably even under 150 mM NaCl treatment the OE lines maintained good leaf condition and vigorous growth whereas wild-type plants displayed severe growth dwarfism and leaf senescence.

3.12. Overexpressing *PavNHX37* Tobacco Enzyme Activity Assay

The enzyme activity assay results after NaCl treatment of overexpressing tobacco showed that proline accumulation in overexpressing plants was significantly higher than that in wild-type plants while MDA content was significantly lower. This indicated enhanced osmotic regulation capacity and reduced oxidative damage. Meanwhile the activities of SOD POD and CAT antioxidant enzymes were significantly increased in overexpressing plants. Under 150 mM NaCl treatment the activities of these three enzymes reached 3.0-fold 3.2-fold and 1.2-fold those of wild-type plants respectively. Overexpression of the target gene significantly enhanced salt tolerance in tobacco plants by strengthening osmotic protection reducing oxidative damage and increasing reactive oxygen species scavenging capacity (Fig. 13).

4. Discussion

In this study 37 NHX gene family members were identified in sweet cherry for the first time through genome-wide homology alignment combined with domain validation. This number is significantly higher than that in cotton which has 24 members [11]. The 37 *PavNHX* genes were unevenly distributed on eight chromosomes. Chromosomes 2 and 6 each contained 10 members suggesting that these chromosomes may be enriched regions for the NHX gene family [21]. The amino acid lengths (150–1223 amino acids) and molecular weights (15.97–132.07 kD) of *PavNHX* proteins varied considerably. Their secondary structures were dominated by α -helices (42.00%–68.68%) and random coils (19.95%–39.27%). This is highly consistent with the structural

features of NHX proteins as transmembrane transporters: α -helices typically form transmembrane domains to mediate ion transmembrane transport while random coils may participate in protein-protein or protein-molecule interactions [22]. Furthermore, subcellular localization prediction showed that all *PavNHX* members were localized to the plasma membrane except *PavNHX21* which was localized to the vacuole. This result is consistent with the classification of the oat NHX family into plasma membrane (PM) vacuolar (Vac) and endosomal (Endo) subfamilies [23].

Phylogenetic analysis divided the *PavNHX* family into three groups. Group I contained eight sweet cherry members and eight Arabidopsis members. This suggests that genes in this group are highly conserved during evolution and may perform basic functions in plant ion transport [5]. Group II (6) and Group III (22) contained only sweet cherry members. They are speculated to be sweet-cherry-specific expanded subfamilies which is similar to the conserved subfamily results of the mung bean NHX family [24]. Synteny analysis showed that sweet cherry shared 30 syntenic gene pairs with peach which belongs to the same *Prunus* genus. This number is significantly higher than the numbers with grape (16) and Arabidopsis (17). This pattern is consistent with the phylogenetic relationships among these species [25].

The tissue expression profiles of *PavNHX* genes showed significant specificity. High-expression cluster genes such as *PavNHX24* and *PavNHX34* were consistently highly expressed in vegetative organs including annual shoots and young leaves. Low-expression cluster genes such as *PavNHX29* and *PavNHX30* were upregulated at specific stages of stem development or during late fruit maturation. In tobacco *NtNHX1-3* showed high expression in leaves but low or no expression in other tissues [26]. As photosynthetic organs and primary sites of salt stress response leaves require sustained ion transport capacity to maintain physiological functions [27]. Promoter cis-element analysis revealed that all *PavNHX* genes contained MeJA responsiveness elements salicylic acid responsiveness elements ABA responsiveness elements and drought-inducible MYB binding sites. Salt stress often occurs together with drought stress. ABA as a core stress-responsive hormone can maintain ion balance by regulating NHX gene expression [28]. This is consistent with the salt-stress response patterns of *PavNHX* genes observed in this study.

qRT-PCR analysis of 12 representative *PavNHX* genes under salt stress revealed distinct expression patterns. Six genes including *PavNHX03* and *PavNHX17* showed decreased expression with increasing salt concentration possibly due to feedback inhibition. *PavNHX37* expression under 150 mM salt stress was 8.9-fold that of the control making it the most significantly salt-stress-responsive gene. This finding is similar to results in quinoa [29]. Following salt stress treatment wild-type tobacco exhibited varying degrees of wilting and chlorosis while overexpressing tobacco lines did not. Enzyme activity assays showed significantly increased activities of SOD POD and CAT decreased MDA content and increased proline accumulation. Proline accumulation is one of the important physiological responses of plants to stress [30]. These results suggest that *PavNHX37* may enhance salt tolerance through a dual mechanism: on one hand maintaining ion homeostasis via Na^+/H^+ exchange and on the other hand regulating the antioxidant system to scavenge ROS and accumulating osmolytes to alleviate stress damage [31]. Furthermore, although *PavNHX37* is not a core node in the protein-protein interaction network it interacts with multiple family members. This implies that *PavNHX37* may participate in a complex ion transport regulatory network and optimize salt tolerance through synergistic actions with other NHX proteins [32].

5. Conclusions

In this study, 37 NHX gene family members were systematically identified for the first time in sweet cherry. Their molecular characteristics, phylogenetic relationships, and tissue-specific expression patterns were elucidated, and *PavNHX37* was screened as a key gene regulating salt tolerance. Overexpression of *PavNHX37* in tobacco significantly enhanced salt tolerance by maintaining ion homeostasis and increasing antioxidant enzyme activities. This study provides a theoretical basis and genetic resources for improving salt tolerance in sweet cherry.

Declarations

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Authors' contributions

Z.G, Y.Z., and S.H. conceived and designed the research. X.A., Z.G., Y.Z., S.H performed the experiments. X.A. and Z.G. performed the data analysis and wrote the manuscript. S.H. and Z.G. supervised the research. X.A. revised the manuscript. All authors read and approved the final manuscript.

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Data availability

The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Ethics approval and consent to participate

The authors declare that no studies involving human participants or animals were conducted for this article. All methods complied with applicable guidelines and regulations, and Tianshui Normal University approved every experimental protocol.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Figures

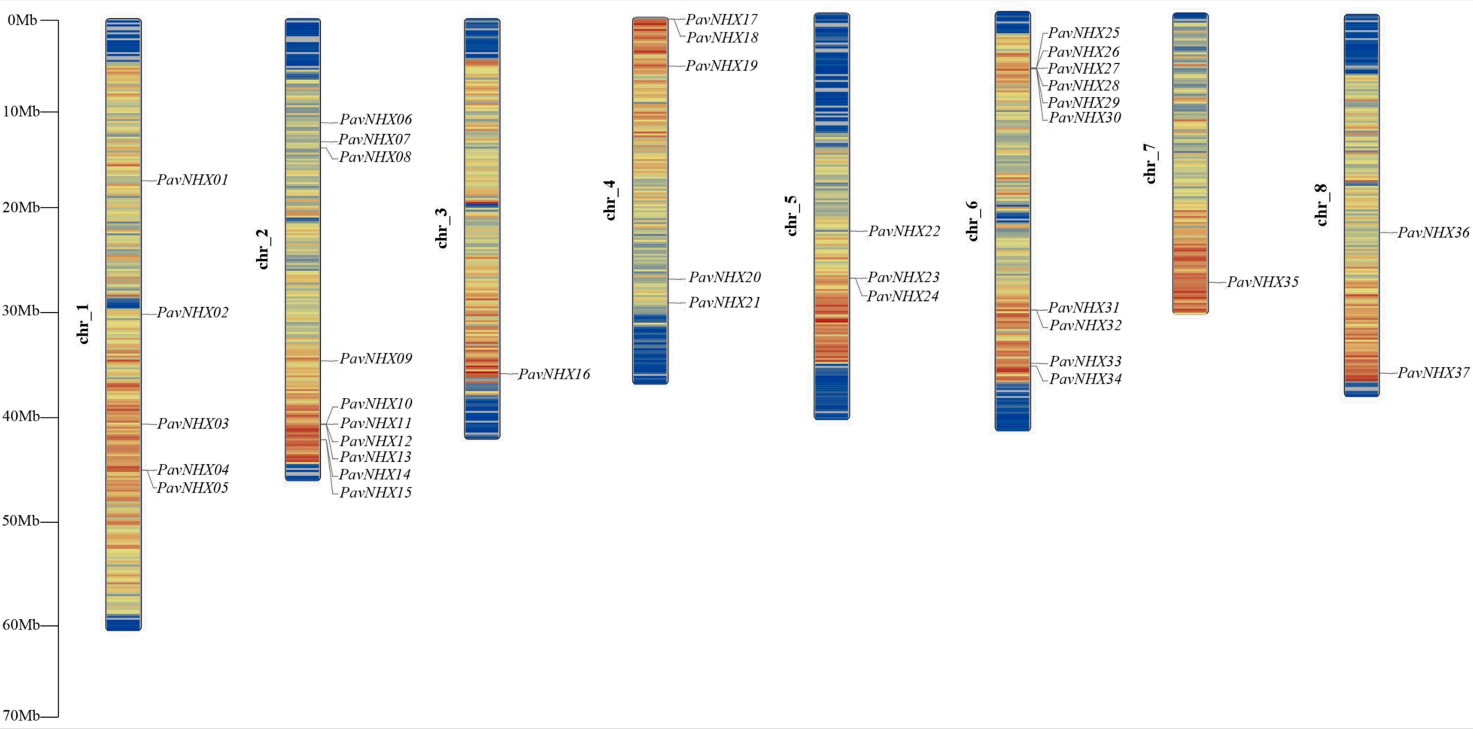


Figure 1

Chromosomal localization of 37 *PavNHX* genes on eight sweet cherry chromosomes

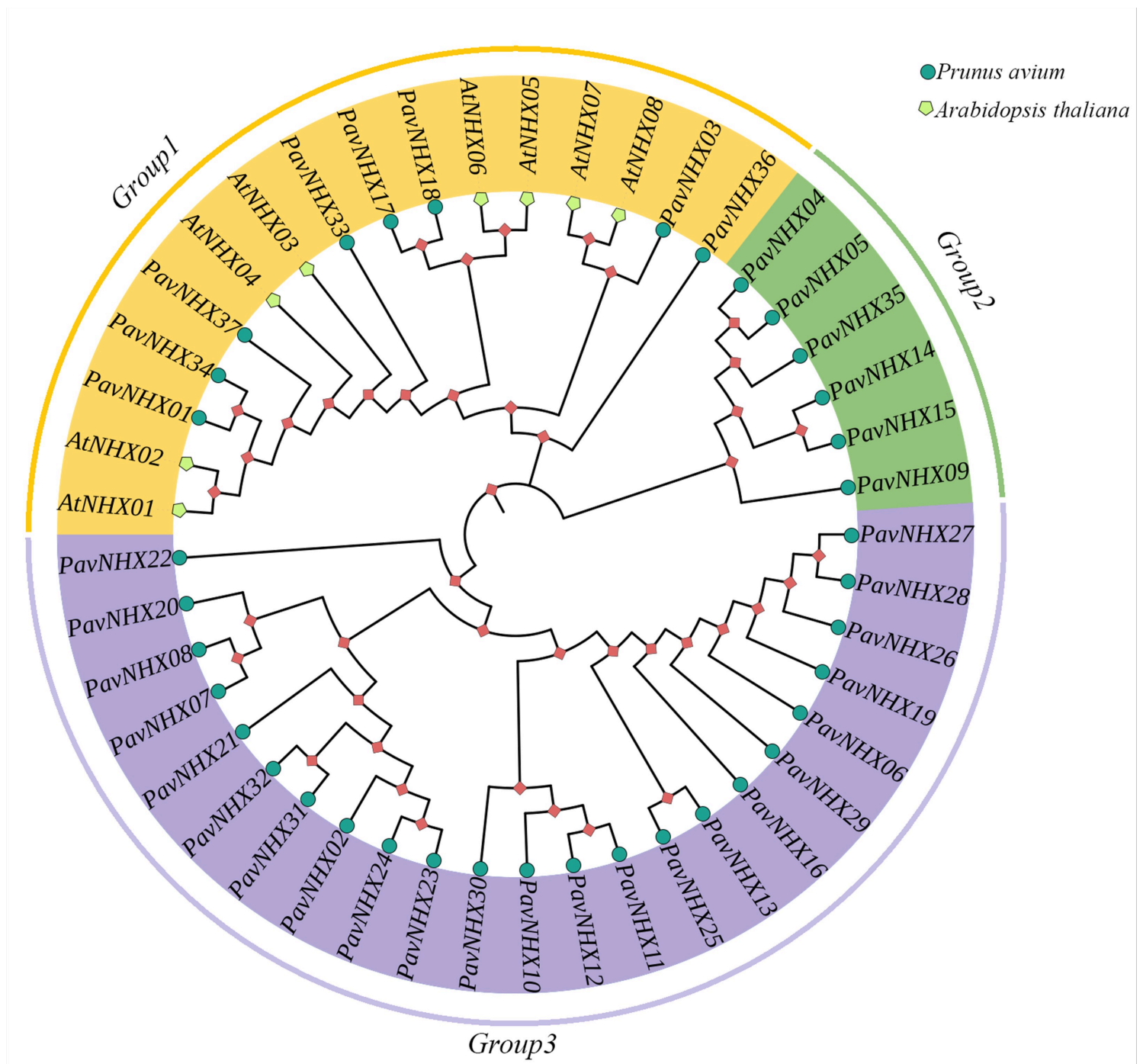


Figure 2

Phylogenetic tree analysis of the NHX gene family in sweet cherry.

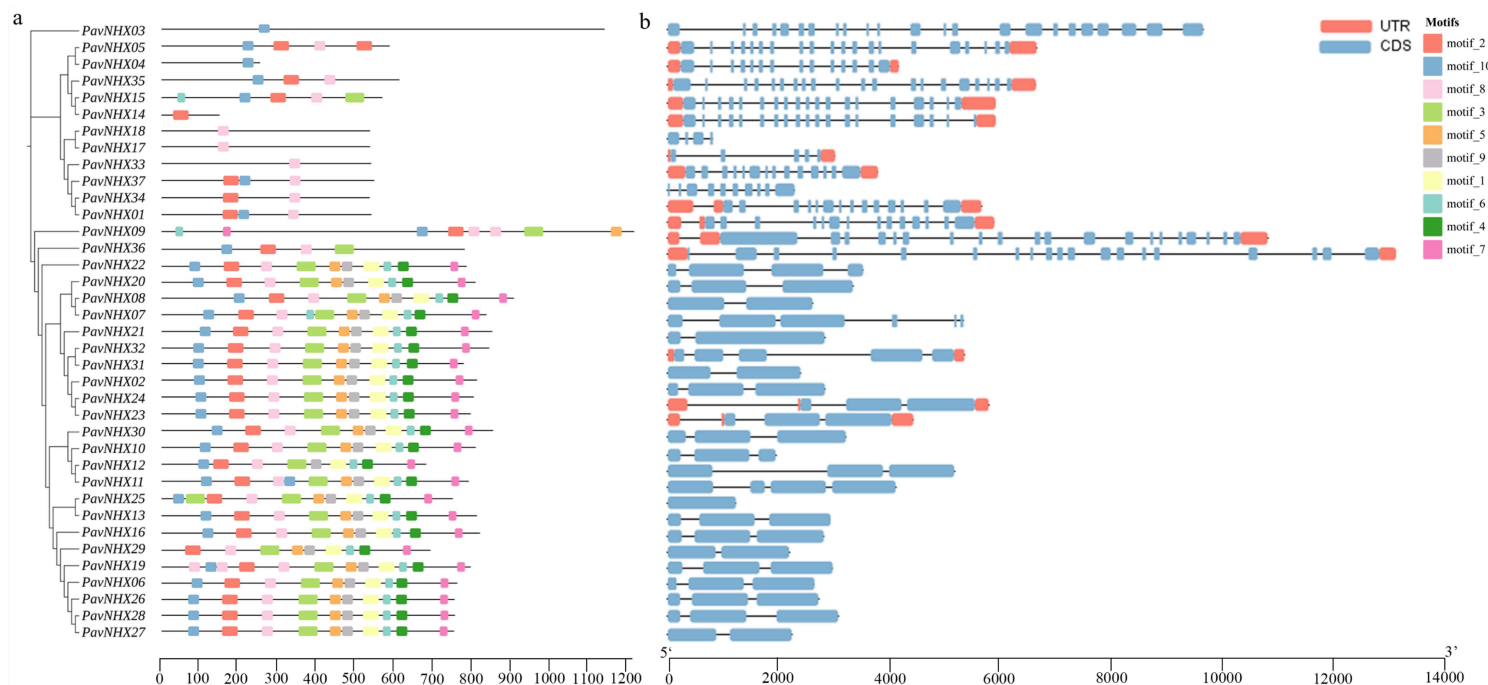


Figure 3
Conserved motifs and gene structures of *PavNHX* family genes in sweet cherry. (a) Conserved protein motifs; ten distinct motifs are colored as indicated, scale=amino acids; (b) Gene structure; CDS (blue boxes), UTR (red boxes), introns (gray lines), scale=base pairs (bp).

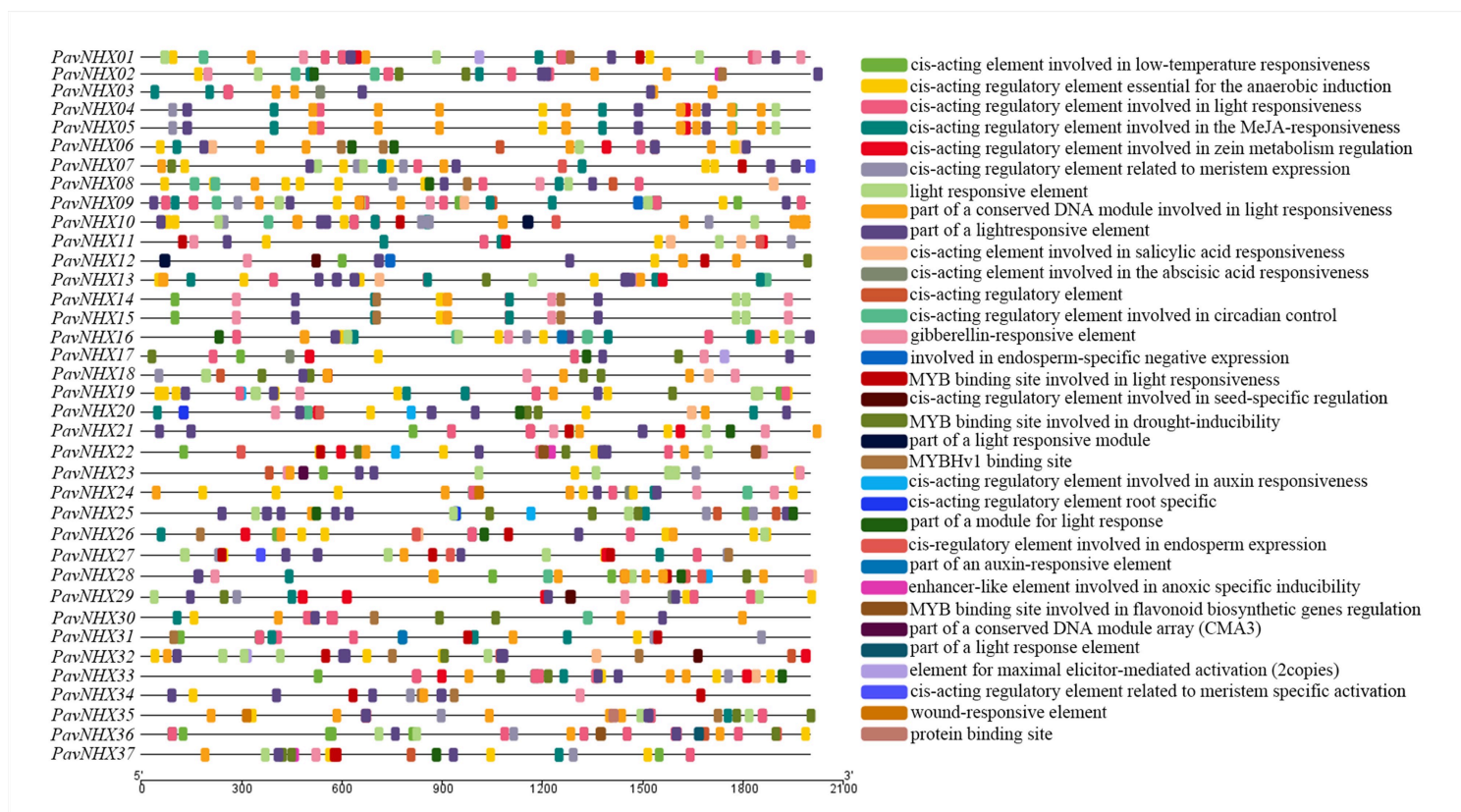


Figure 4
Cis-acting regulatory elements in *PavNHX* promoters. The 2000 bp upstream regions of *PavNHX* genes contain diverse hormone, light, and stress-responsive elements. Scale bar: position relative to the transcription start site (bp).

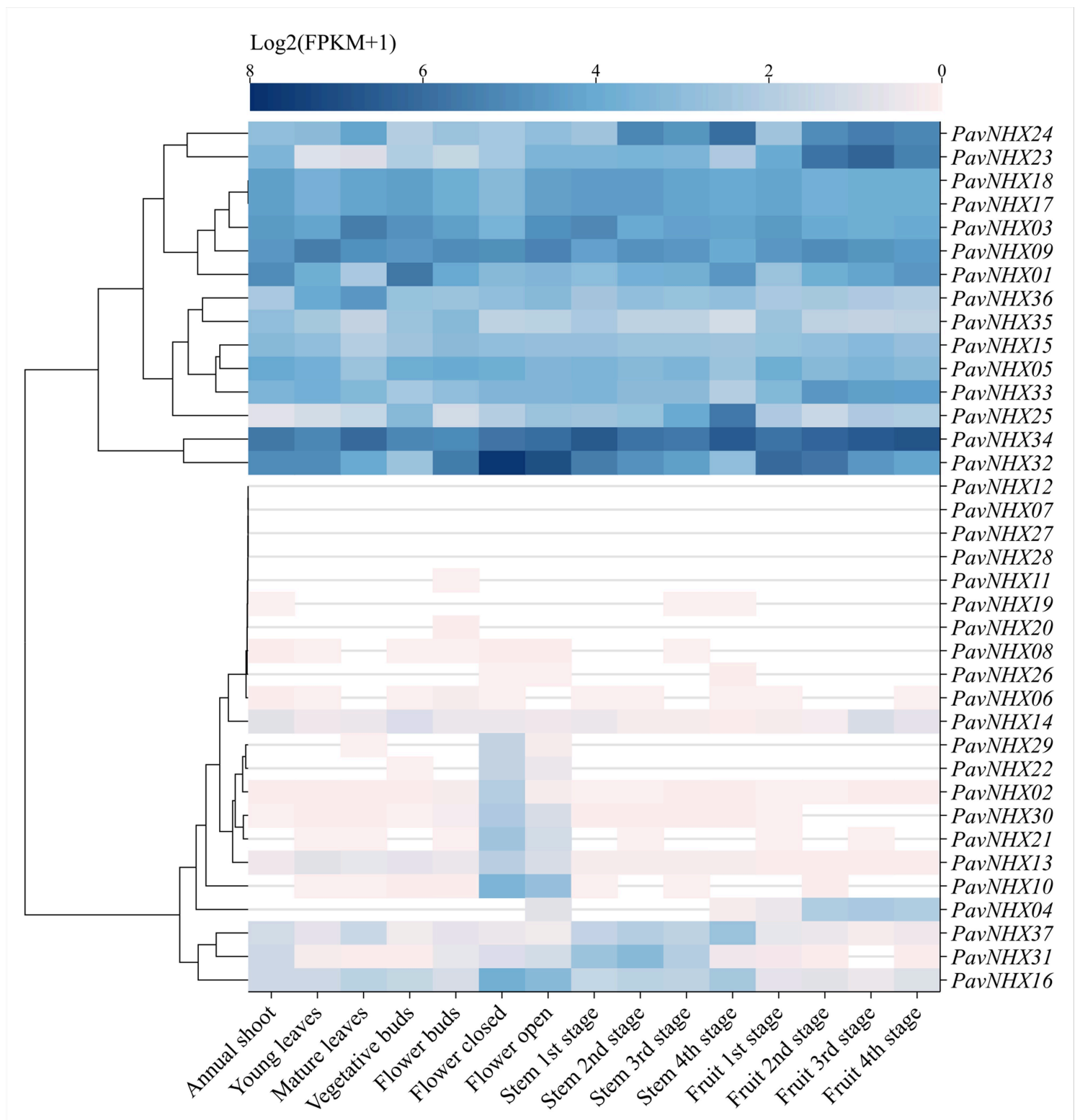


Figure 5

Expression heatmap of 37 *PavNHX* genes across 15 sweet cherry tissues and fruit developmental stages.

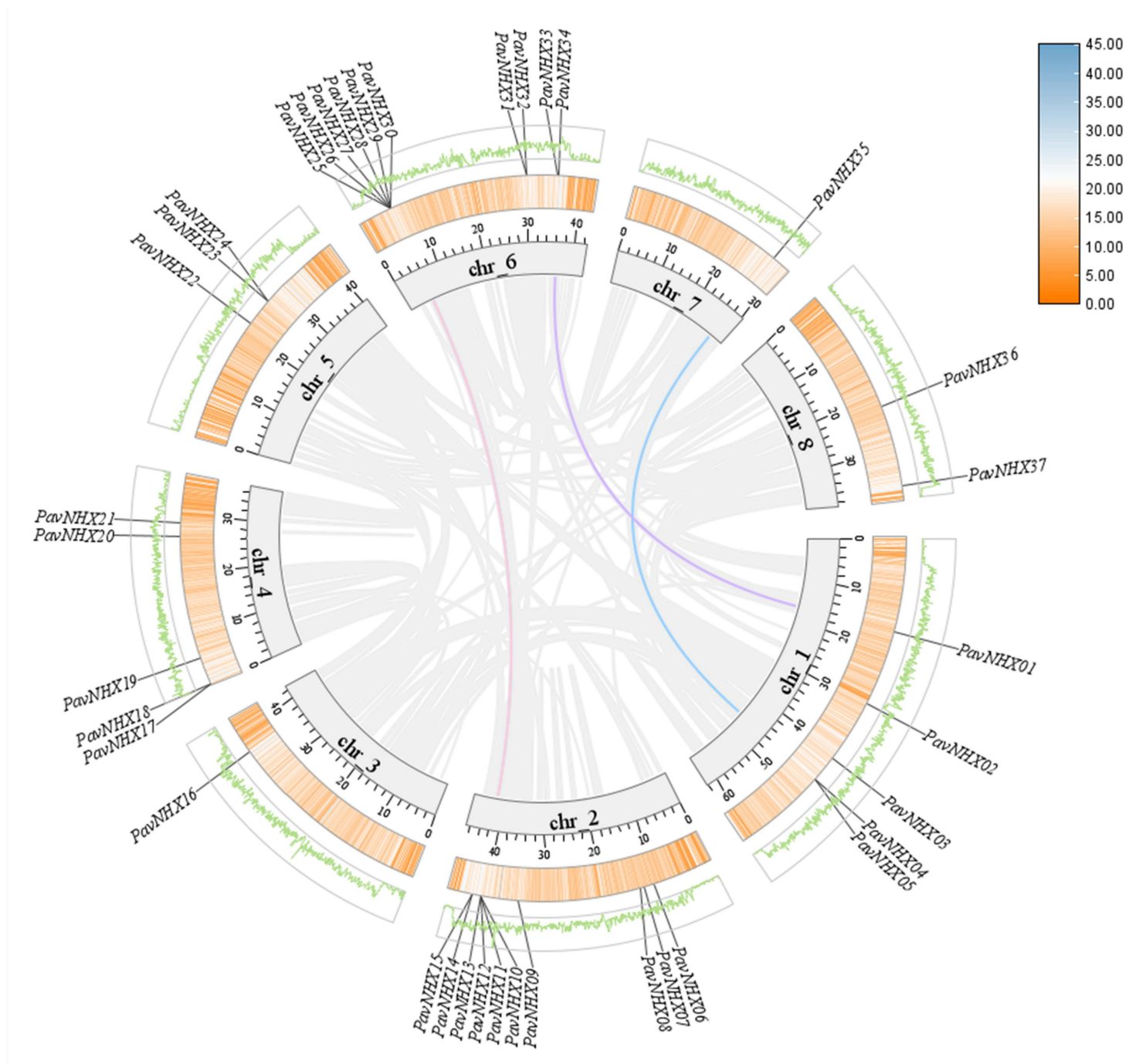


Figure 6

Intraspecific synteny analysis of the sweet cherry *NHX* gene family. Circular plot of sweet cherry chromosomes showing *PavNHX* gene positions, collinear blocks (gray lines), and segmental duplication pairs (colored lines).



Figure 7

Syntenic analysis of NHX genes between *Prunus avium* and five plant species. Chromosomes of *P. avium* (top) and other species (bottom) are shown as horizontal bars. Pink lines highlight syntenic NHX gene pairs; gray lines indicate other collinear genomic regions.

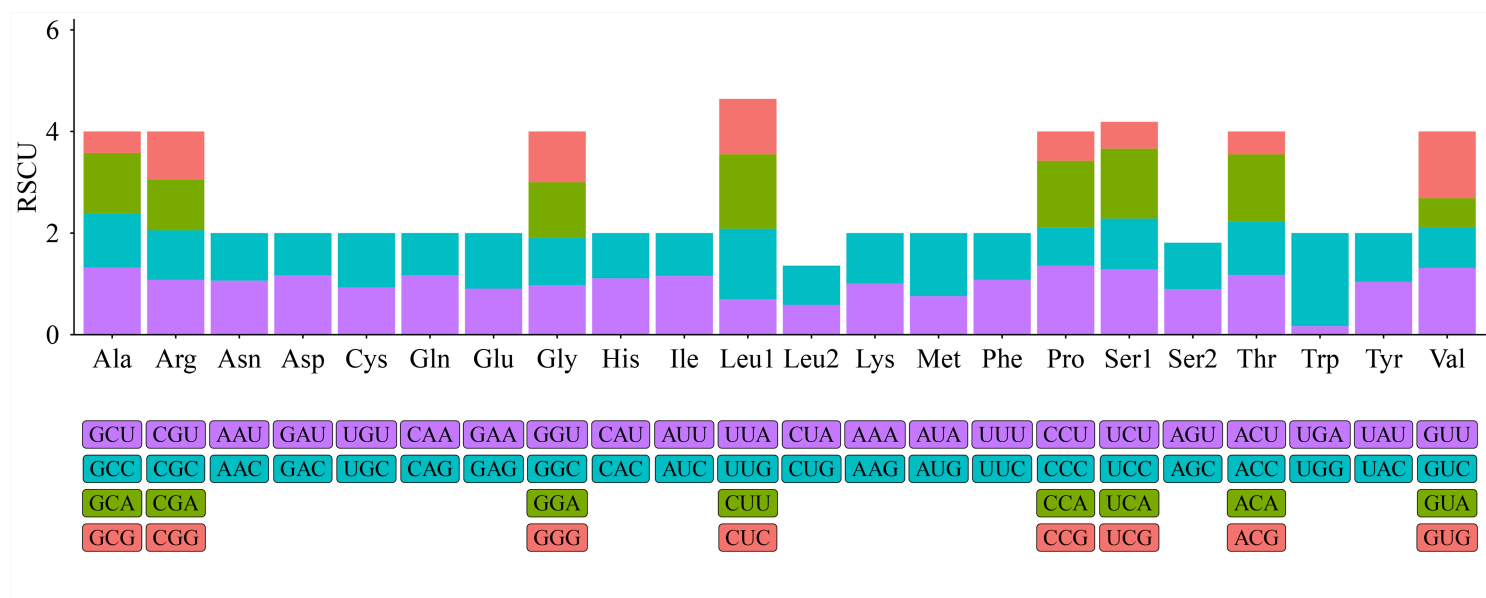


Figure 8

Codon usage bias of *PavNHX* genes.

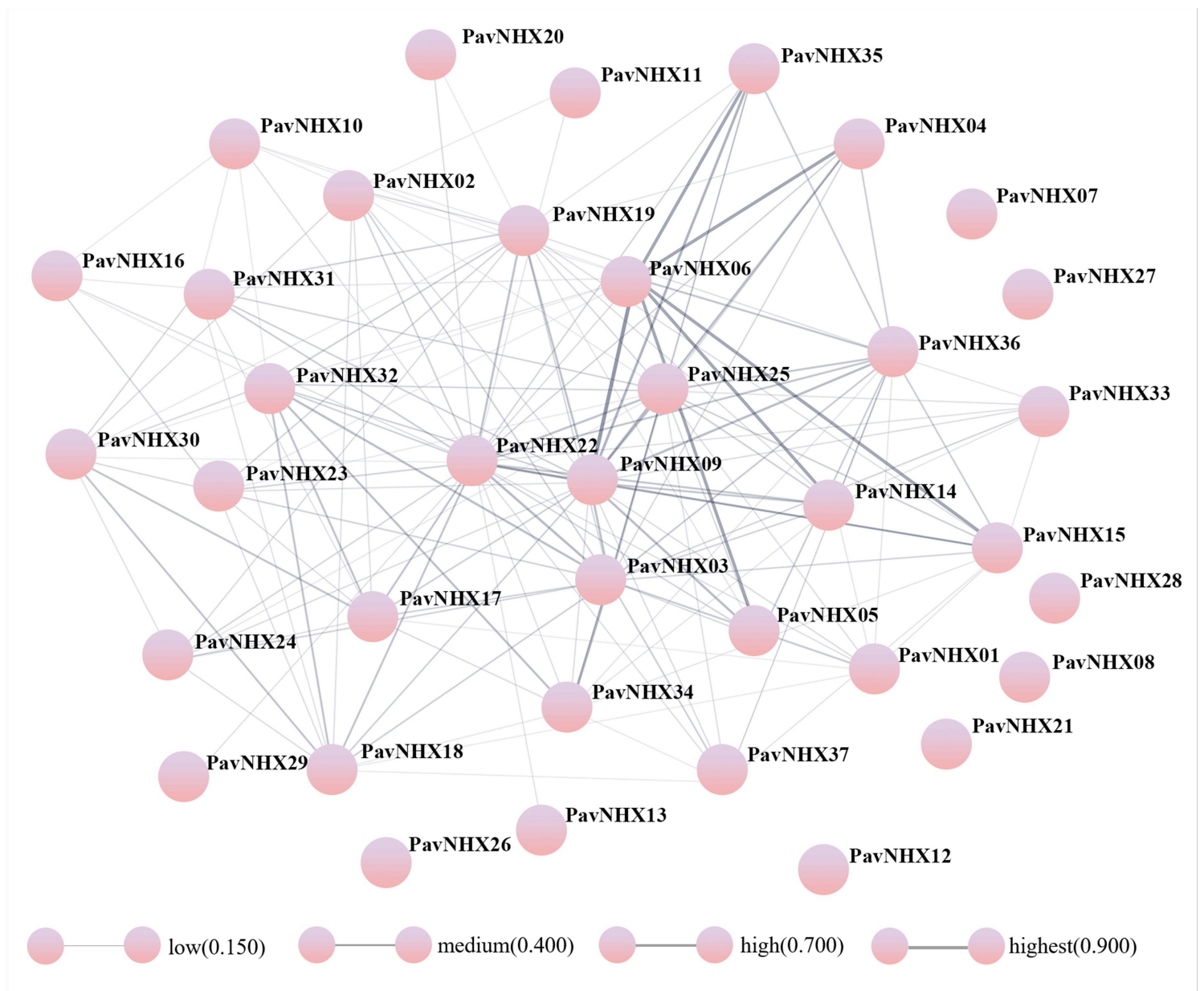


Figure 9

Interaction network of *PavNHX* genes. Nodes represent individual *PavNHX* genes; edges represent predicted functional interactions. Edge thickness indicates interaction confidence (low to highest, 0.150–0.900). Genes with thicker edges show stronger predicted associations.

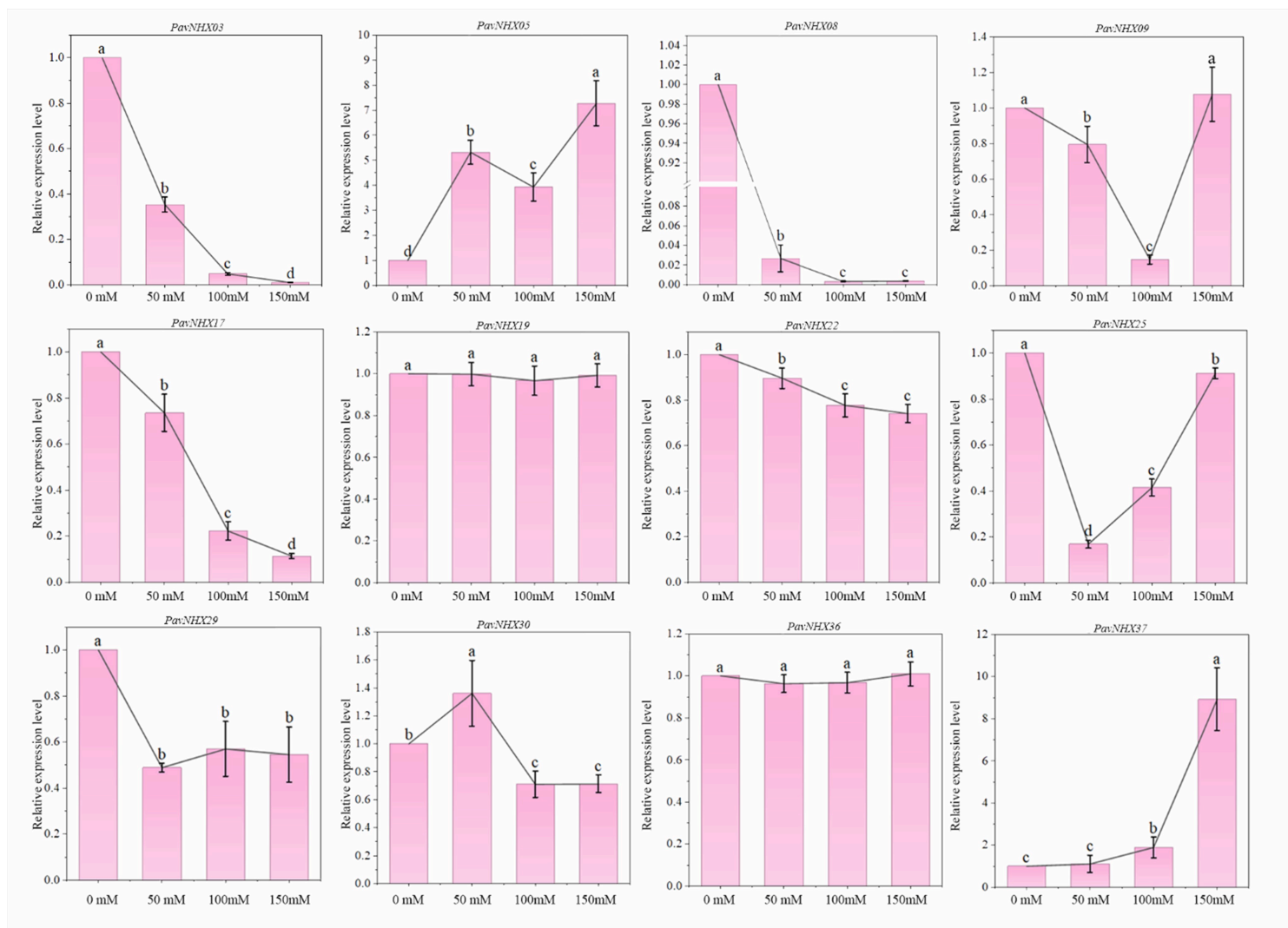


Figure 10

Expression profiles of selected *PavNHX* genes under different NaCl concentrations. The relative expression levels of 12 *PavNHX* genes were analyzed by qRT-PCR under 0, 50, 100, and 150 mM NaCl treatments. Each bar represents the mean $\pm$ standard error of three biological replicates. Different lowercase letters above the bars indicate significant differences ($p < 0.05$) according to one-way ANOVA followed by Tukey's multiple range test.

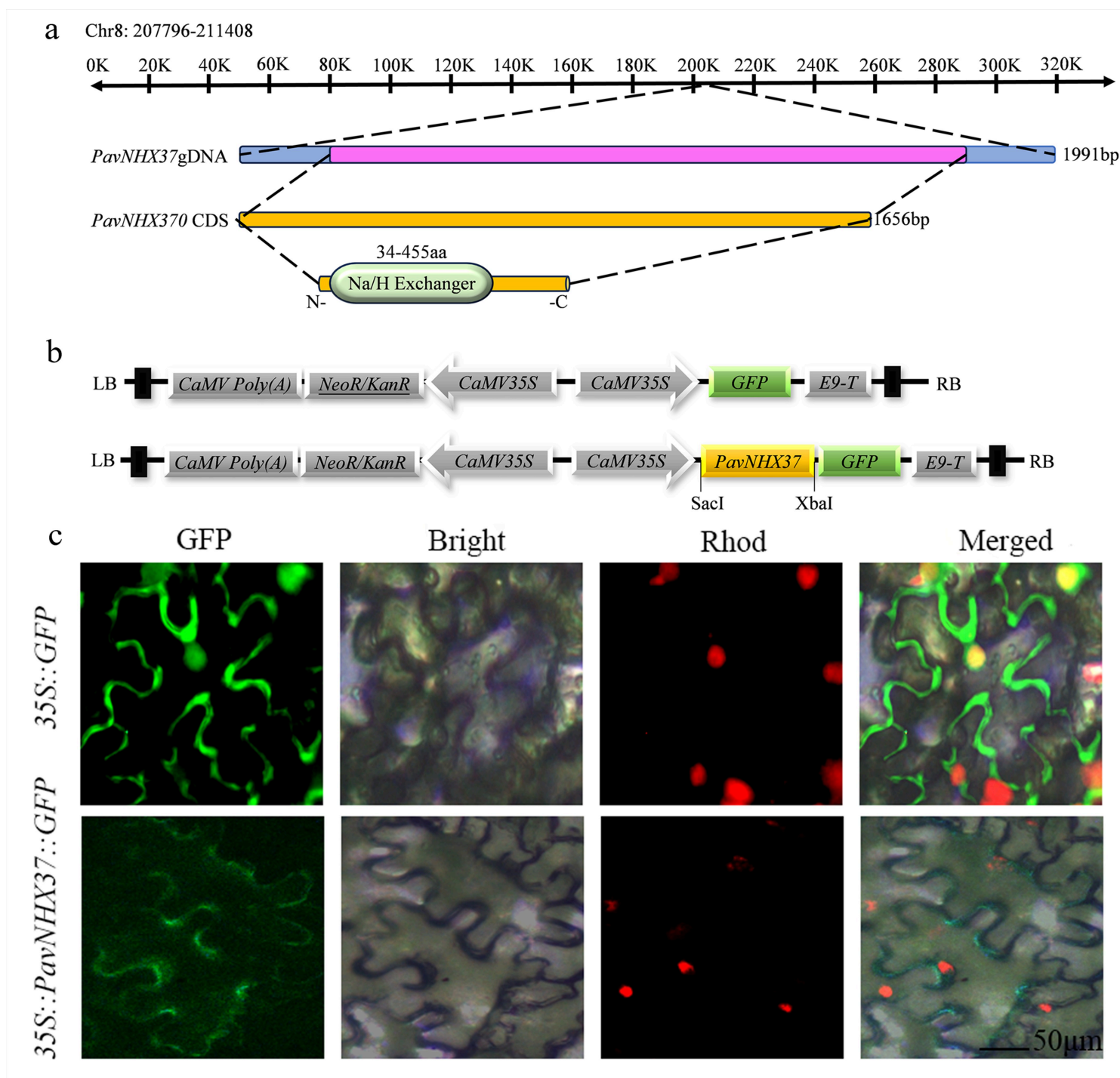


Figure 11

Characterization and subcellular localization of *PavNHX37*. (a) Gene structure of *PavNHX37* on Chr8, showing gDNA, CDS, and conserved Na⁺/H⁺ exchanger domain; (b) Schematic of the 35S::*PavNHX37*::GFP fusion and control vectors; (c) Subcellular localization in tobacco. Scale bar = 50µm.

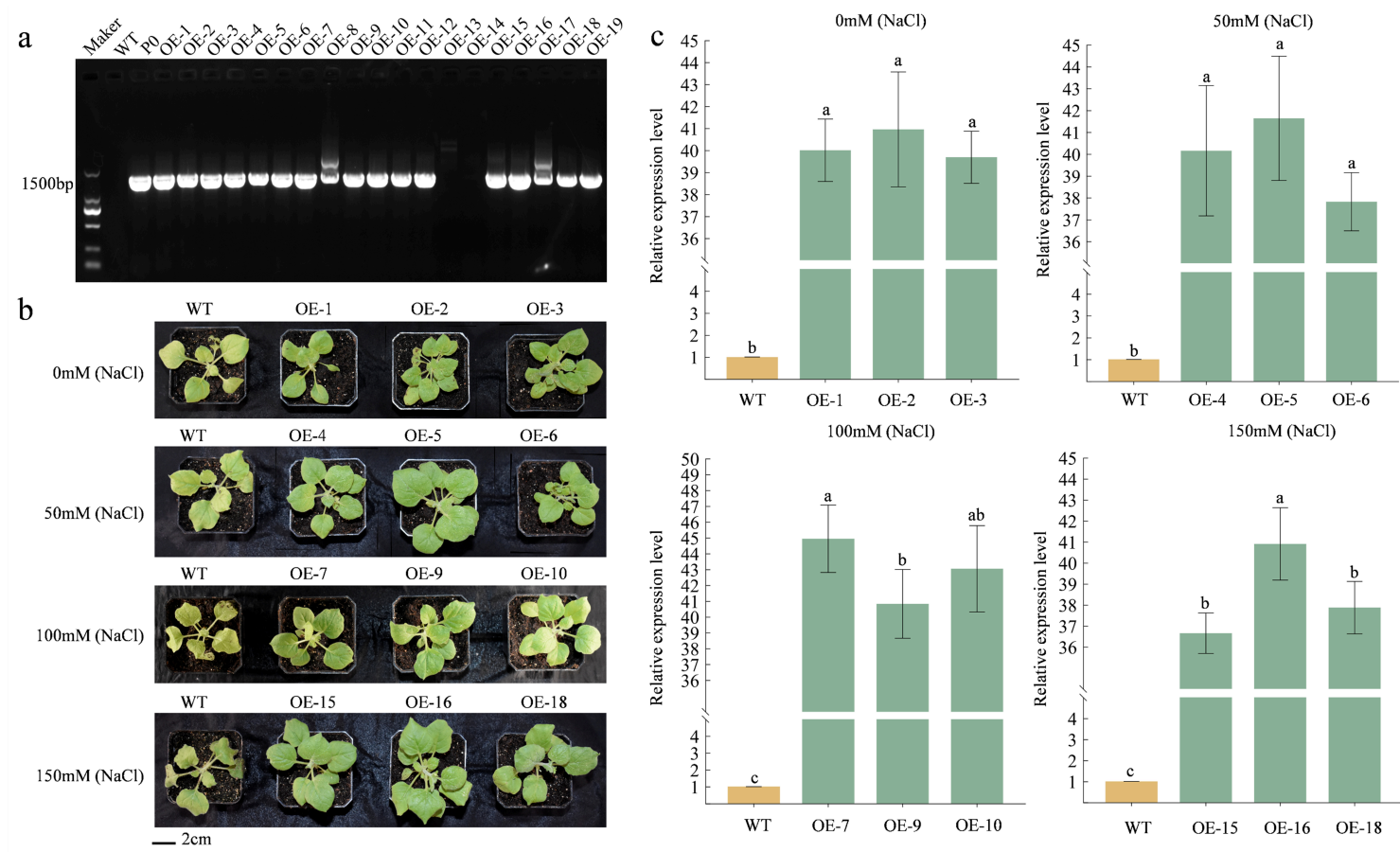


Figure 12

Salt tolerance of *PavNHX37*-overexpressing tobacco plants. (a) PCR identification of transgenic lines; (b) Phenotypes of WT and OE plants under 0–150 mM NaCl treatments (scale bar = 2cm); (c) Relative *PavNHX37* expression in WT and OE lines under salt stress. Data are mean $\pm$ SE (n=3); different letters denote significant differences ($p < 0.05$).

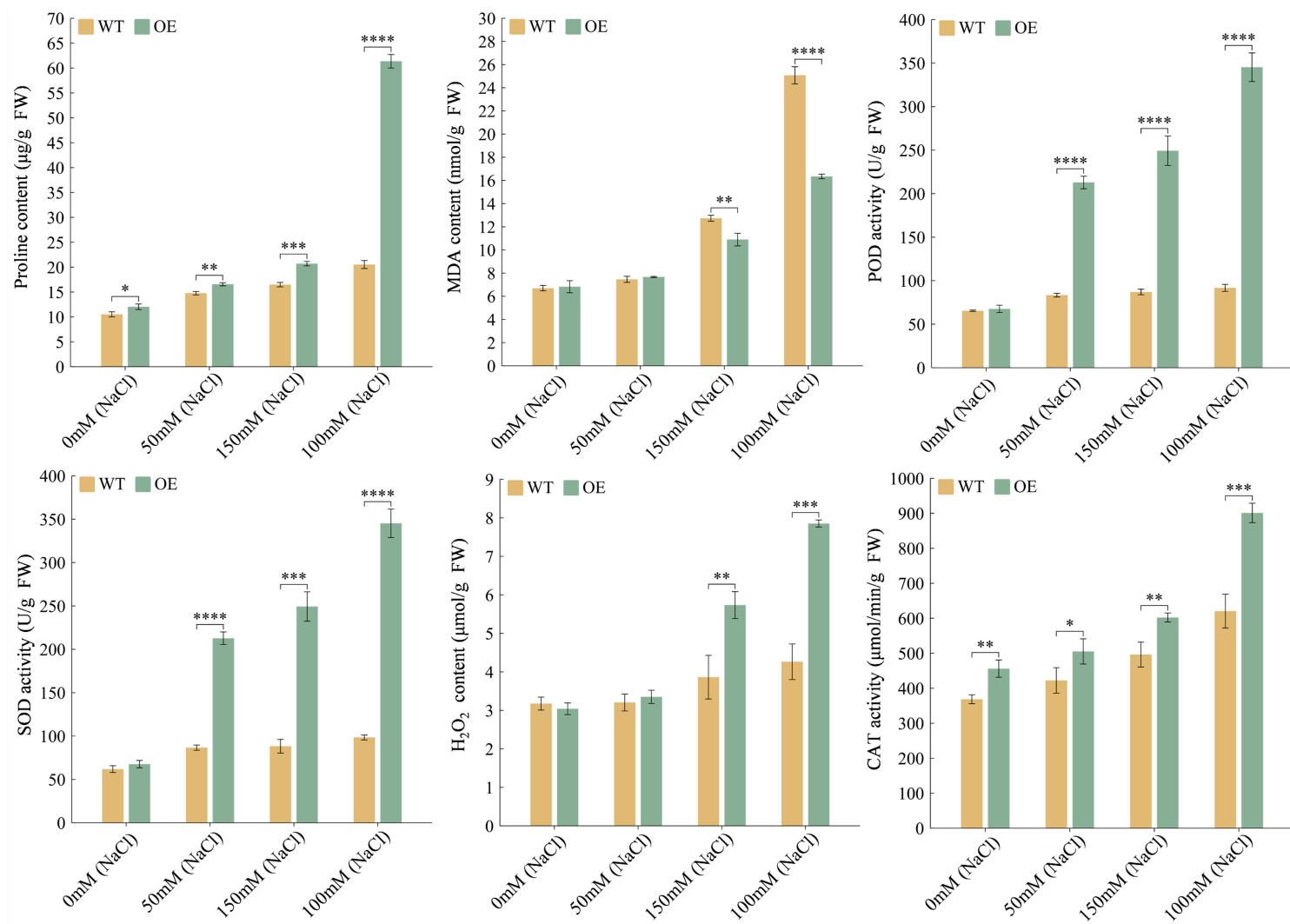


Figure 13

Physiological responses of *PavNHX37*-overexpressing tobacco under salt stress. Pro, MDA, H_2O_2 contents, and SOD, POD, CAT activities in WT and OE plants under 0–150 mM NaCl treatments (mean $\pm$ SE, $n=3$). Asterisks denote significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).