Molecular Characterization and Expression Analysis of the Na\(^+\)/H\(^+\) Exchanger Gene Family in Capsicum annuum L.

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The Na\(^+\)/H\(^+\) exchangers (NHXs) are a class of transporters involved in ion balance during plant growth and abiotic stress. We performed systematic bioinformatic identification and expression-characteristic analysis of CaNHX genes in pepper to provide a theoretical basis for pepper breeding and practical production. At the whole-genome level, the members of the CaNHX gene family of cultivated and wild pepper were systematically identified using bioinformatics methods. Sequence alignment and phylogenetic tree construction were performed using MEGA X software, and the gene functional domain, conserved motif, and gene structure were analyzed and visualized. At the same time, the co-expression network of CaNHX genes was analyzed, and salt-stress analysis and fluorescence quantitative verification of the Zunla-1 cultivar under stress conditions were performed. A total of 9 CaNHX genes were identified, which have typical functional domains of the Na\(^+\)/H\(^+\) exchanger gene. The physical and chemical properties of the protein showed that the protein was hydrophilic, with a size of 503–1146 amino acids. Analysis of the gene structure showed that Chr08 was the most localized chromosome, with 8–24 exons. Cis-acting element analysis showed that it mainly contains cis-acting elements such as light response, salicylic acid response, defense, and stress response. Transcriptome and co-expression network analysis showed that under stress, the co-expressed genes of CaNHX genes in roots and leaves were more obvious than those in the control group, including ABA, IAA, and salt. The transcriptome and co-expression were verified by qRT-PCR. In this study, the CaNHX genes were identified at the genome level of pepper, which provides a theoretical foundation for improving the stress resistance, production, development, and utilization of pepper in genetic breeding.

**Keywords:** Capsicum annuum, CaNHX, phylogenetic tree, co-expression, abiotic stress
INTRODUCTION

Pepper (Capsicum annuum L.), also known variously as capsicum, chili pepper, chile, and chili, is an annual or perennial plant belonging to the Solanaceae family. It is one of the most important vegetable crops in the world (Qin et al., 2015). Capsicum species were first introduced into China during the Ming Dynasty and today, China has the largest planting area and fresh yield in the world (FAO). It is an important cash crop with many varieties, and is considered also of ornamental value, with considerable genetic diversity for research purposes and breeding (Zhang et al., 2016). Many varieties—including Zunla-1, Yunnan Xiaomi Spicy, and Hainan Bell Pepper—are widely planted in China, and their market share is increasing every year.

Peppers contain many substances of nutritional value including vitamin C and vitamin A. The fruits are not only used for food seasoning, but also in the production of food pigments, medicine, and industrial chemicals (Kantar et al., 2016). In medicine, it is widely used for multiple functions, including antibiotic and the prevention and treatment of disease (Saleh et al., 2018). Three pepper genomes (Zunla-1, Chiltepin, and CM334) have been completely sequenced, and with continuing re-sequencing, transcriptome sequencing, and metabolomics based on the whole genome, an increasing amount of genetic data of various pepper varieties has been mined (Kim et al., 2014; Qin et al., 2014). A key component of peppers is their capsaicin. Peppers produced in northwest China contain higher capsaicin. Peppers produced in northwest China contain higher capsaicin and heme levels, due to the dry climate, low rainfall, high solar radiation, and wide temperature difference between day and night (Liu et al., 2012). This study examines how the nutritional content and capsaicin levels in peppers change when Capsicum is stressed by its growing environment. Under drought conditions, the capsaicin content in pepper can be reduced; under certain salt conditions, a significantly higher concentration of salt can promote the yield of capsaicin compared with control and low-salt pepper growth, and photosynthetic efficiency does not necessarily increase with salt (Sarah et al., 2012; Khan et al., 2014).

Plant growth and development depend strongly on environmental factors, such as cold and heat, drought, soil salinity and alkalinity, and other abiotic stresses. When plants are under stress, including some major cash crops, the external environment directly affects plant production. Salt stress is one of the most serious abiotic stresses affecting plant productivity and causes significant crop loss worldwide (Zhang et al., 2018). When plants are in a saline-alkaline soil environment, their ion balance and water balance change significantly. A change in membrane permeability destroys the normal operation of transporters, causing plants to absorb additional sodium ions from the environment, affecting the absorption of other ions and causing nutritional imbalance. Na⁺/K⁺ antiporters play a key role in plant development and tolerance to salt stress (Akram et al., 2020). In response to the external influence on plants, the ions and water in plants are balanced through their own ion channels. In general, the cytoplasmic pH value is above neutral (pH 7.2–7.6), which is controlled by an array of regulating molecules such as Na⁺/K⁺ transporters, cation/proton exchangers like Ca²⁺/H⁺, sodium-proton antiporters (NHX), proton/nutrient transporters, and H⁺-translocating enzymes (Benéina et al., 2009). Studies indicate that NHX antiporters are involved in regulating the ion balance in plants under salt stress. Their primary physiological functions are the regulation of cytoplasmic pH and expulsion of H⁺ generated during metabolism, in exchange for transporting Na⁺ or K⁺ ions into the cytoplasm and vacuoles of plants and animals (Pedersen et al., 2006). This indicates that studying the salt-tolerance mechanism of plants can improve the growth of plants under salt and alkali stress.

Human HsNHE was the first eukaryotic sodium-hydrogen exchanger gene to be identified and cloned, and functions in transport, Na⁺/H⁺ exchange, and pH regulation (Sardet et al., 1989). The first NHX gene identified in plants was the AtNHX1 gene from Arabidopsis thaliana, and the expression of this gene can regulate NaCl in A. thaliana and is a salt-tolerance determiner (Gaxiola et al., 1999; Yokoi et al., 2002; Rodríguez-Rosales et al., 2009). Eight NHX genes were identified in A. thaliana, among which AtNHX1 and AtNHX2 were most common in the buds and roots of seedlings, while AtNHX5 mRNA was expressed in lower abundance in both buds and roots. AtNHX3 was detected in roots, while AtNHX4 and AtNHX6 mRNA were only detected by RT-PCR (Yokoi et al., 2002). To date, NHX genes of several plant species have been identified, such as Vitis vinifera (6 VvNHX genes) (Ayadi et al., 2020), Medicago truncatula (MnNHX1- MnNHX8) (Sandhu et al., 2018), Populus trichocarpa (PtNHX1- PtNHX8) (Tian et al., 2017), Populus euphratica (PeNHX1- PeNHX6) (Ye et al., 2009), Gossypium hirsutum (GhNHX1- GhNHX23) (Fu et al., 2020), Morus alba (MaNHX1- MaNHX7) (Cao et al., 2016), and Beta vulgaris (BvNHX1- BvNHX5) (Wu et al., 2019). This study performed different bioinformatic analyses of CaNHX genes in cultivated and wild peppers, and the CaNHX gene family of pepper was identified at the genome level, providing a theoretical basis for analyzing the function of the gene under salt stress.

MATERIALS AND METHODS

Material and RNA Extraction

In this study, the whole genome data of pepper (C. annuum L. Zunla-1 is hereinafter referred to as pepper) and C. annuum var. glabrisculum Chiltepin were taken as the research object. Zunla-1 pepper material was planted in the greenhouse of the Department of modern agriculture, Zunyi vocational and technical college (Zunyi, Guizhou, 107°04’ E, 27°710’ N). The pepper was treated with 100 Mmol NaCl for 3, 6, 12, 24, and 72 h. The root material of pepper was stored in liquid nitrogen, and 3 samples were taken at each time point as biological replicates. RNA was extracted from the collected samples using the TianGene RNA Extraction Kit (DP432, Beijing, China). We then added root material with a weight of 50–100 ng for aseptic freezing grinding; 450 μL for oscillation mixing. This was transferred to the CS filter column and centrifuged

1http://www.fao.org/

2http://peppersquence.genomics.cn
for 3 min (12,000 rpm). The supernatant was transferred from the collection tube with a pipette gun to the Rnase-free centrifuge tube. Then, the supernatant was added and 0.5 times of anhydrous ethanol was mixed into the centrifuge tube and then transferred to the adsorption column CR3 for centrifugation for 30 s (12,000 rpm). A drop of 80 μL DNase I was added to the center of the collecting tube and left at room temperature for 15 min. 250 μL of protein-removing solution RW1 was added to the adsorption column CR3, and left to stand at room temperature for 2 min, before being centrifuged for 30 s (12,000 rpm). This procedure was repeated once. We then took an enzyme-free centrifuge tube and placed the adsorption column in a new centrifuge tube for several minutes (until the rinsing solution RW was dried). 50 μL of RNA was stored at −80°C for later use.

Identification of the CaNHX Gene in Pepper

*C. annuum* cv. CM334, *C. chinense* PI1159236, *C. annuum* cv. ECW, *C. annuum* SF and *C. baccatum* PBC81 genomes come from PGP (Pepper Genome Platform). Cuneo, Corno di Carmagnola, Quadrato di Carmagnola, and Tumeticot genomes come from ResEPP (Resequencing Piedmontese Pepper Ecotypes). According to the characteristics of the NHX gene family in *Plasm*1 data, there is an obvious conservative structure of the NHX gene family (PF00999) (El-Gebali et al., 2018). The genome-wide protein sequence of *Capsicum* was searched by HHMMER V3.3 software and verified with the Hmmmer web server, and the sequences with the incomplete conservative structure were removed. Meanwhile, the AtNHX gene of *A. thaliana* was used for blast comparison, and the E-value was maintained at 1e−20 for comparison. Selecting the intersection of HHMMER identification and BLAST alignment, 9 candidate genes of CaNHX were finally identified for subsequent analysis (Potter et al., 2018). The physicochemical properties of the pepper CaNHX protein were analyzed using the online tool ExPASy (Artimo et al., 2012). Prediction of Plant-mPloc by subcellular localization of the CaNHX gene in pepper was performed using online tools (Chou and Shen, 2010).

Analysis of Phylogeny and Characteristics of the CaNHX Genes Family

MEGA X was used to perform multiple sequence alignment analysis on the obtained 9 pepper NHX protein sequences, and the phylogenetic tree (neighbor-joining, bootstrap = 1,000) was constructed, and other parameters were left at default settings (Kumar et al., 2018). The online tool Itol was used for the presentation and form of the pepper NHX phylogenetic tree (Letunic and Bork, 2019). The batch CD-search tool in NCBI was used to visually analyze the NHX gene structure of the NHX gene. The online tool GSDS11 was used for the visualization of pepper NHX gene structure (Hu et al., 2014). The online sequence analysis tool MEME Suite was used for motif analysis, with the motif number set at 10 (Bailey et al., 2009). Collinearity analysis of pepper was performed by BLAST for whole-genome protein levels, and the MCScanX tool was used for collinearity analysis (Wang et al., 2012). TBtools were used for the visualization of gene structure, motifs, and collinearity results (Chen et al., 2020).

Ka/Ks and Promoter Analysis of the CaNHX Genes Family

Using BLAST to build a pepper comparison database, and the Ka/Ks Calculator tool to calculate the synonymous substitution rate and nonsensical substitution rate of pepper CaNHX genes, the Ka/Ks ratio of genes was obtained, and evolutionary pressure was analyzed (Wang et al., 2010). The upstream 2,000 bp sequences of NHX genes were compared and extracted using the Bedtools genome analysis tool. The upstream 2,000 bp sequence was predicted and analyzed using the online tool PlantCARE. Visualization was performed with Tbtools, the main action components were discussed (Lescot et al., 2002; Quinlan and Hall, 2010).

Expression Model and Coexpression Analysis

Transcription factors (TFs) in the *C. annuum* genome were identified using the online iTAK Plant Transcription factor and Protein Kinase Identifier and Classifier (Zheng et al., 2016). The expression data obtained from pepper informatic hub were analyzed using temporal and spatial expression patterns and co-expression network associations. The root and leaf tissues of the CM334 pepper cultivar were used for transcriptome and metabolomic analysis, with a total of 574 transcriptome data points. The co-expression results were visualized using Cytoscape 3.7.2 (Liu et al., 2017; Otasek et al., 2019).

cDNA Synthesis and Quantitative RT-PCR Analysis

The First Strand of RNA was synthesized using the Revertaid First Strand cDNA Synthesis Kit (K1622) from Thermo Field (RevertAid First Strand cDNA Synthesis Kit). The fluorescent quantitative primer Actin (GenBank: DQ832719) and Ubiquitin (GenBank: AY496112) were designed by Primer3plus software as the housekeeping gene (Supplementary Table 1). The

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http://www.pepper-genomics.unito.it/
http://pfam.xfam.org/
http://www.ebi.ac.uk/Tools/hmmer/
http://bioinformatics.psb.ugent.be/webtools/plantcare/html/
http://pepperhub.hzau.edu.cn/
fluorescence quantitative instrument for 15 samples was 96 Real-time qTOWER3.0 (Analytikjena, Germany), The fluorescence quantitative reaction system consisted of 10 µL SYBR Primix Ex TaqTM II (ZomBio, Beijing, China), and the upstream and downstream primers of each gene were 1 µL. And ddH2O to 20 µL. The PCR reaction procedure was 95°C for 30 s, 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, for 40 cycles. The quantitative RT-PCR results were analyzed using the 2−△△Ct method (Livak and Schmittgen, 2001). GraphPad Prism v8 was used to visualize the fluorescence quantitative results.

RESULTS

Identification and Physicochemical Properties of CaNHX Gene Family
According to the characteristics of the NHX gene family in the Pfam database, it contains Na_H_Exchanger (PF00999) functional domain. First, a total of 42 NHX genes were identified in pepper by the Hmmsearch identification method. Then, 8 AtNHX genes of A. thaliana were compared with the pepper genome by the BLASTP method. Combined with the two identification methods, the incomplete genes were removed by using the Hmmer online website. Nine NHX genes were obtained for subsequent analysis. These 9 CaNHX genes sequences were used for subsequent analysis and named CaNHX1-CaNHX9 in turn (Supplementary Table 2 and Table 1). The physical and chemical properties of the protein showed that the size was 360–1181 aa, the molecular weight was 398.06–129.91 kDa, the isoelectric value was 5.44–8.79, GRAVY was less than 1, and it was a hydrophilic protein. After subcellular localization of pepper CaNHX gene, it was found that the subcellular localization of CaNHX2 and CaNHX4 was in the Cell membrane, and the other 7 subcellular localization were all in Vacuole.

Phylogeny Analysis of CaNHX Genes With Different Species
The 9 CaNHX genes in C. annuum were identified compared with 8 AtNHXs in A. thaliana, 12 GmNHXs in Glycine max, 8 PtNHXs in P. trichocarpa, 8 VvNHXs in V. vinifera, 7 OsNHXs in O. sativa, 7 ZmNHXs in Zea mays (Figure 1 and Supplementary Table 3). According to the phylogenetic tree, NHX genes were divided into three subgroups, among which subgroup I contained the most genes. Subgroup I and Subgroup III contain four genes, respectively, while subgroup II contains only one gene.

Gene Structure and Conserved Sequence of CaNHX Genes
The NHX gene subfamily classification, gene structure, and motif analysis maps show the characteristics of A. thaliana and pepper gene families (Figure 2). Using TBtools to analyze the gene structure of 17 NHX genes family members in pepper and A. thaliana, the results showed that the exon number of the CaNHX gene family was mainly distributed between 8 and 24, while that of AtNHX gene family members in A. thaliana was between 12 and 23, among which the exon number of Class I subgroup was stable between 12 and 14, while the exon number of Class III was the largest. The NHX protein sequences of C. annuum and A. thaliana were analyzed by MEME web tool. According to the distribution of motif of CaNHX genes family members, the motif number is consistent with the phylogenetic tree. For example, in the Class I subfamily, there are 5 motif sequences, which are motif1, motif2, motif5, motif6, and motif7. It is consistent with the phylogenetic tree classification of the Class I subgroup. The motif of the CaNHX gene in the Class II subfamily all contained motif8 and motif9, of which CaNHX8 was the one with the least motif number. In the Class III subfamily, the motif number of CaNHX1, CaNHX3, CaNHX6, and CaNHX7 remained at 7–8, of which motif8 and motif9 did not exist in the Class III subfamily. Motif7 is a typical amidolide-binding site (LFIYLPPI), which is a motif contained in the genes of salt-tolerant plants and transgenic NHX plants, and contains the motif in CaNHX2, CaNHX3, CaNHX4, and CaNHX5 (Figures 2, 3).

Table 1: Family information and subcellular localization of CaNHX gene in pepper.

| Gene_id       | Gene_name | Chr | aa  | Kd       | pl  | GRAVY | Sub. localization |
|---------------|-----------|-----|-----|----------|-----|-------|-------------------|
| Capana01g001309 | CaNHX1    | Ch01 | 511 | 56256.55 | 6.56 | 0.474 | Vacuole           |
| Capana05g000031 | CaNHX2    | Ch05 | 947 | 100455.39| 5.44 | 0.242 | Cell membrane.    |
| Capana06g000399 | CaNHX3    | Ch06 | 527 | 58411.65 | 8.79 | 0.522 | Vacuole           |
| Capana06g000122 | CaNHX4    | Ch08 | 1181| 129915.14| 6.26 | 0.090 | Cell membrane.    |
| Capana08g000123 | CaNHX5    | Ch08 | 624 | 67507.96 | 7.62 | 0.619 | Vacuole           |
| Capana08g0001332 | CaNHX6    | Ch08 | 482 | 53412.57 | 6.45 | 0.590 | Vacuole           |
| Capana10g000166 | CaNHX7    | Ch10 | 512 | 57296.70 | 8.59 | 0.452 | Vacuole           |
| Capana09g000346 | CaNHX8    | Ch00 | 459 | 51409.16 | 6.36 | 0.000 | Vacuole           |
| Capana09g0004012 | CaNHX9    | Ch00 | 360 | 39805.86 | 6.63 | 0.720 | Vacuole           |
FIGURE 1 | Phylogenetic analysis of CaNHX genes in C. annuum. Square: C. annuum; Star: A. thaliana; Horizontal hexagon: G. max; Right triangle: P. trichocarpa; Rhombus: V. vinifera; Octagon: O. sativa; Up pointing pentagram: Z. mays.

FIGURE 2 | Gene structure and conserved sequence of C. annuum and A. thaliana. (A) Refined relationship between C. annuum and A. thaliana. (B) Functional structure domain. (B) NHX genes. (C) NHX genes structure. (D) Conservative motifs. (D) NHX genes. (E) Motifs.
one CaNHX gene (Figure 4). To study the whole genome duplication (WGD) event, 42 cultivars were identified (contains the confirmed 9 CaNHX genes) by Hmmsearch in cultivated pepper and 37 cultivars identified by hmmsearch in wild pepper were analyzed together (Supplementary Table 3). There are more collinearity relationships among the 9 pepper genes identified, among which CaNHX4 and CaNHX6 are on Chr08 of Zunla-1, while the collinearity block of this gene is on wild type Chr01. There is a collinearity block between CaNHX7 and wild-type Chr00 on Zunla-1 chromosome Chr10. Among them, CaNHX1, CaNHX5, and CaNHX8 have no collinearity block relationship, while CaNHX2, CaNHX3, and CaNHX9 also have collinearity, but their chromosomal positions do not change. The results indicated that the cultivated pepper Zunla-1 and the wild pepper were from the same ancestor, and there was a certain gene replication event. At the same time, we performed collinearity analysis on the genome of pepper varieties with Zunla-1 and other pepper genomes, including Chiltepin, Corno,
FIGURE 4 | Chromosome location and collinearity analysis of CaNHX genes in pepper.

Cuneo, Quadrato, tumaticot, CM334, ECW, PBC81, and SF (Figure 5). The collinearity analysis between Zunla-1 and Corno, Cuneo, Quadrato, and Tumaticot showed that the chromosomal position relationship of Corno, Cuneo, Quadrato, and Tumaticot was the same. The results showed that the four pepper varieties were derived from the same ancestor and had less variation during the species evolution. However, the position relationship between the four pepper varieties and Zunla-1 changed greatly, which indicated that they had a far evolutionary relationship with more variation. Zunla-1 showed significant variation with Chiltepín, CM334, ECW, PBC81, and SF, and the changes of gene position were obvious, indicating that Zunla-1 was far related to the other five varieties of pepper. Three pairs of homologous loci were obtained by analyzing the Ka/Ks ratio of the CaNHX gene, and their Ka/Ks were less than one, indicating that the gene was mainly purified during the evolution of the CaNHX gene in pepper (Table 2).

Promoter Analysis of C. annuum CaNHX Genes

By analyzing the upstream 2,000 bp sequence of the CaNHX genes, the cis-acting elements of the gene were predicted. In addition to a large number of basic elements—CAAT-box and TATA-box—there are also G-Box, GAG-motif, chs-CMA1a, TCT-motif, GATA-motif, GT1-motif, and AE-box in the CaNHX gene family, and TCA-elements in the salicylic acid reaction. Also present were cis-acting elements, TC-rich repeats, meristem expression elements, CAT-box, MYB binding site elements, MBS, MeJA response elements, GCTCA-motif, auxin response elements, TGA-elements, etc. (Figure 6 and...
Supplementary Table 7). The analysis showed that CaNHXs may be regulated by such things as light and salicylic acid, and may participate in defense mechanisms through these cis-acting elements, thus playing a role in protecting plant growth.

Co-expression Network of CaNHX Gene Under Stress Treatment

According to established methods, the co-expression network related to the CaNHX gene was extracted. Under stress, 10 groups of data were obtained: ABA, IAA, GA3, SA, JA, sodium chloride, mannitol, hydrogen peroxide, heat stress, cold stress, plus control groups. The co-expressed gene of the CaNHX gene was extracted by script, and the co-expressed gene related to the NHX gene under stress was obtained (Supplementary Table 8).

TABLE 2 | Nucleotide substitution rate of Pepper CaNHX gene.

| Collateral homologous gene site | Non-synonymous substitution rate (Ks) | Synonymous substitution rate (Ks) | Selective pressure ratio (Ks/Ka) |
|---------------------------------|--------------------------------------|----------------------------------|-------------------------------|
| CaNHX1-CaNHX6                   | 0.201701088                          | 2.493573377                      | 0.080888371                   |
| CaNHX2-CaNHX8                   | 0.026980027                          | 0.041710828                      | 0.646835085                   |
| CaNHX4-CaNHX5                   | 0.155507334                          | 0.327196446                      | 0.475272076                   |

Expression Pattern of Pepper NHX Genes Under Hormone and Abiotic Stress

The expression profile of the CaNHX gene in pepper was analyzed using an online database (Figure 8). The results showed that the expression of CaNHX3, CaNHX4, and CaNHX5 in roots was upregulated compared with that in leaves during IR (IAA root stress) and GR (GA3 root stress), while the expression of CaNHX2, CaNHX6, CaNHX7, CaNHX8, and CaNHX9 was lower in leaves. Under hormone stress, CaNHX1 expression was upregulated. Under abiotic stress, CaNHX1 and CaNHX9 genes were upregulated in HR (heat root stress), while CaNHX2, CaNHX3, and CaNHX4 were upregulated in RR (H2O root stress), FR (cold root stress), and MR (mannitol root stress), whereas CaNHX5 was upregulated in FL (cold leaf stress), CaNHX6 was upregulated in HL (heat leaf stress), CaNHX7 was upregulated in RL (H2O leaf stress), CaNHX7 was upregulated in RL and CaNHX8 in FL. At the same time, expression analysis of the CaNHX gene family under salt stress showed that CaNHX1, CaNHX3, CaNHX4, CaNHX5, and CaNHX9 were upregulated in roots, and their expression tended to be consistent over time. CaNHX6, CaNHX7, and CaNHX8 were upregulated in NL (NaCl stress and cold stress, but other co-expression genes were also present under NaCl stress. Under salt stress, a total of 4 CaNHX genes were co-expressed with transcription factors, among which CaNHX9 co-expressed the most with 11 transcription factors, followed by CaNHX4 with 7 transcription factors, and CaNHX1 with only 1 NAC co-expressed with CaNHX1 was the least.
leaf stress), while the root expression of CaNHX2 was higher in the blank control than in the leaves.

Quantitative RT-PCR Analysis

The pepper at the 6-leaf growth stage was subjected to 100 Mmol salt stress, and the samples at different times (3, 6, 12, 24, and 72 h) were taken for fluorescence quantitative analysis (Figure 9). Results show that, under salt stress, when processing CaNHX1, CaNHX2, CaNHX6, and CaNHX9 showed a trend of increased expression, the five time node, CaNHX1, CaNHX9 two genes in a state of relative balance, express no obvious floating. The expression of CaNHX2 and CaNHX6 began to be down-regulated over time after initial stress treatment and then began to be up-regulated after 24 h. The expression of CaNHX3, CaNHX4, CaNHX5, and CaNHX8 genes was less obvious than others. CaNHX1 with CaNHX9 fluorescence quantitative results agree

![Figure 6](https://example.com/image.png)
FIGURE 7 | (A,B) Co-expression network of C. annuum CaNHX gene under NaCl stress (Diamond: CaNHX genes; Circular: TFs; Node Fill Color Mapping: Degree).

FIGURE 8 | Expression pattern of CaNHX gene in pepper under different stress in root and leaf. (A) Hormonal stress. (B) Abiotic stress. (C) Salt stress.
with the transcriptome data, in response to salt stress were a higher expressed state, CaNHX2, CaNHX6, CaNHX7, CaNHX8 increase is not obvious in the transcriptome, which no expression in fluorescence quantitative CaNHX7 gene, do not make the same amount in the transcriptome. In conclusion, two genes, CaNHX1 and CaNHX9, were stably expressed in pepper under salt stress, which was consistent with transcriptome results. It was speculated that pepper could adjust its own ion balance by up-regulating the expression of CaNHX1 and CaNHX9 genes in the process of salt stress, so that pepper could adapt itself to the changes in the environment.

**DISCUSSION**

**NHX Gene Family in Pepper**

We identified 9 CaNHX genes in the pepper genome (zunla-1), 6 in C. annuum chiltepin, 9 in C. chinense. PI159236, and 9 in C. annuum. Cv.CM334. There were 9 identified in C. nnuum. Cv. ECW, 10 identified in C. accentum. PBC81, 8 identified in C. nnuum. SF, 8 identified in Corno, 8 identified in Cuneo, 9 identified in Quadrato, and 8 identified in Tumaticot. The CaNHX gene was found to contain up to 9 genes in different varieties of pepper. So far, NHX genes have been identified in many species, with the most identified in *Gossypium hirsutum* and *G. barbadense* (23 GhNHX and 24 GbNHX, respectively) (Fu et al., 2020).

However, in subcellular localization, NHX genes can be classified into three subgroups according to their subcellular localization according to previous reports which were divided into three categories, namely Vac-Class, Endo-class, and PM-class, among which Vac-class is located in Vacuole, Endo-class in Endoretinal Reticum, and PM-class in Plasma membrane (Wu et al., 2019). Other NHX gene species based on subcellular localization also include *A. thaliana*, *P. trichocarpa*, *G. hirsutum*, *V. vinifera*, *Triticum aestivum*, *Oryza sativa*, *Sorghum bicolor*, *Cucurbita maxima*, *Solanum lycopersicum*, *Panicum virgatum*, *Eutrema halophilum*, *Spinacia oleracea*, and *Hordeum vulgare*. According to the classification of grapes by Ayadi et al. (2020) the *VvNHX* genes of grapes are divided into two categories, namely Group I Vacuolar (*VvNHX1–VvNHX5*) and Group II Endosomal (*VvNHX6*). However, CaNHX genes in pepper are not classified according to their subcellular location. CaNHX2 and CaNHX4 are located in the Cell membrane, while the other subcellular locations of CaNHX gene family members are located in Vacuole. There is no PM-Class and Endo-class in pepper, which is quite different from previous studies.

The CaNHX gene in Zunla-1 can be divided into three subfamilies, namely Class I, Class II, and Class III. In the identified NHX gene families, NHX contains a complete functional domain. These CaNHX genes can be divided into 3 categories, which are the same as *A. thaliana*, beet, and other plants reported by predecessors (Wu et al., 2019). In addition to CaNHX7, CaNHX8, and CaNHX9, the other six genes also have the typical amiloride-binding site of the NHX gene (FFI/LY/FLLPPI), but this structure does not exist in the Class II subgroup. At the same time, the intermediate residues LF/AV/IY, LF in Class I, LA in Class II, and IY in Class III, PagNHX gene also had the same motif and residues in pomegranate. Not only pepper but also *A. thaliana* had an NHX gene without an amiloride-binding site (Counillon et al., 1993; Dong et al., 2021). In the wheat TanHX gene, it was found that under salt stress, the expression of TaNHX2 and TaNHX3 genes were higher in leaves and roots, and the expression of TaNHX1 was higher only in roots. All three TaNHX genes contained an amiloride-binding site (LFIYLLPPI) (Brini et al., 2005; Yu et al., 2007; Lu et al., 2014). It is speculated that the NHX gene containing an amiloride-binding site is more suitable for the growth of salinization conditions.

Plants are affected by the external environment during their growth, such as abiotic stress, drought, high temperature, salt, and alkali, etc. Transcription is particularly important in the response of plants to environmental changes. There are many cis-acting elements in the pepper CaNHX genes family, such as hormones and stress elements. Studies show that stress-related elements (such as high temperature, low temperature, drought, injury, and defense) and hormone-related elements (such as Auxin, Ethylene, GA, SA, MeJA, and ABA) are identified in the
promoter of PtNHXs, and there are also cis-acting elements such as ABA and ABRE in sugar beet, which indicate that they can pass through during plant growth (Tian et al., 2017; Wu et al., 2019). In the transcriptional data of pepper, it was found that the co-expression networks of pepper under biotic and abiotic stress had higher gene network abundance than those under untreated conditions. In the co-expression network, CaNHX1, CaNHX3, CaNHX4, and CaNHX9 were found to be co-expressed with transcription factors, among which CaNHX3 was co-expressed with transcription factor WRKY, indicating that the cis-acting element of CaNHX3 was G-box, which has been found in studies. The G-box is an element associated with WRKY transcription factors under stress conditions. CaNHX3 was upregulated in several periods.

Expression Profiles of NHX Genes in Pepper

Up to now, the function of the CaNHX gene in pepper has not been analyzed, and no report on the CaNHX gene in pepper has been reported. With the transformation of salt-tolerant transgenic plants, the NHX gene will provide more benefits for agricultural development in soil salinization. Transgenic technology has become one of the important ways to obtain salt-tolerant plants. The number of flowering was more, and the growth of plants is inhibited, thus affecting the yield of plants. Cao et al. (2011) through the overexpression of CaNHX3 was G-box, which has been found in studies. The G-box is an element associated with WRKY transcription factors under stress conditions. CaNHX3 was upregulated in several periods.

We found that the expression of CaNHXs was mainly concentrated in roots under hormone stress, while under abiotic stress, there were up-regulated expressions of CaNHX2, CaNHX3, and CaNHX4 genes in roots. In the cis-acting elements of the CaNHX gene family, it was found that CaNHX was expressed under various hormones and stress. However, under salt stress, most of the CaNHX genes were up-regulated, which indicated that CaNHX genes in Pepper could condition its ion balance by expressing NHX. The function of plant vacuole NHX antipporter has been identified and expressed in an exogenous system to enhance the salt tolerance of plants. Akram et al. (2020) found that the NHX gene was up-regulated under salt stress, and Yokoi et al. (2002) found that AtNHX1 had higher transcript abundance during salt stress (Yarra, 2019). The results indicated that the expression of NHX genes responded to salt stress during plant growth, which played a very important role in plant growth.

Transcriptome analysis found that the CaNHX gene had multiple expression patterns under single or multiple stress conditions. Meanwhile, the fluorescence quantitative verification in this study showed that the results were consistent with the transcriptome results, in which CaNHX1 and CaNHX9 were up-regulated under salt stress. These results indicated that the CaNHX gene provided a guarantee for the normal growth of pepper and the balance of ion channels of plant stress resistance.

CONCLUSION

In the pepper genome, 42 CaNHX genes were identified by a Hidden Markov Model database search (hmmsearch). Of these, 9 genes with complete functional domains were identified by BLASTP. We constructed a phylogenetic tree and found that the 9 CaNHX genes were divided into three categories: Class I, Class II, and Class III. The exon number of the Class I subgroup was relatively stable, and the genes were distributed on six chromosomes; these were for hydrophilic proteins. There was a motif amilorid-binding site of the NHX gene (FFI/ILY/FLLPPI) associated with salt tolerance in the pepper CaNHX gene. There are many elements in the CaNHX gene, such as hormone stress, salt stress, and so on, and it was found that the CaNHX gene is associated with many genes in the co-expression process, and salt stress conditions are also associated with many genes. Transcriptome analysis showed that the CaNHX gene was up-regulated under various abiotic stresses, which was verified in combination with fluorescence quantification in this study and found to be consistent with transcriptome results. In this study, the whole gene of Pepper was identified at the genome level, which provided a theoretical basis for the genetic breeding of pepper under stress.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.
AUTHOR CONTRIBUTIONS

XL, SY, ZZ, and CQ conceived and designed the experiments, and drafted the manuscript.YL, HQ, TL, JI, XC, ZX, YC, and JZ performed the experiments. SY and CQ analyzed the data. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2021.680457/full#supplementary-material

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