Article

Genome-Wide Identification and Functional Characterization of the Cation Proton Antiporter (CPA) Family Related to Salt Stress Response in Radish (Raphanus sativus L.)

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Abstract: The CPA (cation proton antiporter) family plays an essential role during plant stress tolerance by regulating ionic and pH homeostasis of the cell. Radish fleshy roots are susceptible to abiotic stress during growth and development, especially salt stress. To date, CPA family genes have not yet been identified in radish and the biological functions remain unclear. In this study, 60 CPA candidate genes in radish were identified on the whole genome level, which were divided into three subfamilies including the Na\(^+\)/H\(^+\) exchanger (NHX), K\(^+\) efflux antiporter (KEA), and cation/H\(^+\) exchanger (CHX) families. In total, 58 of the 60 RsCPA genes were localized to the nine chromosomes. RNA-seq. data showed that 60 RsCPA genes had various expression levels in the leaves, roots, cortex, cambium, and xylem at different development stages, as well as under different abiotic stresses. RT–qPCR analysis indicated that all nine RsNHXs genes showed upregulated trends after 250 mM NaCl exposure at 3, 6, 12, and 24h. The RsCPA31 (RsNHX1) gene, which might be the most important members of the RsNHX subfamily, exhibited obvious increased expression levels during 24h salt stress treatment. Heterologous over-and inhibited-expression of RsNHX1 in Arabidopsis showed that RsNHX1 had a positive function in salt tolerance. Furthermore, a turnip yellow mosaic virus (TYMV)-induced gene silence (VIGS) system was firstly used to functionally characterize the candidate gene in radish, which showed that plant with the silence of endogenous RsNHX1 was more susceptible to the salt stress. According to our results we provide insights into the complexity of the RsCPA gene family and a valuable resource to explore the potential functions of RsCPA genes in radish.

Keywords: CPA gene family; RsNHX1; over-expression; virus-induced gene silence; salt resistance; radish

1. Introduction

Plants respond to salt stress by regulating the cell ion and pH balance through a variety of mechanisms, which are mainly dependent on ion transporters in cell membranes and organelle membranes [1,2]. Cation proton antiporters (CPAs) are mainly involved in the exchange and transport of monovalent cations in plants, which not only reverses the transport of Na\(^+\) with H\(^+\) but also exchanges and transports monovalent cations, such as K\(^+\) and Li\(^+\) [3–5]. The CPA family is divided...
into two main superfamilies, named CPA1 and CPA2 [6]. The CPA1 superfamily contains the Na⁺/H⁺ exchanger family (NHX), which is predicted to have 10–12 membrane-spanning domains in plants and it has been confirmed with an important effect on salt tolerance of plants [2,7]. CPA2 consists of the K⁺ efflux reverse transporter family (KEA) and cation/H⁺ exchanger family (CHX), which is predicted to have 8–14 transmembrane domains [2,7], all of which contain the Na⁺/H⁺ exchanger domain (PF00999) [8]. In addition, CPA proteins are mainly localized on the plasma, vacuole membrane and organelle membrane of plant cells [2,9], which can play essential roles in plants responding to environmental stress and maintaining the homeostasis of pH and ions [8,10].

Identification and characterization of the CPA family has been extensively reported in several plant species, including Arabidopsis thaliana [11], rice (Oryza sativa) [12], grape (Vitis vinifera) [13], and pear (Pyrus bretschneideri) [14]. In recent years, increasing evidence has indicated that some CPAs, especially NHXs, respond to salt stress, cell expansion, pH and ion balance regulation, osmotic regulation, and vesicle transport, as well as protein processing and floral organ development [15–19]. The previous studies showed that the NHX subfamily consisted of eight members (AtNHX1–AtNHX8) in Arabidopsis, which were mainly involved in the exchange and transport of Na⁺ and H⁺ [20–22]. Recent reports further revealed that the NHX1 gene in Vigna unguiculata, Malus domestica, Sesuvium portulacastrum, Arachis hypogaea, and Helianthus tuberosus can regulate salt tolerance [12,23–26]. For example, VviNHX1 displayed higher salt tolerance through over-expression in Arabidopsis [23]. Over-expression of SpNHX1 yeast cells grew better and accumulated more Na⁺ than control, indicating that SpNHX1 was a key response gene to salt stress [25]. Furthermore, over-expression of AtNHX7 (AtSOS1) limited Na⁺ accumulation in xylem and stem, and improved salt tolerance in transgenic Arabidopsis thaliana [27]. Interestingly, the AtCHX17 mutant accumulated less K⁺ than the control in salt stress and K⁺ deficient environment, indicating that the AtCHX gene was involved in the exchange and transport of K⁺ rather than Na⁺ [28]. In addition, K⁺/H⁺ reverse transporters AtKEA1–AtKEA3 played an important role in chloroplast function, osmoregulation, photosynthesis, and pH regulation [29,30].

Radish (Raphanus sativus L.) belongs to the Brassicaceae family and is one of the most economically important annual or biennial root vegetable crops, which is widely cultivated all over the world with high nutritional and medicinal value. The fleshy taproot is the edible organ of the radish, which has different sensitivities to salt stress [31,32]. Although some CPA genes have been proved to be related to salt stress response in other plant species, such as Arabidopsis, pear (Pyrus bretschneideri), grape (Vitis vinifera) and Helianthus tuberosus, the information of CPA genes identification at the whole genome level in radish is still limited [11–14]. The radish genomes were released to provide a helpful resource to identify the CPA gene family at the whole genome level [33]. Our study aimed to systematically identify CPA family members from the radish genome, map RsCPAs onto chromosomes, and investigate gene structure and conserved motifs. Moreover, the expression patterns of RsCPAs in different developmental stages and tissues were analyzed and also explored for differentially sensitive genes under abiotic stress, especially in salt stress. Furthermore, the biological function of RsNHX1 was validated for heterologous over-expression and inhibited-expression, as well as through turnip yellow mosaic virus (TYMV)-mediated silencing (VIGS) in radish. The outcomes of our study lay the foundation for further characterization of these CPA genes for roles in radish salt-tolerance processes.

2. Results

2.1. Identification and Classification of RsCPA Members in Radish

The Hidden Markov Model (HMM) profile was firstly performed to search the whole genome protein sequence of radish with the Na⁺/H⁺ exchanger domain (PF00999), and a total of 61 putative CPA proteins were obtained. Following, the CDD and InterPro tools were employed for detecting the completeness of the Na⁺/H⁺ exchanger domain and then one was excluded. Finally, 60 non-redundant
and complete RsCPA members were identified among the radish genome, which were correspondingly named as RsCPA01–RsCPA60 (Table S1).

Through the physical and chemical properties analysis, the protein sizes of RsCPA ranged from 231 to 1172 amino acids (AAs) with molecular weight (MWs) from 25.97 to 126.11 kDa and the theoretical isoelectric point (pI) varied from 4.98 to 9.21. In addition, the instability coefficient reached from 27.56 to 46.90 and 42 members were <40.00, which were considered as stable proteins. The aliphatic index varied from 95.31 to 127.56, indicating that most RsCPA proteins contained a lot of aliphatic amino acids. The grand average of hydropathicity (GRAVY) ranged from 0.048 to 0.798, suggesting that all RsCPA proteins were hydrophobic proteins (Table S1).

2.2. Phylogenetic Analysis of RsCPA Members

To investigate the classification of the CPA subfamily of radish and the evolutionary relationship with other species, full-length CPA protein sequences of radish, Arabidopsis, and Brassica rapa were extracted and aligned to construct a neighbor-joining (NJ) phylogenetic tree (Figure 1). A total of 166 CPA protein members in these three species (containing 60 radish, 42 Arabidopsis, and 64 Brassica rapa) were categorized into three subfamilies, namely NHX, KEA, and CHX. Among them, the NHX group had 28 members containing nine, eight, and 11 members of radish (15%), Arabidopsis (19.05%), and Brassica rapa (17.19%), respectively. The KEA group included 29 members with ten, six, and 13 members of radish (16.67%), Arabidopsis (14.29%), and Brassica rapa (20.31%), respectively. The CHX group was the most abundant subfamily and had 109 members with 41, 28, and 40 members in radish (68.33%), Arabidopsis (66.67%), and Brassica rapa (62.5%), respectively (Table 1). The phylogenetic relationships indicated that the CPA proteins in radish had stronger homology with Brassica rapa than Arabidopsis.

| Species                  | CHX Number | NHX Number | KEA Number | Total Gene Number |
|--------------------------|------------|------------|------------|-------------------|
| Arabidopsis thaliana     | 28         | 8          | 6          | 42                |
| Raphanus sativus         | 41         | 9          | 10         | 60                |
| Brassica rapa            | 40         | 11         | 13         | 64                |
| Pyrus bretscheideri      | 27         | 14         | 12         | 53                |
| Malus domestica          | 42         | 12         | 7          | 61                |
| Prunus persica           | 26         | 6          | 5          | 37                |
| Fragaria vesca           | 24         | 6          | 5          | 35                |
| Prunus mume              | 23         | 7          | 4          | 34                |
| Vitis vinifera           | 17         | 8          | 4          | 29                |
| Oryza sativa             | 18         | 8          | 4          | 30                |
| Zea mays                 | 16         | 11         | 6          | 33                |
| Sorghum bicolor          | 17         | 7          | 4          | 28                |
| Selaginella              | 3          | 7          | 4          | 14                |
| Ostreococcus             | 0          | 6          | 4          | 10                |
| Chlorophyta reinhardtii  | 0          | 9          | 3          | 12                |
| Physcomitrella patens    | 5          | 10         | 7          | 22                |
| Populus trichocarpa      | 29         | 8          | 7          | 44                |

Table 1. The number of cation proton antiporter (CPA) genes in 17 plant species.
Meanwhile, compared with several dicotyledon and monocotyledon crops, the number of CPA gene in radish was closer to *Malus domestica* and *Pyrus bretschneideri*, while it was significantly different from that in *Prunus persica*, *Fragaria vesca*, *Prunus mume*, and *Vitis vinifera*. Especially compared with monocotyledonous plants, such as *Oryza sativa*, *Zea mays*, and *Sorghum bicolor*, the difference of CPA gene numbers was very significant (Table 1).

### 2.3. Gene Structure and Motif Composition Analysis

All the 60 RsCPA members were divided into three subfamilies, including 9 RsNHXs, 10 RsKEAs, and 41 RsCHXs (Figure 2a). The distributions of RsCPA protein motifs were conducted by Multiple Em for Motif Elicitation (MEME) and 20 conserved motifs were generated (Figure 2b, Figure S1). Most RsCPA members in the same subfamily had similar motif compositions, suggesting that these proteins might have conservative functions. Among them, motif 1 and 12 were found in the CHX, KEA, and NHX subfamilies, indicating that were highly conserved in all RsCPA proteins. Additionally, some motifs were distributed in two subfamilies. For instance, motif 5, 11, and 17 were distributed in the CHX and NHX subfamilies, while motif 14 was distributed in the CHX and KEA subfamilies. Intriguingly, several motifs were only detected in specific RsCPA subfamilies. For example, the RsCHX subfamily independently contained diverse motifs, such as motif 3, 4, 6, 7, 8, 10, 13, 18, and 19. However, the KEA subfamily exclusively contained the motif 15 and 20, as well as the RsNHX subfamily that specifically contained motif 16 (Figure 2b).
Figure 2. Conserved motif and gene structure distribution of RsCPA proteins. (a) Phylogenetic tree of RsCPA proteins. The scale bar indicates 200 aa; (b) Conserved motif distribution of RsCPA proteins; (c) Exon-intron structure of CPA genes in radish. The scale bar indicates 1 kb.

Furthermore, exon-intron analysis was investigated to obtain the structure information of RsCPA genes. As shown in Figure 2c, the gene structures involved in the same subfamily were similar, while the lengths of the exon and intron were different. Compared with the RsCHXs subfamily, the gene structures of RsNHXs and RsKEAs were more complex. Among them, the exon numbers of RsCHXs were generally one to five. However, the exon numbers of RsNHXs and RsKEAs ranged from 10 to 19 and 17 to 21, respectively. Additionally, all members in RsNHX subfamily as well as most RsKEA ones (except RsCPA01) had UTR (untranslated region), whereas several members of the RsCHX subfamily had no UTR region (Figure 2c).

2.4. Promoter Elements and Transmembrane Region Analysis

The putative promoter sequence (2000 bp upstream region of transcription initiation site) of RsCPA genes was submitted to PlantCARE to search for cis-acting elements. A total of 103 cis-acting elements were identified. Except basic promoter elements, such as the CAAT box and TATA box, 18 other important cis-acting elements related to plant growth and development and various stresses were explored. In Figure 3, it was shown that the distribution pattern of cis-acting elements were diverse, indicating that the expression of RsCPA genes might be regulated by various factors. In the aspect of hormone regulation, Abscisic Acid (ABA), auxin, Gibberellin A₃ (GA₃), and Methyl Jasmonate (MeJA) responsiveness elements frequently existed. While in terms of stress, anaerobic induction, defense and stress, low temperature, and wound-responsive elements were resided. Moreover, the zein metabolism regulation element existed in 14 promoters of RsCPA genes, suggesting that RsCPA genes might participate in the process of metabolic regulation. Intriguingly, there were 12 promoters of the RsCPA gene involved in endosperm expression, among them, RsCPA27 and RsCPA38 participated in
endosperm specific negative expression (Figure 3 and Figure S2). Additionally, the transmembrane regions of RsCPA proteins showed that all RsCPA proteins, except RsCPA07, contained transmembrane regions that varied from three to 14 (Table S2).

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Furthermore, an interaction of CPA orthologs co-regulatory network was constructed based on the stress-inducible RsCPA orthologs in Arabidopsis (Figure S3). It was found that the combination score of the ATCHX1 and KEA4 protein was highest at 0.868, suggesting that it was involved in a stronger relationship between some specific RsCPA proteins, such as RsCPA45, RsCPA47, RsCPA03, RsCPA01, and RsCPA02.

2.5. Chromosomal Localization and Gene Distribution Analysis

A total of 58 RsCPA genes (96.67%) were successfully mapped to the R1–R9 chromosomes of radish by TBtools, except RsCPA12 and RsCPA51 (Figure 4a). R1 and R4 harbored the most RsCPA genes (Ten, 17.67%), followed by R5 and R6 (Eight, 13.37%), while R3 and R8 contained the least RsCPA genes (Two, 3.33%). Genome duplication events have facilitated the expansion of plant gene families, including whole-genome duplication (WGD)/segmental duplication, dispersed duplication (DD), tandem duplication (TD), proximal duplication (PD), and transposed duplication (TRD) [34–36]. The duplication types driving expansion of the RsCPA gene family was explored by Multiple Collinearity Scan toolkit (MCScanX). Each RsCPA gene was mapped on the radish genome based on the position coordinates to deduce the evolutionary relationship (Figure 4b). Totally, 24 pairs of CPA genes in radish had collinear relationships. Of these, 36 (60%) RsCPA genes were duplicated and retained in the WGD event, indicating that the WGD/segmental duplication type played an important role in expansion of the RsCPA gene family.
were 57 pairs of collinear genes in radish and Arabidopsis. AtCHX6B genes, for instance, Arabidopsis orthologous gene pairs, one radish gene corresponded to multiple RsCPA genes (Ten, 17.67%), followed by R5 and R6 (Eight, 13.37%), while R3 and R8 contained the least five pairs of syntenic orthologous genes (one to one) were identified, including RsCPA20–AtCHX15, RsCPA26–AtKEA2, RsCPA44–AtCHX28, and RsCPA45–AtCHX2. These genes could be traced back to a common ancestor in Arabidopsis and radish. Among the two synthetic orthologous gene pairs, one radish gene corresponded to multiple Arabidopsis genes, such as RsCPA26–AtKEA1/2, RsCPA27–AtCHX13/14/21/25/26, and RsCPA32–AtNHX1/2/6. Accordingly, there also existed syntenic orthologous gene pairs with one Arabidopsis gene corresponding to multiple radish genes, for instance, AtCHX6B–RsCPA39/42/43/53/55, AtNHX6–RsCPA32/36/60, AtKEA1–RsCPA24/26, among others. In addition, the gene pairs of two or three Arabidopsis genes corresponding to the same two radish gene pairs were also found, containing AtCHX3/4/6B–RsCPA39/43, AtCHX13/14/26–RsCPA27/28, AtCHX6B/7–RsCPA42/53, and AtNHX1/2–RsCPA31/32. A series of synteny events indicated that many CPA genes appeared before the divergence of the Arabidopsis and radish lineages (Figure 5, Table S3).

The non-synonymous/synonymous substitution ratio (Ka/Ks) for the 24 gene pairs was calculated to determine the selection pressure among duplicated RsCPA genes. Most of the RsCPA duplication genes (except RsCPA11–RsCPA23 and RsCPA21–RsCPA34) had a Ka/Ks < 1, indicating that they had experienced strong purifying selective pressure (Table S4).

Figure 4. Chromosomal distribution and chromosomal relationships of RsCPA genes. (a) Chromosomal distribution of RsCPA genes; (b) Genome distribution and collinearity of the RsCPA family. Red lines indicate the collinear relationship among genes.

Figure 5. Evolution analysis of the RsCPA gene family. Synteny blocks of CPA genes between radish and Arabidopsis. Colored lines connecting two chromosomal regions indicate syntenic regions between Arabidopsis (Chr1–5) and radish (R1–9) chromosomes.
2.7. Spatial and Temporal Expression Profiles of RsCPA Genes

According to the reads per kilobase per million (RPKM) values, the heatmap was generated to characterize the divergence in expression patterns of RsCPA genes among special tissues (cortical, cambium, xylem, root tip, and leaf) and different development stages (40, 60, and 90 d) (Figure 6). In general, the RPKM value varied from 0 to 103.06 and all RsCPA genes exhibited diverse expression patterns. Most of the RsNHX and RsKEA genes showed high expression levels among the five tissues, while the RsCHX genes had extremely low expression or were hardly expressed, as well as a few that showed tissue-specific expression (Figure 6a). It was found that RsCPA58 (RsCHX) was only highly expressed in leaves rather than other tissues, indicating that it might be a leaf-specific gene. Moreover, RsCPA35 was highly expressed in roots after 7 days, while it was down-regulated in roots at other development stages, suggesting it might be a spatiotemporal-specific gene. Furthermore, 68.33, 43.33, 28.33, 30, and 83.33% of the RsCPA genes showed a higher transcriptional abundance value (RPKM value > 10) in the five tissues, respectively (Figure 6b). Notably, RsCPA24, RsCPA29, RsCPA34, and RsCPA35 had abundant expression (RPKM value > 10) in five tissues (Figure 6c). These RsCPA genes might play various functions in the development of different tissues during various stages.

Figure 6. Expression profile of RsCPA genes in different stages and tissues. (a) RsCPA genes expression heatmap in six stages (7, 14, 20, 40, 60, and 90 days) and five tissues (cortical, cambium, xylem, root tip, and leaf). Expression values were calculated by (reads per kilobase per million) RPKM. The scale represents relative expression values; (b) Number of RsCPAs with a high transcriptional abundance level (RPKM > 10) in each tissue; (c) Venn diagram of overlapping RsCPAs that are abundantly expressed (RPKM > 10) in different tissues.
2.8. The RsCPA Genes Expression Levels under Abiotic Stresses

Based on our previous RNA-Seq. data in radish taproots, the differential expression levels of RsCPA genes under various abiotic stresses were investigated, including heavy metal (HM, such as Cadmium (Cd), Chromium (Cr), and lead (Pb)), temperature, and salt exposure. As a result, a total of 35, 38, 35, 33, and 29 RsCPA genes were differentially expressed during Cd, Pb, Cr, heat, and salt stress (Fold change >1, p-value < 0.05), respectively (Figure S4). For instance, RsCPA13 was up-regulated in response to Cd, Pb, Cr, and salt stresses, while it was down-regulated under high temperatures. RsCPA08 was up-regulated in response to Cd and Pb, whereas it was down-regulated under Cr and salt stress. Additionally, RsCPA35 was up-regulated in response to Cd, Pb, high temperature, and salt stresses, but it was down-regulated under Cr stress.

Furthermore, RT–qPCR was conducted to explore the expression levels of RsNHXs subfamily genes under the stress of salt exposure. On the whole, all of the RsNHX genes were significantly up-regulated under the 250 mM salt stress treatment. The expression level of RsCPA13, RsCPA29, RsCPA31, and RsCPA35 were highly increased during the 24 h salt exposure. Moreover, several genes were significantly up-regulated after 12 h salt exposure, such as RsCPA21 and RsCPA34. Notably, the expression level of RsNHXs recovered to normal levels after 96 h salt exposure, implying that RsNHXs might play a crucial role in the process of salt stress response (Figure S5).

2.9. Ectopic Expression of the RsNHX1 Gene in Arabidopsis Can Influence Salt Tolerance

To confirm the biologic function of RsNHX1 gene in the salt stress response of plant, over-expression (OE–RsNHX1) and amiRNA-induced inhibit-expression (amiR–RsNHX1) constructs were introduced into wild-type Arabidopsis (WT). Four and six independent transgenic lines were respectively generated for OE–RsNHX1 (#2, 4, 5 and 6) and amiR–RsNHX1 vectors (#1, 2, 3, 4, 5, and 6) (Figure S6). Following, each of the six OE–RsNHX1 and amiR–RsNHX1 transgenic lines were compared with nine WT lines under the 200 mM NaCl stress. It was found that the OE–RsNHX1 transgenic lines were slightly yellowed and grew better than the control lines, while most of the amiR–RsNHX1 transgenic lines turned markedly yellow and their growth was inferior to control lines (Figure 7a). For salt stress assays at the seedling stage, the germination ratio of two transgenic seedlings and WT seedlings were counted under 0, and 100 mM NaCl treatment (Figure 7b). As shown in Figure 7b,c, the germination rate of OE–RsNHX1 seedlings treated with 50 mM NaCl was higher than the WT, while amiR–RsNHX1 seedlings treated with 100 mM NaCl was significantly inferior to the WT. Furthermore, the root length of transgenic seedlings and the WT were measured under 0 and 100 mM NaCl treatment (Figure 7d). The root of OE–RsNHX1 seedlings exhibited continuing elongation, whereas the amiR–RsNHX1 seedlings were significantly inhibited under 100 mM NaCl exposure (Figure 7e). These results indicated that RsNHX1 might play a positive role in the salt tolerance of radish.
Three weeks after particle gun bombardment in radish seedlings, typical phenotype of chlorophyll photobleaching and TYMV spots were separately observed on the leaves of pTY–yellow mosaic virus (TYMV)-induced gene silencing (VIGS) technique to silence and pTY–S plants, indicating that TYMV–VIGS system was effective in radish and pTY–S vectors. Phytoene desaturase (PDS) was employed as a reporter gene to test whether the TYMV–derived vector can silence the endogenous gene of radish. Plants treated with the pTY–S virus vector designed to silence RsNHX1 expression (pTY–RsNHX1), with the wide type (WT), empty pTY–S silencing vector as well as the pTY–RsPDS gene served as mock, empty vector, and positive controls, respectively. Three weeks after particle gun bombardment in radish seedlings, typical phenotype of chlorophyll photobleaching and TYMV spots were separately observed on the leaves of pTY–RsPDS, pTY–RsNHX1, and pTY–S plants, indicating that TYMV–VIGS system was effective in radish (Figure 8a).

2.10. Functional Analysis of RsNHX1 in Radish Confirms that It Can Positively Regulates Salt Tolerance

We further identify the function of RsNHX1 gene responding to salt stress in radish by using turnip yellow mosaic virus (TYMV)-induced gene silencing (VIGS) technique to silence RsNHX1 expression. Phytoene desaturase (PDS) was employed as a reporter gene to test whether the TYMV–derived vector can silence the endogenous gene of radish. Plants treated with the pTY–S virus vector designed to silence RsNHX1 expression (pTY–RsNHX1), with the wide type (WT), empty pTY–S silencing vector as well as the pTY–RsPDS gene served as mock, empty vector, and positive controls, respectively. Three weeks after particle gun bombardment in radish seedlings, typical phenotype of chlorophyll photobleaching and TYMV spots were separately observed on the leaves of pTY–RsPDS, pTY–RsNHX1, and pTY–S plants, indicating that TYMV–VIGS system was effective in radish (Figure 8a).
were identified from the radish genome, which showed more members than other reported species. Furthermore, gene structure and motif analysis indicated that the RsCPAs family harbored KEA (29, 17.47%), and NHX (28, 16.87%), which was largely consistent with previous studies in Arabidopsis, Brassica rapa, and Vitis vinifera species indicating that the CPA gene family in radish may be duplicated and expanded. With the completion of genome sequencing in many plant species, CPA family members have been reported in various plant biology processes, including salt stress, cell expansion, pH and ion balance regulation, osmotic regulation, vesicle transport, protein processing, and floral organ development [15–19].

The total RNA was extracted from the diseased leaves of the suspected radish. Gel electrophoresis showed that the 488 bp PCR products were amplified in two pTY–S plants, the 522bp PCR products were amplified in four pTY–RsPDS plants and five pTY–RsNHX1 plants, indicating that they were successfully silenced in radish plants (Figure 8b). Furthermore, RT-qPCR revealed that the expression of positive pTY–RsNHX1 was significantly decreased compared to the WT and positive pTY–S plants (Figure 8c). Subsequently, the positive pTY–RsNHX1 plants showed more severe yellowing and wilting than positive pTY–S plants after 7 days of 250mM NaCl exposure (Figure 8d), showing that RsNHX1 might be a salt sensitive gene.

3. Discussion

3.1. Genome-Wide Identification and Phylogenetic Analysis of CPA Genes in Radish

The CPA gene encoded a conserved Na+/H+ domain and played an important role in diverse biology processes, including salt stress, cell expansion, pH and ion balance regulation, osmotic regulation, vesicle transport, protein processing, and floral organ development [15–19]. With the completion of genome sequencing in many plant species, CPA family members have been reported in various plant species, including model plant Arabidopsis (42) and Oryza sativa (30), as well as several horticulture plants, such as Vitis vinifera (29) and Pyrus bretschneideri (53) [11–14]. Herein, 60 RsCPA members were identified from the radish genome, which showed more members than other reported species indicating that the CPA gene family in radish may be duplicated and expanded.

Phylogenetic analysis indicated that a total of 166 CPAs among the three species containing radish, Brassica rapa, and Arabidopsis were divided into three subfamilies: CHX (109, 65.66%), KEA (29, 17.47%), and NHX (28, 16.87%), which was largely consistent with previous studies in Arabidopsis, Vitis vinifera, and Pyrus bretschneideri [11–14]. Through the phylogenetic relationships analysis, it was showed that RsCPAs exhibited closer relations to BraCPAs than AtCPAs, demonstrating that the CPA proteins in radish had stronger homology with the Brassica rapa rather than Arabidopsis (Figure 1). Furthermore, gene structure and motif analysis indicated that the RsCPAs family harbored
similar exon–intron structure and shared motif composition with other species, such as *Vitis vinifera* and *Pyrus bretschneideri* [13,14] (Figure 2).

### 3.2. Evolutionary Characterization of the RsCP A Family

The expansion of the gene family was mainly caused by gene duplication [34,35]. In the process of plant evolution, duplicated genes could obtain new functions or segment existing functions to improve the adaptability of plants [34]. For instance, expansion of the RsHSE gene family was primarily driven by WGD or segmental duplication, which might be largely related with gene duplication [36]. It was previously reported that WGD and PDs event were mainly involved in the expansion of the CPA family in *Arabidopsis* [14]. In the present study, the predicted gene duplication was also found in radish among the CPA genes, indicating that the WGD event played an important role in the expansion of the CPA gene family (Figure 4b). In addition, Ka/Ks could be used to identify whether selective pressure existed on the RsCPA gene family, including positive, negative, and neutral selection. Herein, except RsCPA11–RsCPA23 and RsCPA21–RsCPA34, all of the RsCPA duplication genes displayed a Ka/Ks < 1, suggesting that they had experienced strong purifying selective pressure. A similar Ka/Ks was also reported in the *Pyrus bretschneideri* and cotton, further confirming that the evolutionary pattern of CPA genes was very conservative (Table S4) [14,37].

Combined with evolutionary classification and synteny analysis, a large number of the RsCPAs were identified as orthologous genes in *Arabidopsis*. For example, eleven pairs seemed to be single radish-to-*Arabidopsis* pairs, presuming that these genes might exist in the genome of the last common ancestor of the two species. There also existed more complex relationships, such as single radish and multiple *Arabidopsis* genes, one *Arabidopsis* and multiple radish genes, and two *Arabidopsis* and multiple radish genes, etc. This phenomenon was similar to the evolutionary relationship of bZIP genes in radish and *Arabidopsis* [38]. The close relationship of orthologous genes may exhibit similar functions in different species. For instance, *AtNHX1* gene was reported high salt sensitivity [24], accordingly in our study its orthologues comparing RsCPA31, RsCPA32, and RsCPA34 also exhibited high induction under 250 mM NaCl salt treatments (Figure S5). The collinearity-orthologues analysis of radish and *Arabidopsis* could provide a valuable reference for further exploring the functions of these highly homologous genes in radish.

### 3.3. Roles of RsCP A Genes in Response to Different Abiotic Stresses

Increasing evidences indicated that CPA genes played vital roles in a variety of abiotic stresses, including HM, temperature, and salt exposure [39–42]. For instance, *AtCHX17* mutant accumulated less K+ than wide type under the salt stress and K+ deficiency environment, indicating that *AtCHX17* was involved in the absorption and transport of K+ [28]. Among the CPA genes in other plants, it was *NHX1* that could decrease the salt-tolerance ability of kallar grass at the concentration of 100 and 150 µM cadmium concentrations [43]. Moreover, *NHX1* regulated Cd2+ and H+ flow during short-term Cd2+ shock and confirmed that it could enhance tolerance during Cd2+ stress [44]. Recent studies have shown that *NHX2* homologues had a high expression under salinity stress at higher time intervals in *G. barbadense* and *G. hirsutum* [45]. The expressions of *NHX* were up-regulated in root tissues of wheat under salinity stress [46]. In this study, the transcriptome data of the radish taproot showed that nearly one-half of RsCPA genes displayed diverse expression profiles under HM, heat, and salt stress, indicating that they might play important roles in the plant response to abiotic stress. For example, RsCPA09 was significantly up-regulated under HM and heat stress, while exhibited down-regulated under salt stress, indicating that the expression of RsCPA09 might be a repress factor during salt stress. While RsCPA13 and RsCPA31 were all up-regulated under the HM and salt stress, indicating these two genes might play positive roles in response the various abiotic stresses of radish. A recent study in genome-wide identification of the *Gossypium hirsutum* NHX genes showed that most of the *GhNHX* genes were affected by salinity through salt-induced expression patterns analysis [47]. Here, according to the RT–qPCR analysis of *RsNHX* subfamily genes, we found all of them were significantly
up-regulated under the 250 mM salt treatment, which indicated that the RsNHX genes may be the critical characters for the salt response of radish.

3.4. Potential Functions of RsNHXs Genes in Salt Stress

Emerging evidence indicates that the Plant NHX proteins play critical roles for salt tolerance through biological function verification. For instance, over-expression of the soybean gene GmNHX1 in Arabidopsis thaliana could enhance salt tolerance through maintaining higher Na\(^{+}\) efflux rate and K\(^{+}/Na^{+}\) ratio, while silencing it may cause soybean plants became more susceptible to salt stress [48]. Similar, over-expression of wheat TaNHX2 gene in transgenic sunflower improved salinity stress tolerance and growth performance, which showed better growth performance and accumulated higher Na\(^{+}\), K\(^{+}\) contents in leaves and roots under 200 mM NaCl salt stress [49]. Additionally, a yeast functional complementation test proved that GhNHX4A can partially restore the salt tolerance of the salt-sensitive yeast mutant AXT3, while silencing it decreased the resistance of cotton [47]. In the present study, ectopic over-expression and inhibited-expression of the RsNHX1 gene in Arabidopsis significantly affected salt tolerance. Soil culture experiments showed that the growth of Arabidopsis OE–RsNHX1 responded more positively and amiR–RsNHX1 responded more negatively than the non-transgenic control plants. Furthermore, a TYMV-based VIGS system was used to functionally characterize the RsNHX1 gene, which was the first time to be employed for silencing the endogenous gene of radish. The expression levels of RsNHX1 gene were successfully silenced in pTY–RsNHX1 lines and the seedlings showed more salt damage than controls, which clarified that RsNHX1 may be a potential regulator in response to salt stress of radish.

According to our results, 60 CPA candidate genes of radish were firstly identified on the whole genome level. These genes could be clustered into three subfamilies, including nine RsNHXs, ten RsKEAs, and 41 RsCHXs. 58 genes were mapped to the nine chromosomes based on radish genome sequences. All the 60 RsCPA genes had various expression levels in the leaves, roots, cortex, cambium, and xylem at different development stages, as well as under various abiotic stresses. RT–qPCR analysis indicated that all nine RsNHXs genes showed upregulated trends after 250 mM NaCl exposure. The RsCPA31 (RsNHX1) gene, which might be the most important members of the RsNHX subfamily, exhibited obvious increased expression levels during 24h salt stress treatment. Heterologous over-expression and inhibited expression of RsNHX1 in Arabidopsis showed that RsNHX1 had a positive function in salt tolerance. Meanwhile, TYMV-based gene silence system was firstly used to functionally characterize the candidate gene in radish, and the silence of endogenous RsNHX1 in radish was more susceptible to the salt stress. The results would be useful to understand the complexity of the RsCPA gene family and could provide a valuable resource to explore the potential functions of RsCPA genes in radish.

4. Materials and Methods

4.1. Sequence Collection, CPA Identification, and Phylogenetic Analysis

Whole genome sequences of CPA were obtained from the radish genome database (RGD, http://radish-genome.org/) [33]. The CPA protein sequence of Arabidopsis was downloaded from TAIR10 (http://www.arabidopsis.org) [50,51]. The CPA protein sequence of Brassica rapa was available from Brassica database (BRAD, http://brassicadb.org/brad/) [52]. CPA family candidate genes with the Na\(^{+}/H^{+}\) exchanger domain (PF00999) were obtained from the Pfam database (http://pfam.xfam.org) [53]. Then, the Hidden Markov Model (HMM) search was carried out by HMMER 3.0 [54,55]. Subsequently, CDD (https://www.ncbi.nlm.nih.gov/cdd) and InterPro (http://www.ebi.ac.uk/interpro/interproscan/) were employed to verify the integrity of the Na\(^{+}/H^{+}\) exchanger domain [56,57]. In addition, the ExPASy ProtParam (https://www.expasy.org/) was performed to predict the physical and chemical properties of RsCPA proteins, such as the number of amino acids (AA), MW, pI, and instability index [58].
All CPA protein sequences in radish, *Brassica rapa* and *Arabidopsis* were imported to generate the phylogenetic tree using MEGA 6.0 with the neighbor-joining (NJ) and bootstrap value set to 1000 replicates [59,60]. The phylogenetic tree was visualized using Evolview (http://www.evolgenius.info/evolview/) [61].

4.2. Gene Structure, Motif Composition, and Promoter Element Analysis

The structure information of *RsCP A* genes were displayed by TBtools (https://github.com/CJ-Chen/TBtools) and the conserved motifs were identified by MEME (http://meme.nbcr.net/meme/meme) [62,63]. Moreover, the 2000 bp upstream sequence (putative promoter region) of all *RsCP A* genes were extracted by TBtools [62]. The cis-acting regulatory elements were predicted through PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [64]. Additionally, TransMembrane prediction was analyzed by Hidden Markov Models Server v.2.0 (TMHMM, http://www.cbs.dtu.dk/services/TMHMM/) [65] and the protein–protein relationships of stress-inducible *RsCP A* orthologs was evaluated by STRING 9 (https://string-db.org) [66].

4.3. Synteny Analysis and Chromosomal Localization

Synteny analysis was performed by the method described in the Plant Duplicate Gene Database (PlantDGD, http://pdgd.njau.edu.cn:8080/) [67]. The collinear block was identified by *RsCP A* duplication events in the MCScanX [68]. The data were integrated and plotted by using Circos [69]. Based on the annotation information from RGD and duplications of the *RsCP A* genes, the corresponding location distributions of *RsCP A* genes in chromosomes were displayed by TBtools [62].

4.4. Expression Analysis of *RsCP As* Based on the RNA-Seq Data

Illumina RNA-Seq data were downloaded from the NODAI Radish Genome Database and used for the transcriptional profiling of *RsCP A* genes in five tissues (cortical, cambium, xylem, root tip, and leaf) and six stages (7, 14, 20, 40, 60, and 90 days after sowing) [70]. The RPKM method was used to analyze the expression level for each *RsCP A* gene and the heatmap was displayed by TBtools [62]. Furthermore, the expression patterns of *RsCP A* genes during abiotic stress, including heavy metal (HM, such as Cd, Cr and Pb), high temperature, and salt stress were extracted and analyzed from the transcriptome data of radish taproots [71–75].

4.5. Plant Material, Salt Stress Treatment, and RT-qPCR Expression Analysis

The radish variety ‘NAU–XBC’ was used, which is an advanced inbred line with white flesh and white skin in taproot, and sensitive to salt stress. The seeds were germinated at 25 °C in the dark for 3 days and then cultivated in the growth chamber with a day/night temperature of 25/18 °C (16/8 h), 60% humidity, and 12,000 lx light. The three-week-old radish seedlings were grown in a plastic container with half-strength Hoagland nutrient solution, as previously described [76]. One week later, seedlings were treated with 250 mM NaCl and the NaCl-free solution was used as a control. Leaves were collected in triplicate at 0, 3, 6, 12, 24, 48, and 96 h after NaCl treatment and immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA was isolated using the TRIzol reagent RNAsimple total RNA kit (Tiangen, Beijing, China) and reverse transcribed into cDNA using the PrimeScript™ RT reagent kit (Takara, Dalian, China) according to the instructions. RT–qPCR analysis was carried out on a LightCycler® 480 System (Roche, Mannheim, Germany). The 2−ΔΔCT formula was used to calculate the relative expression level [77]. *RsActin* was regarded as the internal reference gene. The primers used for RT–qPCR are listed in Table S5.

*Arabidopsis thaliana* (ecotype: Col-0) plants were used for heterologous over-expression and inhibited expression *RsNHX1*. In addition, the advanced inbred line ‘NAU–YH’ seedlings were used to perform the VIGS experiment [78].
4.6. Genetic Transformation and Generation of RsNHX1 Transgenic Lines in Arabidopsis

To generate over-expression lines, the ORF sequence of RsNHX1 was cloned into the pCAMBIA2301 vector, using with BamHI/KpnI restriction sites, and driven by a 35S promoter [79]. For amiRNA construction, the natural miR319a sequence as the backbone was exchanged with the amiRRsNHX1 via the overlapping PCR method [80]. The 418 bp specific sequence was inserted with the XbaI and SacI digestion sites and then transferred into the pCAMBIA2301 vector [76]. These fusion plasmids were introduced into Arabidopsis using A. tumefaciens-mediated transformation with the floral dip method [81,82]. Transgenic lines were screened by 1/2 Murashige and Skoog (1/2 MS) solid medium with 100 mg·L⁻¹ kanamycin. Following, the 14-day-old wide type and transgenic line seedlings were planted in sterilized soil one week later and treated with 200 mM NaCl solution every other day [83]. Additionally, the seeds were sown on 1/2 MS solid media containing 0 and 100 mM NaCl to calculate the germination ratio as well as the root length after two weeks [24].

4.7. VIGS-Mediated Silencing of RsNHX1 in Radish

The VIGS experiment was conducted to functionally characterize RsCPA, as previously described [78]. The 40 bp specific sequence and its reverse complementary fragment of RsNHX1 and RsPDS were separately synthesized and phosphorylated, and inserted into the pTY–S vector by digestion with SnaBI and transformed to obtain positive clones. The primers used for vector construction to identify RsNHX1-silenced plants are listed in Table S6. The pTY–S empty vector and self-hybridized palindromic oligonucleotide of RsPDS-silencing vector were regarded as negative and positive controls, respectively. Particle bombardment was performed on the two-four fully expanded leaves from ‘NAU–YH’ plants using the PDS–1000/He bio-gun (Bio–Rad, Hercules, CA, USA) to trigger RsNHX1 silencing [84]. Five plants were bombarded in each experiment. Three weeks later, the inoculated plant leaf phenotype was observed and sampled to analyze the downstream gene level and silencing efficiency. RT–qPCR was used to further confirm RsNHX1-silencing. The primers used for RT–qPCR are listed in Table S5. Afterwards, the two positive plants were treated with 250 mM NaCl solution to observe the phenotypes.

Supplementary Materials: Supplementary Materials can be found at http://www.mdpi.com/1422-0067/21/8262/s1. Figure S1. LOGO of 20 amino acid motifs in RsCPA proteins. Figure S2. Number of cis–acting elements on promoters of RsCPA genes. Figure S3. Functional interaction networks of 60 RsCPA proteins. Figure S4. RNA–Seq of RsCPA genes under different treatments in the radish taproot. Figure S5. The expression levels of RsNHX genes at different times under 250 mM NaCl treatment. Figure S6. PCR analysis of over–expression and inhibited–expression in T3 transgenic Arabidopsis plants. Table S1. Basic data for the CPA proteins in radish. Table S2. Number of transmembrane regions in RsCPA proteins. Table S3. Synteny blocks of CPA genes between radish and Arabidopsis genomes. Table S4. Ka/Ks analysis of 24 RsCPA duplicated gene pairs. Table S5. Specific primers of RsCPA genes for qRT–PCR. Table S6. Specific primers of RsNHX1 for vector construction.

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Abbreviations

AA Amino Acids
ABA Abscisic Acid
AtCPA Arabidopsis thaliana Cation proton antiporter gene
BraCPA Brassica rapa Cation proton antiporter gene
BRAD Brassica database
Cd Cadmium
Chr(s) Chromosome(s)
CHX Cation/H+ exchanger
CPA Cation proton antiporter
Cr Chromium
DD Dispersed duplication
GA3 Gibberellin A3
GRAVY Grand average of hydropathicity
HM Heavy metal
HMM Hidden Markov Model
KEA K+ efflux antiporter
MCScanX Multiple Collinearity Scan toolkit
MeJA Methyl Jasmonate
MEME Multiple Em for Motif Elicitation
MW Molecular weight
NHX Na+/H+ exchanger family
NJ Neighbor-joining
ORF Open reading frame
Pb Lead
PD Proximal duplication
pI Theoretical isoelectric point
PlantDGD Plant Duplicate Gene Database
RGD Radish Genome Database
RPKM Reads per kilobase per million reads
RsCPA Raphanus sativus Cation proton antiporter gene
RT-qPCR Real-Time Quantitative Polymerase Chain Reaction
TD Tandem duplication
TMHMM TransMembrane prediction was analyzed by Hidden Markov Models
TRD Transposed duplication
TYMV Turnip yellow mosaic virus
UTR Untranslated region
VIGS Virus-induced gene silencing
WGD Whole-genome duplication
WT Wide type

References

1. Zhu, J.K. Abiotic stress signaling and responses in plants. Cell 2016, 16, 313–324. [CrossRef] [PubMed]
2. Chanroj, S.; Wang, G.Y.; Venema, K.; Zhang, M.W.; Delwiche, C.F.; Sze, H. Conserved and diversified gene families of monovalent cation/H+ antiporters from algae to flowering plants. Front. Plant Sci. 2012, 9, 50–55. [CrossRef] [PubMed]
3. Ye, C.Y.; Yang, X.; Xia, X.; Yin, W.L. Comparative analysis of cation/proton antiporter superfamily in plants. Gene 2013, 521, 245–251. [CrossRef] [PubMed]
4. An, R.; Chen, Q.J.; Chai, M.F.; Lu, P.L.; Su, Z.; Qin, Z.X.; Chen, J.; Wang, X.C. AtNHX8, a member of the monovalent cation: Proton antiporter-1 family in Arabidopsis thaliana, encodes a putative Li+/H+ antiporter. Plant J. 2007, 49, 718–728. [CrossRef]
5. Very, A.A.; Sentenac, H. Molecular mechanisms and regulation of K+ transport in higher plants. Annu. Rev. Plant Biol. 2003, 54, 575–603. [CrossRef]
11. Mäser, P.; Thomine, S.; Schroeder, J.I.; Ward, J.M.; Hirschi, K.; Sze, H.; Talke, I.N.; Amtmann, A.; Zhou, Y.; Yang, C.; Hu, Y.; Zhu, X.P.; Hu, D.G.; Hao, Y.J. The novel Na
12. Bassil, E.; Coku, A.; Blumwald, E. Cellular ion homeostasis: Emerging roles of intracellular NHX Na
13. Ma, Y.; Wang, J.; Zhong, Y.; Cramer, G.R.; Cheng, Z.M.M. Genome-wide analysis of the cation
14. Zhou, H.S.; Qi, K.J.; Liu, X.; Yin, H.; Wang, P.; Chen, J.Q.; Wu, J.Y.; Zhang, S.L. Genome-wide identification
15. Banjara, M.; Zhu, L.F.; Shen, G.X.; Payton, P.; Zhang, H. Expression of an Arabidopsis sodium/proton antiporter gene (AtNHX1) in peanut to improve salt tolerance. Plant Biotechnol. Rep. 2012, 6, 59–67. [CrossRef]
16. Bassil, E.; Tajima, H.; Liang, Y.C.; Ohto, M.A.; Ushijima, K.; Nakano, R.; Esumi, T.; Coku, A.; Belmonte, M.; Blumwald, E. The Arabidopsis Na+/H+ antiporters NHX1 and NHX2 control vacuolar pH and K+ homeostasis to regulate growth, flower development, and reproduction. Plant Cell 2011, 23, 3482–3497. [CrossRef] [PubMed]
17. Bassil, E.; Coku, A.; Blumwald, E. Cellular ion homeostasis: Emerging roles of intracellular NHX Na+/H+ antiporters in plant growth and development. J. Exp. Bot. 2012, 63, 5727–5740. [CrossRef]
18. Yokoi, S.; Quintero, F.J.; Cubero, B.; Ruiz, M.T.; Bressan, R.A.; Hasegawa, P.M.; Pardo, J.M. Differential expression and function of Arabidopsis thaliana NHX Na+/H+ antiporters in the salt stress response. Plant J. 2002, 30, 529–539. [CrossRef] [PubMed]
19. Li, H.T.; Liu, H.; Gao, X.S.; Zhang, H.X. Knock-out of Arabidopsis AtNHX4 gene enhances tolerance to salt stress. Biochem. Biophys. Res. Commun. 2009, 382, 637–641. [CrossRef]
20. Mishra, S.; Alavilli, H.; Lee, B.; Panda, S.K.; Sahoo, L. Cloning and characterization of a novel vacuolar Na+/H+ antiporter gene (VuNHX1) from drought hardy legume, cowpea for salt tolerance. Plant Cell Tissue Organ 2015, 120, 19–33. [CrossRef]
21. Sun, M.H.; Ma, Q.J.; Liu, X.; Zhu, X.P.; Hu, D.G.; Hao, Y.J. Molecular cloning and functional characterization of MdNHX1 reveals its involvement in salt tolerance in apple calli and Arabidopsis. Sci. Hortic. 2017, 215, 126–133. [CrossRef]
22. Zhou, Y.; Yang, C.; Hu, Y.; Zhu, X.P.; Hu, D.G.; Hao, Y.J. The novel Na+/H+ antiporter gene SpNHX1 from Sesuvium portulacastrum confers enhanced salt tolerance to transgenic yeast. Acta Physiol. Plant. 2018, 40, 61. [CrossRef]
26. Zhang, W.W.; Meng, J.J.; Xing, J.Y.; Yang, S.; Guo, F.; Li, X.G.; Wan, S.B. The K+/H+ antiporter AhNHX1 improved tobacco tolerance to NaCl stress by enhancing K+ retention. J. Plant Biol. 2017, 60, 259–267. [CrossRef]

27. Qiu, Q.S.; Guo, Y.; Dietrich, M.A.; Schumaker, K.S.; Zhu, J.K. Regulation of SOS1, a plasma membrane Na+/H+ exchanger in Arabidopsis thaliana, by SOS2 and SOS3. Proc. Natl. Acad. Sci. USA 2002, 99, 8436–8441. [CrossRef] [PubMed]

28. Cellier, F.; Conéjéro, G.; Ricaud, L.; Luu, D.T.; Lepetit, M.; Gosti, F.; Casse, F. Characterization of AtCHX17, a member of the cation/H+ exchangers, CHX family, from Arabidopsis thaliana suggests a role in K+ homeostasis. Plant J. 2004, 39, 834–846. [CrossRef]

29. Kunz, H.H.; Gierth, M.; Herdean, A.; Satoh-Cruz, M.; Kramer, D.M.; Spetec, C.; Schroeder, J.I. Plastidial transporters KEA-1, -2, and-3 are essential for chloroplast osmoregulation, integrity, and pH regulation in Arabidopsis. Proc. Natl. Acad. Sci. USA 2014, 111, 7480–7485. [CrossRef]

30. Wang, L.; Jun, L.; Shi, W.M.; Yan, H.; Han, L. Identification and localized expression of putative K+/H+ antiporter genes in Arabidopsis. Acta Physiol. Plant. 2015, 37, 101.

31. Sun, X.C.; Xu, L.; Wang, Y.; Yu, R.G.; Zhu, X.W.; Luo, X.B.; Gong, Y.Q.; Wang, R.H.; Limera, C.; Zhang, K.Y.; et al. Identification of novel and salt-responsive miRNAs to explore miRNA-mediated regulatory network of salt stress response in radish (Raphanus sativus L.). BMC Genom. 2015, 16, 1–16. [CrossRef]

32. Sun, X.C.; Wang, Y.; Xu, L.; Li, C.; Zhang, W.; Luo, X.B.; Jiang, H.Y.; Liu, L.W. Unraveling the root proteome changes and its relationship to molecular mechanism underlying salt stress response in radish (Raphanus sativus L.). Front. Plant Sci. 2017, 8, 1192. [CrossRef]

33. Jeong, Y.M.; Kim, N.; Ahn, B.O.; Oh, M.; Chung, W.H.; Chung, H.; Jeong, S.; Lim, K.B.; Hwang, Y.J.; Kim, G.B.; et al. Elucidating the triplicated ancestral genome structure of radish based on chromosome-level comparison with the Brassica genomes. Theor. Appl. Genet. 2016, 129, 1357–1372. [CrossRef]

34. De, G.A.; Lanave, C.; Saccone, C. Genome duplication and gene-family evolution: The case of three OXPHOS gene families. Gene 2008, 421, 1–6.

35. Airoldi, C.A.; Davies, B. Gene duplication and the evolution of plant MADS-box transcription factors. J. Genet. Genom. 2012, 39, 157–165. [CrossRef] [PubMed]

36. Tang, M.J.; Xu, L.; Wang, Y.; Cheng, W.W.; Luo, X.B.; Xie, Y.; Fan, L.X.; Liu, L.W. Genome-wide characterization and evolutionary analysis of heat shock transcription factors (HSFs) to reveal their potential role under abiotic stresses in radish (Raphanus sativus L.). BMC Genom. 2019, 20, 772. [CrossRef]

37. Fu, X.; Lu, Z.; Wei, H.; Zhang, J.; Yang, X.; Wu, A.; Ma, L.; Kang, M.; Lu, J.; Wang, H.; et al. Genome-Wide identification and expression analysis of the NHX (Sodium/Hydrogen Antiporter) gene family in cotton. Front. Genet. 2020, 11, 964. [CrossRef]

38. Fan, L.X.; Xu, L.; Wang, Y.; Tang, M.J.; Liu, L.W. Genome- and Transcriptome-wide characterization of bZIP gene family identifies potential members involved in abiotic stress response and anthocyanin biosynthesis in radish (Raphanus sativus L.). Int. J. Mol. Sci. 2019, 20, 6334. [CrossRef]

39. Chen, H.T.; Chen, X.; Wu, B.Y.; Yuan, X.X.; Zhang, H.M.; Cui, X.Y.; Liu, X.Q. Whole-Genome identification and expression analysis of KEA and NHX antiporter family under abiotic stress in soybean. J. Integr. Agric. 2015, 14, 1171–1183. [CrossRef]

40. Jha, A.; Joshi, M.; Yadav, N.S.; Agarwal, P.; Jha, B. Cloning and characterization of the Salicornia brachiata Na+/H+ antiporter gene SbNHX1 and its expression by abiotic stress. Mol. Biol. Rep. 2011, 38, 1965–1973. [CrossRef]

41. Hamed, A.; Hamid, R.K.; Hadi, H.; Iraj, T. Assessment of the vacuolar Na+/H+ antiporter (NHX1) transcriptional changes in Leptochloa fusca L. in response to salt and cadmium stresses. Mol. Biol. Res. Commun. 2015, 4, 133–142.

42. Sharma, H.; Taneja, M.; Upadhyay, S.K. Identification, characterization and expression profiling of cation–proton antiporter superfamily in Triticum aestivum L. and functional analysis of TaNHX4-B. Genomics 2020; 112, 356–370. [CrossRef]

43. Yang, L.; Han, Y.J.; Wu, D.; Yong, W.; Liu, M.M.; Wang, S.T.; Liu, W.X.; Lu, M.Y.; Wei, Y.; Sun, J.S. Salt and cadmium stress tolerance caused by overexpression of the Glycine Max Na+/H+ antiporter (GmNHX1) gene in duckweed (Lemma tritiumfera 5511). Aquat. Toxicol. 2017, 192, 127–135. [CrossRef] [PubMed]
44. Yang, L.; Wei, Y.; Li, N.; Zeng, J.Y.; Han, Y.J.; Zuo, Z.J.; Wang, S.T.; Zhu, Y.R.; Zhang, Y.; Sun, J.S.; et al. Declined cadmium accumulation in Na+/H+ antiporter (NHX1) transgenic duckweed under cadmium stress. *Ecotoxicol. Environ. Saf.* 2019, 182, 109397. [CrossRef] [PubMed]

45. Akram, U.; Song, Y.H.; Liang, C.Z.; Abid, M.A.; Askari, M.; Myat, A.A.; Abbas, M.; Malik, W.; Ali, Z.; Guo, S.D.; et al. Genome-wide characterization and expression analysis of NHX gene family under salinity stress in *Gossypium barbadense* and its comparison with *Gossypium hirsutum*. *Genes* 2020, 11, 803. [CrossRef]

46. Tiwari, B.K.; Aquib, A.; Anand, R. Analysis of physiological traits and expression of NHX and SOS3 genes in bread wheat (*Triticum aestivum* L.) under salinity stress. *J. Pharmacogn. Phytochem.* 2020, 9, 362–366.

47. Ma, W.; Ren, Z.; Zhou, Y.; Zhao, J.; Zhang, F.; Feng, J.; Liu, W.; Ma, X. Genome-wide identification of the *Gossypium hirsutum* NHX genes reveals that the endosomal-type GhNHX4A is critical for the salt tolerance of cotton. *Int. J. Mol. Sci.* 2020, 21, 7712. [CrossRef]

48. Sun, T.J.; Fan, L.; Yang, J.; Cao, R.Z.; Yang, C.Y.; Zhang, J.; Wang, D. A glycine max sodium/hydrogen exchanger enhances salt tolerance through maintaining higher Na+ efflux rate and K+/Na+ ratio in *Arabidopsis*. *BMC Plant Biol.* 2019, 19, 469. [CrossRef] [PubMed]

49. Mushke, R.; Rajesh, Y.; Kirti, P.B. Improved salinity tolerance and growth performance in transgenic sunflower plants via ectopic expression of a wheat antiporter gene (*TaNHX2*). *Mol. Biol. Rep.* 2019, 46, 5941–5953. [CrossRef] [PubMed]

50. Berardini, T.Z.; Reiser, L.; Li, D.H.; Mezheritsky, Y.; Muller, R.; Strait, E.; Huala, E. The arabidopsis information resource: Improved gene annotation and new tools. *Nucleic Acids Res.* 2012, 40, D1202–D1210. [CrossRef]

51. Lamesch, P.; Berardini, T.Z.; Li, D.H.; Swarbreck, D.; Wilks, C.; Sasidharan, R.; Muller, R.; Dreher, K.; Alexander, D.L.; Garcia-Hernandez, M.; et al. The Arabidopsis Information Resource (TAIR): Improved gene annotation and new tools. *Nucleic Acids Res.* 2008, 32, D138.

52. Cheng, F.; Liu, S.Y.; Wu, J.; Fang, L.; Sun, S.L.; Liu, B.; Li, P.X.; Hua, W.; Wang, X.W. BRAD, the genetics and genomics database for Brassica plants. *BMC Plant. Biol.* 2011, 11, 36. [CrossRef]

53. Finn, R.D.; Tate, J.; Coggill, P.C.; Sammut, S.J.; Hotz, H.R.; Ceric, G.; Forslund, K.; Eddy, S.R.; Sonnhammer, E.L.L. The Pfam protein families database. *Nucleic Acids Res.* 2006, 35, D219–D225. [CrossRef]

54. Marchler-Bauer, A.; Anderson, J.B.; Cherukuri, P.F.; DeWweese-Scott, C.; Geer, L.Y.; Gwadz, M.; He, S.Q.; Hurwitz, D.J.; Jackson, J.D.; Ke, Z.X. CDD: A Conserved Domain Database for protein classification. *Nucleic Acids Res.* 2005, 33, D192–D196. [CrossRef]

55. Zdobnov, E.M.; Apweiler, R. InterProScan—An integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 2001, 17, 847–848. [CrossRef]

56. Marchler-Bauer, A.; Anderson, J.B.; Cherukuri, P.F.; DeWweese-Scott, C.; Geer, L.Y.; Gwadz, M.; He, S.Q.; Hurwitz, D.J.; Jackson, J.D.; Ke, Z.X. CDD: A Conserved Domain Database for protein classification. *Nucleic Acids Res.* 2005, 33, D192–D196. [CrossRef]

57. Zdobnov, E.M.; Apweiler, R. InterProScan—An integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 2001, 17, 847–848. [CrossRef]

58. Marchler-Bauer, A.; Anderson, J.B.; Cherukuri, P.F.; DeWweese-Scott, C.; Geer, L.Y.; Gwadz, M.; He, S.Q.; Hurwitz, D.J.; Jackson, J.D.; Ke, Z.X. CDD: A Conserved Domain Database for protein classification. *Nucleic Acids Res.* 2005, 33, D192–D196. [CrossRef]

59. Zdobnov, E.M.; Apweiler, R. InterProScan—An integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 2001, 17, 847–848. [CrossRef]

60. Olivier, G.; Mike, S. Neighbor-Joining Revealed. *Mol. Biol. Evol.* 2006, 23, 1997–2000.

61. Zhang, H.K.; Gao, S.H.; Lercher, M.J.; Hu, S.N.; Chen, W.H. EvolView, an online tool for visualizing, annotating and managing phylogenetic trees. *Mol. Biol. Evol.* 2006, 23, 2725–2729. [CrossRef]

62. Zdobnov, E.M.; Apweiler, R. InterProScan—An integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 2001, 17, 847–848. [CrossRef]

63. Marchler-Bauer, A.; Anderson, J.B.; Cherukuri, P.F.; DeWweese-Scott, C.; Geer, L.Y.; Gwadz, M.; He, S.Q.; Hurwitz, D.J.; Jackson, J.D.; Ke, Z.X. CDD: A Conserved Domain Database for protein classification. *Nucleic Acids Res.* 2005, 33, D192–D196. [CrossRef]

64. Zdobnov, E.M.; Apweiler, R. InterProScan—An integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 2001, 17, 847–848. [CrossRef]

65. Ottoman, G.; Schuster, C.; Christian, H.; Ron, D.A.; Amos, B. ExPaSy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* 2003, 31, 3784–3788.

66. Tamura, K.; Stecher, G.; Peterson, D.; Filipski, A.; Kumar, S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 2013, 30, 2725–2729. [CrossRef]

67. Olivier, G.; Mike, S. Neighbor-Joining Revealed. *Mol. Biol. Evol.* 2006, 23, 1997–2000.

68. Zhang, H.K.; Gao, S.H.; Lercher, M.J.; Hu, S.N.; Chen, W.H. EvolView, an online tool for visualizing, annotating and managing phylogenetic trees. *Nucleic Acids Res.* 2012, 40, 569–572. [CrossRef]

69. Chen, C.J.; Xia, R.; Chen, H.; He, Y.H. TBtools, a toolkit for biologists integrating various biological data handling tools with a user-friendly interface. *BioRxiv* 2018, 289660. [CrossRef]

70. Bailey, T.L. MEME-ChIP: Motif analysis of large DNA datasets. *Bioinformatics* 2011, 27, 1696–1697.

71. Stephane, R.; Patrice, D.; Marc, VM.; Pierre, R. PlantCARE, a plant cis-acting regulatory element database. *Nucleic Acids Res.* 1999, 27, 295–296.

72. Chen, Y.J.; Yu, P.; Luo, J.C.; Jiang, Y. Secreted protein prediction system combining CJ-SPHMM, TMHMM, and PSORT. *Mamm. Genome* 2003, 14, 859–865. [CrossRef]

73. Zdobnov, E.M.; Apweiler, R. InterProScan—An integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 2001, 17, 847–848. [CrossRef]

74. Marchler-Bauer, A.; Anderson, J.B.; Cherukuri, P.F.; DeWweese-Scott, C.; Geer, L.Y.; Gwadz, M.; He, S.Q.; Hurwitz, D.J.; Jackson, J.D.; Ke, Z.X. CDD: A Conserved Domain Database for protein classification. *Nucleic Acids Res.* 2005, 33, D192–D196. [CrossRef]

75. Zdobnov, E.M.; Apweiler, R. InterProScan—An integration platform for the signature-recognition methods in InterPro. *Bioinformatics* 2001, 17, 847–848. [CrossRef]
