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Cation/Proton Antiporter Genes in Tomato: Genomic Characterization, Expression Profiling, and Co-Localization with Salt Stress-Related QTLs

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Abstract: The cation/proton antiporter (CPA) family represents a class of transmembrane transporter proteins that play a crucial role in plants during high salinity stress by maintaining the cell's ionic balance and pH homeostasis. So far, the *CPA* genes have not been systematically characterized in tomato *(Solanum lycopersicum)*. In this study, we identified and analyzed 33 putative *CPA* genes in tomato. Phylogenetic analysis showed that tomato CPAs could be classified into three subgroups, i.e., CHX (18 genes), KEA (8 genes), and NHX (7 genes). *CPA* genes within each subgroup shared similar motifs, conserved catalytic domains and gene structure. Further analysis revealed that the *CPA* genes were unevenly distributed on the chromosomes and segmental duplication events played a major role in the expansion of the *CPA* gene family in tomato. Gene expression analysis exhibited that *CPA* genes were differentially expressed in different tissues, various stages of fruit development, and differentially regulated in response to abiotic stresses, especially salt stress. Further, co-localization of tomato *CPA* genes with quantitative trait loci (QTL) of salt stress-related phenotypes revealed their broader functions in salt stress tolerance. Finally, predicted protein–protein interactions of tomato CPAs, gene ontology analysis, and the presence of putative cis-elements in the promoter further support the diverse role of tomato CPAs in plant development and plant stress tolerance. In brief, this study highlights the potential role of tomato CPAs in plant development and abiotic stress tolerance, especially in salt stress, and provides comprehensive information to explore new candidate genes for salt tolerance in tomato.

Keywords: CPA; CHX; KEA; NHX; ion transporter; tomato; stress response; stress QTLs; expression analysis

1. Introduction

The cation/proton antiporters (CPAs) are responsible for maintaining the pH homeostasis and ion concentrations in all living species, including plants and animals. *CPA* genes play critical roles in many aspects of plant growth, development, signal transduction, and stress responses [1]. The CPA members are structurally divided into two broad categories, designated as CPA1 and CPA2 [2]. The CPA1 includes the Na^+/H^+ exchanger (NHX) family, while CPA2 consists of K^+ efflux antiporter (KEA) and cation/H^+ exchanger (CHX) family of proteins [3,4].

The CPA superfamily is functionally involved in the exchange and transport of monovalent cations in plants [5], therefore, can be divided into two main types, i.e., transport of sodium ion (Na+) or potassium ion (K^+) and a cation exchange for one or two protons [6]. In plants, the salt tolerance mechanism is mainly based on maintaining the ion homeostasis, osmotic balance, and cellular tolerance. Plants respond to the elevated level of ions (Na^+, Cl^-, and K^+) by regulating the transport of water and ions between the plant and the environment, as well as distribution inside the plant, and among the organelles [7]. In plants, ion concentration and pH balance feat are accomplished with a diverse array of transporters, especially the CPA proteins [1] that are localized in the plasma membrane as well as organelle membranes such as endosomes, vacuoles, and chloroplasts [3,7,8]. The CPA gene family has been extensively studied in various plant species, including *Arabidopsis thaliana* [9], radish (*Raphanus sativus*) [10], wheat (*Triticum aestivum*) [5], rice (*Oryza sativa*) [11], grape (*Vitis vinifera*) [12], and pear (*Pyrus bretschneideri*) [13]. However, the function of CPAs in tomato remains largely unknown.

Tomato (*Solanum lycopersicum*) is considered the most important vegetable crop cultivated worldwide. Tomato has a relatively small and compact genome of approximately 950 Mb and a short life cycle [14]. Tomato is a rich source of nutrients and a model plant for fleshy fruit development. In the last decade, the genome of tomato and its wild relatives have been completely sequenced by the international genome sequencing consortium [15]. However, with a continuous expansion in the cultivation of tomato, its production has been seriously impaired in recent years by various abiotic stresses, such as temperature, drought, and salinity [16]. The tomato plant is highly vulnerable to salinity stress because high levels of Na+ ions adversely affects cellular metabolism and ion homeostasis. Therefore, the identification of potential genes that could confer resistance to abiotic stresses is of the utmost importance for molecular breeding of tomato and a comprehensive understanding of CPA gene family functions in tomato would be an ideal beginning based on the reported role of CPA genes in other plant species.

In the present study, a comprehensive genome-wide analysis of the *Solanum lycopersicum* CPAs, hereafter referred to as *Sl*CPAs, gene family was performed and identified 33 *SlCPA* genes. Moreover, they were further analyzed to determine their phylogenetic relationship, physiochemical parameters, conserved motifs, subcellular localization, and gene structure. The transcriptome of *SlCPA* genes was evaluated in different tissues, various fruit developmental stages, and under abiotic stresses. Furthermore, we carried out the co-localization of *SlCPA* genes with QTLs of salt stress-related phenotypes. Finally, protein–protein interactions among the *Sl*CPAs and the associated proteins were predicted. This study provides a fundamental understanding of *Sl*CPAs in conferring abiotic stress resistance in tomato and will be useful for the long-term improvement of stress tolerance in tomato.

2. Materials and Methods

2.1. Identification of CPA Gene Family in Tomato

Genomic and amino acid sequences of tomato CPA family members were retrieved from the Phytozome database (https://phytozome.jgi.doe.gov/pz/portal.html, accessed on 25 march 2021). The *Arabidopsis* CPA protein sequences were obtained from TAIR10 database (http://www.arabidopsis.org, accessed on 25 March 2021) [17]. HMM profile (Hidden Markov Model) was built from Arabidopsis CPA proteins to identify tomato CPAs

using HMMER 3.0. Then, the HMM profile was used to query the tomato protein database (E-value less than e^{-10}) [18,19]. The putatively identified *Sl*CPA sequences were verified by BLASTp search at e-value < 1.0. The *Sl*CPA protein sequences were verified by a conserved domain search (PF00999) using InterPro webtool (http://www.ebi.ac.uk/x/pfa/iprscan/, accessed on 30 March 2021) [20]. We eliminated the proteins without the CPA-conserved domain. The CDA-Hit-v4.6.648 (cluster database at high identity with tolerance) with a threshold identity of 90% further removed the redundant proteins sequences and provided 33 *Sl*CPA representative proteins for further study.

2.2. Physiochemical Properties and Sequence Analysis

The physiochemical details of *Sl*CPA proteins were predicted using the ExPASy ProtParam tool (http://web.expasy.org/protparam/, accessed on 10 April 2021).) [21]. The various properties include isoelectric point, length of protein, protein molecular weights, theoretical pI, GRAVY, and instability index. The subcellular localization of *Sl*CPA proteins were detected using CELLO2GO (http://cello.life.nctu.edu.tw/cello2go/, accessed on 10 April 2021) [22]. Additionally, transmembrane helices in *Sl*CPA proteins were analyzed by TMHMM Server v.2.0 (http://www.cbs.dtu.dk/services/TMHMM/, accessed on 12 April 2021) [23]. The gene structures of *SlCPAs* (exon/intron) were displayed by the GSDS server (http://gsds.gao-lab.org, accessed on 15 April 2021). The conserved motifs of *Sl*CPA proteins were analyzed using the MEME server with default parameters (http://meme-suite.org/tools/meme, accessed on 15 April 2021) [24].

2.3. Phylogenetic Analysis

All the CPA protein sequences from tomato and *Arabidopsis* were aligned with Clustal X. Phylogenetic tree was built by the neighbor-joining (NJ) method and bootstrap consensus tree values were set to 1000 replicates. The phylogenetic analysis was executed in MEGA 7.0 [25], and the tree visualization was done by Itol v 6 (https://itol.embl.de, accessed on 25 April 2021) [26].

2.4. Chromosomal Localization and Synteny Analysis

The genome and annotation files of tomato and *Arabidopsis* were downloaded from the phytozome website. Gene duplication events, paralogous, and orthologous genes were identified by MCScanX toolkit [27]. The collinear block was identified by *SlCPAs* duplication events in the MCScanX. The synteny blocks were constructed by utilizing TBtools [28]. The chromosomal positions of *SlCPA* genes were identified according to the Phytozome database and the chromosomal map was visualized using the Ritchie-lab phenogram tool (http://visualization.ritchielab.org/phenograms/plot, accessed on 2 May 2021).

2.5. Promoter Analysis

The presence of cis-regulatory elements in the *SlCPA* genes was investigated as described previously [29]. Briefly, promoter sequences (2 kb upstream genomic DNA sequences) of *SlCPA* genes were analyzed using the PlantCARE database [30] and validated in the PLACE database [31] and visualized into TBtools [28].

2.6. Transcriptomic Data Analysis of SlCPA Genes in Different Tissues and under Different Abiotic Stresses

Illumina high throughput RNA-sequencing data of leaves, roots, flower buds, fully opened flowers, and 1 cm, 2 cm, and 3 cm of mature green, breaker, and breaker+10 fruits of tomato cultivar *Heinz* were downloaded from the Tomato Functional Genomics Database (http://ted.bti.cornell.edu/cgi-bin/TFGD/digital/home.cgi, accessed on 10 May 2021). Illumina high throughput RNA-sequencing data of tomato under heat and drought (GEO accession: GSE151277), and salinity (GEO accession: GSE148353) were downloaded from the NCBI GEO database. The expression data in three replicates were used for the transcrip-

tional profiling of *SlCPA* genes in different tissues, developmental stages, and under abiotic stresses. The FPKM (fragments-per-kilobase-per-million) protocol was utilized for the expression analysis of each *SlCPA* gene. The heatmap charts were drawn by the pheatmap package in R Studio.

2.7. Plant Material and Salinity Stress Treatment

The tomato cultivar (*Solanum lycopersicum L. cv.* Rio Grande) was used to analyze the expression patterns of *SlCPA* genes in response to salinity stress. Seeds were planted in the growth room at 25/22 °C (day/night), 60% humidity, 12,000 lx light, and 16/8 h light and dark regime. After germination, seedlings of uniform length were shifted to 1/5 Hoagland solution with pH 5.0 as previously described [32], under the same growth conditions. After six days, the nutrient solution was supplemented with 250 mM NaCl for salinity treatment.

2.8. RNA Isolation, cDNA Synthesis, and qRT-PCR

For RNA isolation, leaf tissues were collected after 0, 3, 6, 12, 24, 48 and 96 h of salinity stress. RNA from leaves was isolated using RNA Plant Mini Kit (Tiangen, Beijing, China). The RNA was converted to cDNA using the PrimeScriptTM RT kit (Takara, Kusatsu shi, Japan). qRT-PCR analysis was performed using LightCycler® 480 System (Roche, Basel, Switzerland) using gene-specific primers (Supplementary Materials, Table S3) as described previously in [33]. The relative expression level was calculated by the $2^{-\Delta\Delta CT}$ formula and the data were normalized with *Actin* (*Act*). Three biological replicates were performed for each sample.

2.9. Gene Ontology and Network Interaction Analysis

Gene ontology (GO) terms of *SlCPA* genes were determined using CELLO2GO web server for protein subcellular localization prediction with functional gene ontology annotation (http://cello.life.nctu.edu.tw/cello2go/, accessed on 25 May 2021). The protein–protein interactions of stress-responsive *Sl*CPAs were carried out using STRING 9 (https://string-db.org, accessed on 25 May 2021) [34].

2.10. Co-Localization of SlCPAs with Salt Stress-Related QTLs

To identify the localization of QTLs for morphological and biochemical traits under salt stress, QTLs and linked molecular markers were retrieved from the Sol Genomics Network website (https://solgenomics.net/search/phenotypes/qtl, accessed on 1 June 2021) and tomato marker database (http://marker.kazusa.or.jp/Tomato/, accessed on 1 June 2021) as well as from the publications [16,35–38]. The markers of respective QTLs were obtained from previous publications and co-localization was shown as described previously [39]. Briefly, each marker sequence or name was BLAST against Sol Genomics Network website (https://solgenomics.net/search/phenotypes/qtl, accessed on 1 June 2021) and tomato marker database (http://marker.kazusa.or.jp/Tomato/, accessed on 1 June 2021) to obtain the physical position. *SlCPA* genes co-localized with salt stress-related QTLs were displayed using the MapChart software [40]. This displayed the *SlCPA* genes distribution along with surrounding QTLs. QTLs co-localized with the genes are indicated by asterisk and purple color.

3. Results

3.1. Identification and Characterization of CPAs in Tomato

To identify the CPAs in tomato, Hidden Markov Model (HMM) search was carried out against the tomato genome. The redundant protein sequences were removed by the CD-Hit program, and 33 non-redundant *CPA* genes were obtained. These tomato *CPA* genes were named *SlCHX1-18*, *SlNHX1-7*, and *SlKEA1-8* according to their distributions and relative linear orders among their respective chromosomes. Basic information of all *Sl*CPA proteins (gene name, chromosome, protein length, molecular weight, theoretical pI, aliphatic index, GRAVY, subcellular localization, and number of predicted transmembrane

helices) is provided (Supplementary Materials Table S1). Notably, all these *Sl*CPA proteins contained a Na^+/H^+ exchanger domain, with the protein length of 229 aa to 1199 aa and the molecular weight ranging from 24 kDa to 128 kDa. The *Sl*CPA proteins contain 7–14 transmembrane domains with membranous subcellular localization.

3.2. Phylogenetic, Conserved Motifs, and Gene Structure Analysis of Tomato CPA Genes

The phylogenetic tree was constructed to explore the evolutionary relationship of full-length CPA protein sequences of tomato and *Arabidopsis*. Based on their evolutionary relationship, similar to *Arabidopsis*, the *Sl*CPAs could be distinctly categorized into three main subgroups, i.e., CHX, KEA, and NHX. The CHXs were further classified into three subgroups—C1, C2, and C3. Similarly, KEAs and NHXs were further divided into two subgroups—K1 and K2, and N1 and N2, respectively (Figure 1).

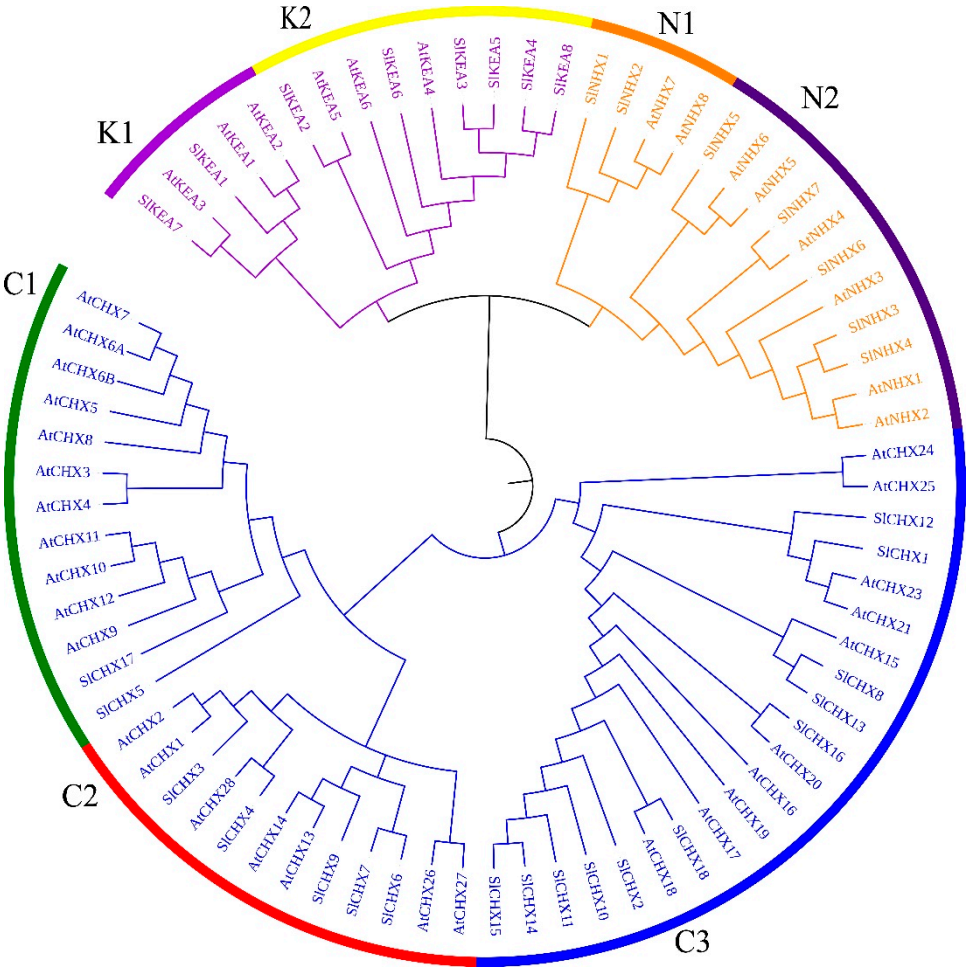

Figure 1. Phylogenetic relationship of proteins from CPA supergene family of *Solanum lycopersicum* and *Arabidopsis*. The members of each gene family are highlighted in different colors. The subgroups in each family are denoted as C1, C2, C3 (CHX), N1, N2 (NHX), and K1, K2 (KEA).

The Na^+/H^+ exchanger domain was predicted in the 33 *Sl*CPA proteins by InterPro scan to verify the integrity of the proteins. Furthermore, the *Sl*CPA protein sequences and structures were also examined by MEME to predict the conserved motif sites (0–10). Tomato CHXs proteins have seven to thirteen conserved motifs, while KEAs proteins have four to seven conserved motifs. In the NHX gene family, SlNHX1, SlNHX3, and SlNHX5 contained two conserved motifs, whereas SlNHX4, SlNHX6, and SlNHX7 have only one conserved motif (Figure 2A). We also noted that tomato NHX, KEA, and CHX subgroups share similar conserved motifs but also have different conserved motifs. For example, four motifs were

common in both the CHX and KEA families, while one to two motifs were exclusively present in the NHX family (Figure 2A). The motif logos discovered in *SlCPA* genes are given in Figure S1 and the motif information is provided in Table S4.

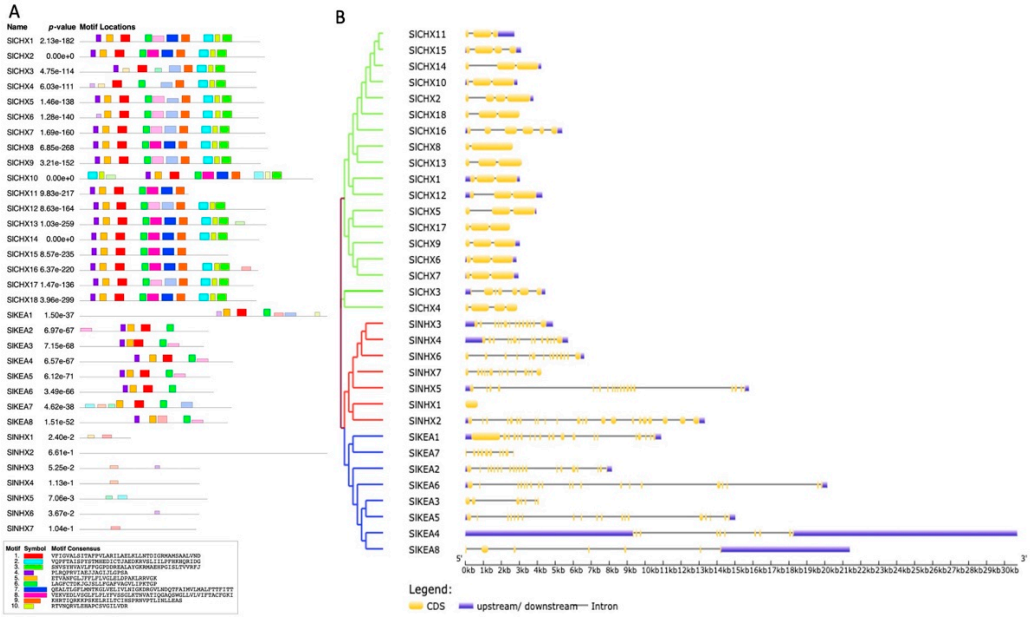

Figure 2. Conserved motif analysis and exon–intron distribution of *Sl*CPA proteins. (**A**) Conserved motif distribution of *Sl*CPA proteins. The 10 motifs are represented by different color ranges. (**B**) Gene structure of *SlCPA* genes. Untranslated regions (UTRs), coding (exons), and non-coding (introns) are denoted by blue and yellow boxes, and black lines, respectively.

The gene structural diversity of *SlCPAs* was further investigated with exon/intron analysis (Figure 2B). The analysis shows that *SlCPAs* vary greatly in the sequence length and the number of introns/exons. The gene structure in the same subgroup (*CHX, KEA,* and *NHX*) was found similar, whereas the number and length of exons/introns were different. For example, *SlNHX1* has only one exon, while *SlNHX2* has thirteen exons. Furthermore, *SlCHXs* have fewer but longer exons, while *SlKEAs* have many but small exons. The UTRs regions were found in most *SlCPAs* except for a few.

3.3. Chromosomal Localization and Duplication Analysis of CPA Genes

The locations of *SlCPA* genes on the chromosomes were obtained from the Phytozome database and 33 *SlCPA* genes were successfully mapped to the 12 chromosomes of tomato (Figure 3). The *SlCPA* genes were found unevenly distributed on the different chromosomes of tomato, ranging from one to eight genes per chromosome. Chromosome eight possessed the highest number of genes (seven), followed by five genes on chromosome one, and four genes on chromosome six. We further investigated the contribution of gene duplication to the expansion of *SlCPA* genes and found five segmental duplications in the tomato genome (*SlNHX4/SlNHX7*, *SlKEA1/SlKEA7*, *SlKEA2/SlKEA3*, *SlCHX2/SlCHX11*, and *SlCHX5/SlCHX17*).

Furthermore, the *CPA* genes of tomato and *Arabidopsis* were compared and analyzed by the synteny block method to explore the evolutionary mechanism of *SlCPA* members. The syntenic map revealed many colinear genes pairs between tomato and *Arabidopsis*. The orthologous genes in the synteny blocks with one-to-one pairing (*SlKEA6-AT5G11800*, *SlCHX16-AT3G53720*, *SlKEA2-AT5G51710*, *SlNHX3-AT3G06370*, and *SlNHX4-AT5G27150*) revealed common ancestors of these genes in *Arabidopsis* and tomato. In addition, there were also gene pairs with one, two, or three *Arabidopsis* genes corresponding to the same or different tomato genes in the synteny blocks (*SlCHX14-AT4G23700/AT5G41610/AT1G64170*, *SlCHX10-AT1G64170/AT4G23700/AT5G41610*, *SlKEA-AT1G01790/AT3G05030/AT4G00630*,

and *SlNHX2-AT1G14660.1*). Such types of synteny events suggested that many *CPA* genes appeared before the divergence of the tomato and *Arabidopsis* lineages (Figure 4).

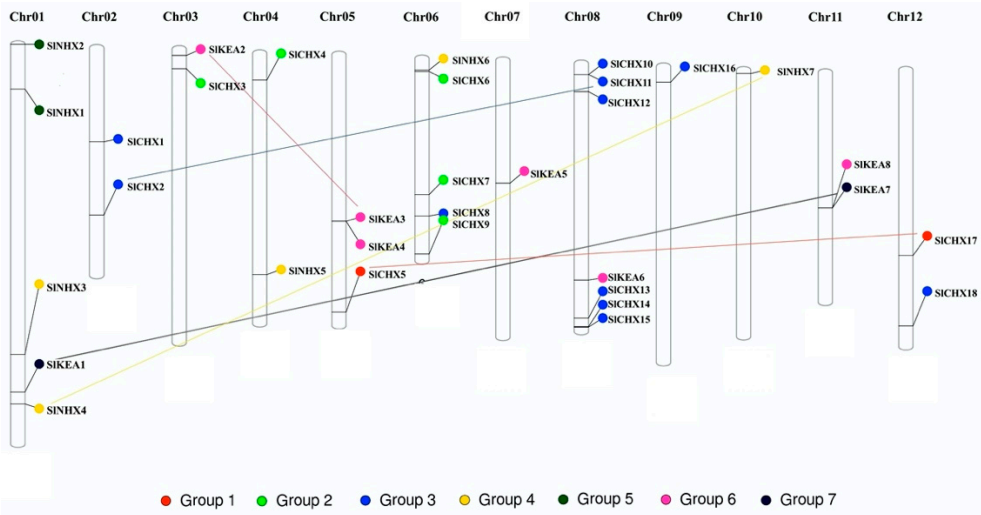

Figure 3. Distribution of tomato *SlCPA* genes on 12 chromosomes of tomato. The chromosome number is indicated at the top of each bar chart. Chromosomal positions of the *SlCPA* genes are displayed by the exact name and the duplicated genes are connected with different colored lines.

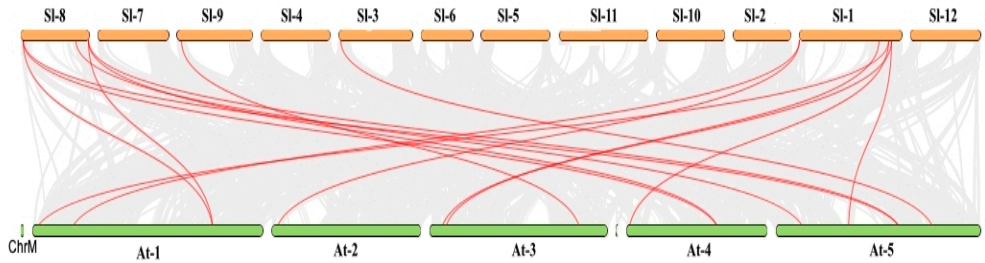

Figure 4. Evolutionary analysis of the *SlCPA* genes. Synteny blocks representation of *CPA* genes between tomato and *Arabidopsis*. The red lines between two chromosomal locations indicate syntenic relationship between tomato (Sl-1 to 12) and *Arabidopsis* (At-1 to 5).

3.4. Putative Cis-Elements in the Promoter Regions of SlCPAs

To gain more insights into the putative functions of *SlCPAs*, the presence of cis-regulatory elements were scanned in the upstream 2 kb promoter regions of the *SlCPA* gene family using the Plant Care database (Figure 5, Supplementary Materials, Table S2). The result revealed that *SlCPAs* carry TATA and CAAT box core cis-elements, light responsive, environmental stress related, development responsive, and phytohormones response elements in their promoters. This suggests that *Sl*CPAs are potentially involved in developmental processes and environmental stress tolerance. Among the light responsive elements, I-box, ATCT-motif, and G-box motif were the most abundant cis-elements found in the *SlCPAs* promoters. Among the environmental stress-related cis-elements, TC-rich repeats, MBS, and LTS cis-elements were abundant in the promoter of *SlCPAs*. Similarly, CGTCA-motif, ABRE, and ARE were found to be the most abundant in *SlCPAs* promoters, while O2-site was the most abundant in development-related cis-elements. Although *CHX*, *KEA*, and *NHX* subgroups of *SlCPA* genes share most of the cis-regulatory elements in their promoter regions, some cis-elements were found absent in certain groups. Notably, cis-elements were also found different in the promoter of duplicated genes, suggesting their unique functions. For example, the promoter of *SlKEA2* has five light responsive ATCT-motifs, while the promoter of *SlKEA3* has only one ATCT-motif cis-element.

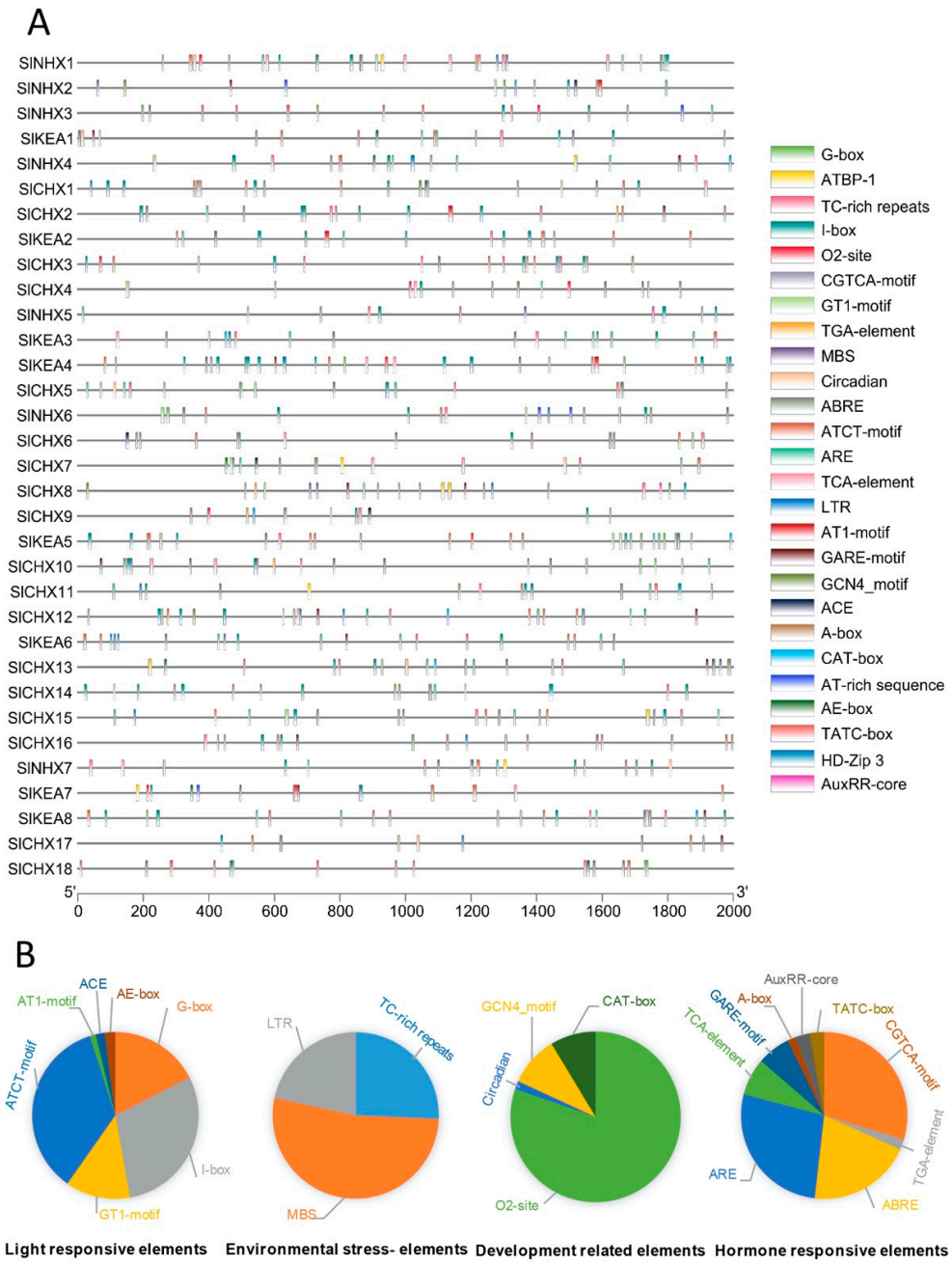

Figure 5. Putative *cis*-regulatory elements in the promoter of *Sl*CPAs. (**A**) Promoter regions 2 kb upstream of *SlCPA* genes analyzed by Plant Care. Different-colored rectangles represent different cis-elements. (**B**) Pie charts represent the classification of putative cis elements into four major categories.

3.5. Expression Analysis of SlCPA Genes in Different Tissues and at Various Stages of Fruit Development

To understand the potential physiological functions of *SlCPAs*, we investigated the expression of *SlCPA* genes in different tissues and at various stages of fruit development by using the publicly available RNA-seq data. The results showed that *SlCPAs* were widely expressed in leaf, root, flowers, and at various stages of fruit development (Figure 6A), suggesting the diverse biological functions of *SlCPAs* in different tissues and at various stages of fruit development. We also noted the tissue-specific gene expression among the different subgroups of *SlCPAs*. For example, *CHX* subgroup showed the highest expression in flower buds and flowers compared with roots and leaves. Similarly, *KEA* and *NHX* subgroups of *SlCPAs* genes, such as *SlKEA2*, *SlKEA4*, *SlKEA5*, *SlKEA6*, *SlNHX2*, *SlNHX4*,

and *SlNHX6*, showed an increase in gene expression with an increase in fruit size. These results suggest that *CHX*, *KEA*, and *NHX* subgroups of *SlCPAs* may have common as well as specific functions in tomato growth and development.

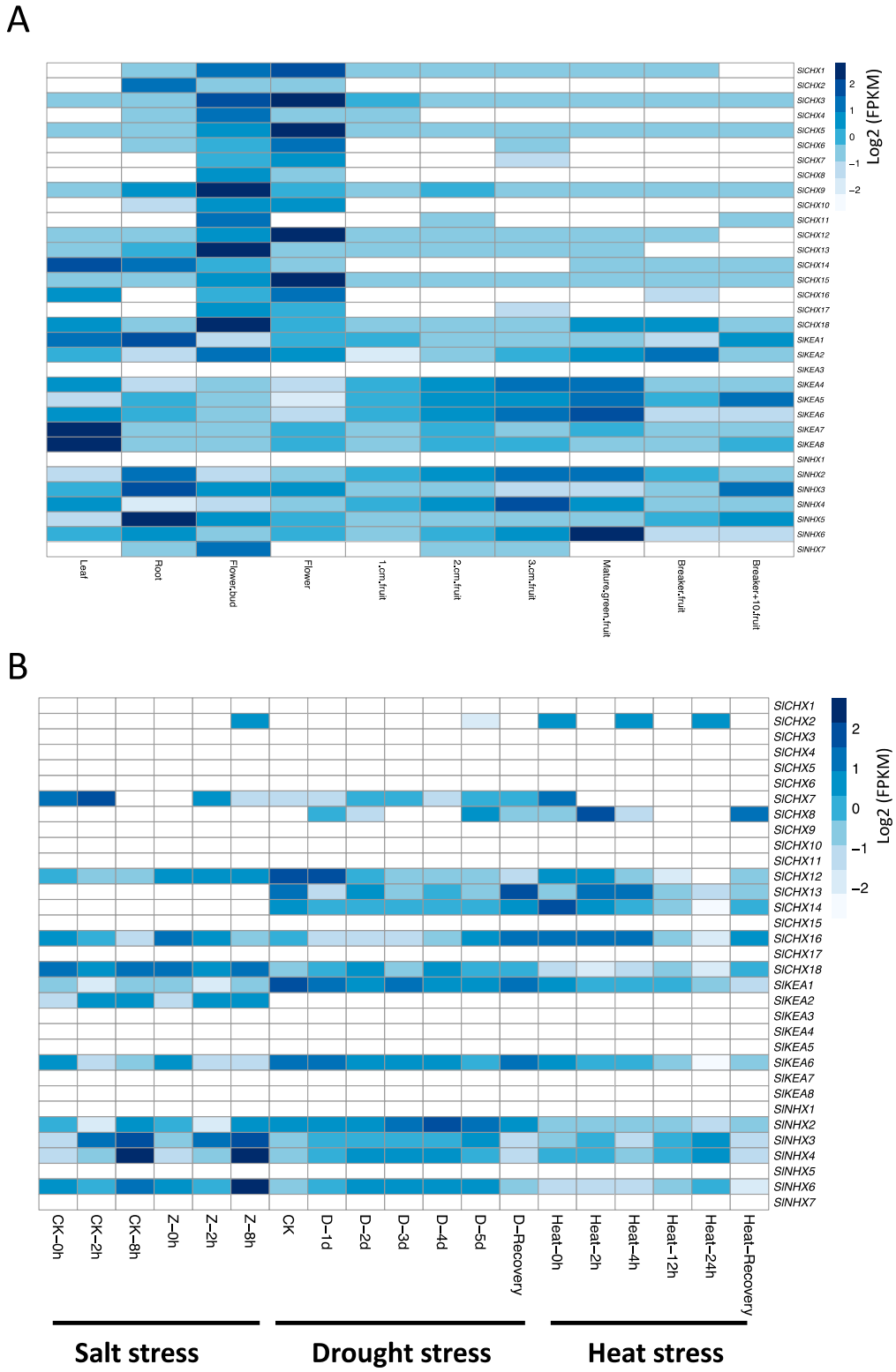

Figure 6. Gene expression analysis of *SlCPA* genes in different tissues and various fruit developmental stages (**A**) and under heat, drought, and salinity conditions (**B**) from RNA-sequencing. The expression values were calculated by Log2 (FPKM) and presented according to the color code. White boxes represent no expression.

3.6. Expression Analysis of SlCPA Genes under Heat, Drought, and Salinity Stresses

The differential expression patterns of putative *SlCPA* genes were further investigated under different abiotic stress conditions using the publicly available RNA-seq data (Figure 6B). Among the 33 *SlCPAs*, only 15 genes showed differential gene expression in response to salt, drought, and heat stress, suggesting their important role in abiotic stress tolerance. However, 16 genes were not expressed in response to salt, drought, and heat stress. Among the 15 differentially expressed genes, we also noted the specific regulation of *SlCPAs* genes in response to particular stress. For example, *SlKEA2* was expressed only in response to salt stress, while *SlCHX8*, *SlCHX813*, and *SlCHX14* were expressed in response to drought and heat stress but not in salt stress. Together, these results indicate their potential role in abiotic stress tolerance.

3.7. Quantitative Expression Analysis of SlCPAs under Salinity Stress

The RNA-seq data were further validated by qRT-PCR analysis by exposing tomato plants to salt stress (Figure 7). Gene expression was analyzed at 0, 3, 6, 12, 24, 48, and 96 h time intervals in response to salt stress. Ten *SlCPA* genes (*SlCHX14, SlCHX16, SlCHX18*, *SlKEA1*, *SlKEA2*, *SlKEA6*, *SlNHX2*, *SlNHX3*, *SlNHX4*, and *SlNHX6*) were selected for qRT-PCR as they were highly upregulated in response to salt, drought, and heat stress (Figure 6B). In general, similar to RNA-seq, all the selected *SlCPA* genes were highly upregulated (five to seven-fold) in response to salt stress at different time intervals. On average, the expression was significantly increased over time, with a maximum after 24 h of salt stress, but then decreased at 96 h. However, the response of some genes was stronger than others, even after 3 h of salt stress. For example, *SlCHX18, SlKEA6*, and *SlNHX3* were almost at the maximum level of their gene expression only after 3 h of salt stress.

3.8. Co-Localization of SlCPA Genes with QTLs (Quantitative Trait Loci) of Salt Stress-Related Phenotypes

To gain more insight into the role of *SlCPA* genes in salt tolerance, *SlCPA* genes were mapped with the previously reported salt-tolerance-related QTLs (Figure 8). These QTLs were reported previously based on the morpho-biochemical traits under salt stress, i.e., time to flower (Flw), time to ripe (RIP), leaf length (Leaf), leaf area (LA), dry shoot weight (DSW), number of fruits ripen (NFR), fruit weight (FW), fruit firmness (Firm), soluble solid content (SSC), Na^+ concentration in leaves (LNC), K^+ concentration in leaves (LKC), Na+ concentration in the shoot (SNC), K^+ concentration in the shoot (SKC), the ratio of K^+/Na^+ in leaves (LKN), Cl^- accumulation in leaves, total Na^+ content (TN), and salt tolerance (ST). The gene names and physical position with respective co-localized QTL and linked marker name and physical position are provided in Table S5.

The co-localization results showed that chromosome one has thirteen salt-related QTLs. Among the thirteen salt-related QTLs on chromosome one, only four were co-localized with *SlNHX1, SlKEA1*, and *SlNHX4.* Both genes on chromosome two (i.e., *SlCHX1* and *SlCHX2*) were co-localized with four QTLs (FW 2.1, NFr2.1, FLW2.1, and SSC2.1). Chromosome five had three *SlCPA* genes but only *SlCHX5* was co-localized with the lkc5.1 and skc5.2 QTLs. Out of five *SlCPA* genes on chromosome six, four *SlCPAs* genes were co-localized with the previously reported QTLs [35,36]. Chromosome seven has only one gene (*SlKEA5*), which showed co-localization with the SSC3.1. Chromosome eight has seven *SlCPA* genes, but only two genes (*SlKEA6* and *SlCHX13*) were co-localized with two QTLs. Chromosome nine has only one *SlCPA* gene (*SlCHX16*), which co-localized with two QTLs. Chromosome 10 also has one *SlCPA* gene (*SlNHX7*), which co-localized with three QTLs. Two genes on chromosome 11 (*SlKEA8* and *SlKEA7*) coincided with the three QTLs (Firm11.2, SSC11.1, and FW11.1). Chromosome 12 has two *SlCPA* genes (*SlCHX17* and *SlCHX18*) but only *SlCHX18* was co-localized with the NFr12 (number of fruits ripen). The co-localization of *SlCPA* genes with salt-related QTLs suggested a comprehensive role of the *SlCPA* genes in different morpho-biochemical traits under salt stress.

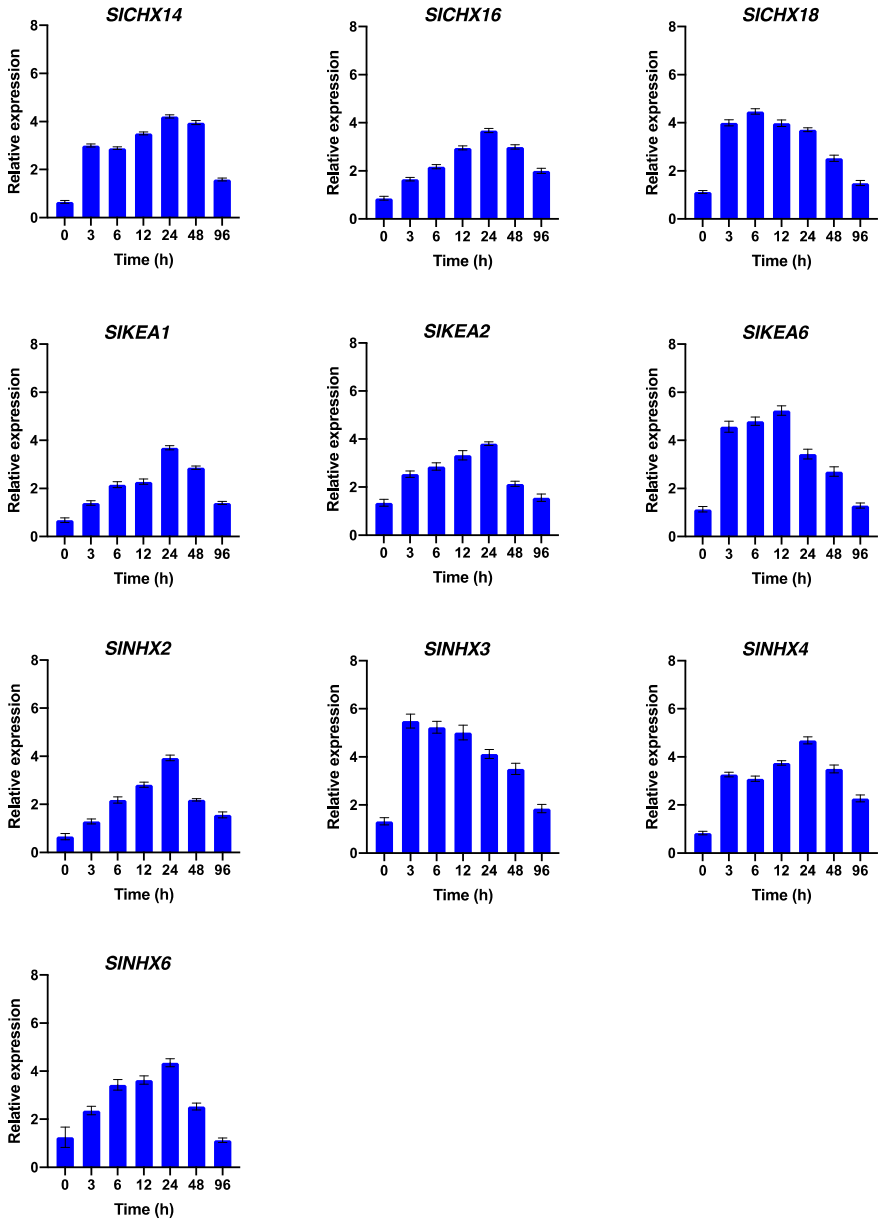

Figure 7. Expression profiles (qRT-PCR) of the *SlCPA* genes at different time intervals (0, 3, 6, 12, 24, 48, and 96 h) under 250 mM NaCl treatments. The relative expressions at different stress treatment times were compared with the control (0 h).

3.9. Protein–Protein Interaction and Gene Ontology Analysis

The protein–protein interactions among the *Sl*CPAs and the associated proteins were predicted by string database and modified in Cytoscape. The majority of the *Sl*CPAs revealed strong protein–protein interaction networks, as depicted in Figure 9. Overall, the CHX subgroup members of *Sl*CPAs possessed more protein–protein interactions than other subgroups. The members of the CPA protein family interact with other proteins, such as plasma membrane H^+ ATPase (LHA1), calcineurin B-like protein 1 (CBL1), CBL-interacting protein kinases (CIPK), salt overly sensitive (SOS1), PIN-Formed 2 (PIN2), HIGH-AFFINITY K^+ TRANSPORTER 1 (HKT1), and HIGH-AFFINITY K^+ TRANSPORTER 2 (HKT2). All these proteins are involved in salinity tolerance and support chloroplast against the reactive oxygen species and other pathways leading to abiotic stress tolerance. Our PPI networks provide essential evidence for understanding the functions of proteins with unknown functions.

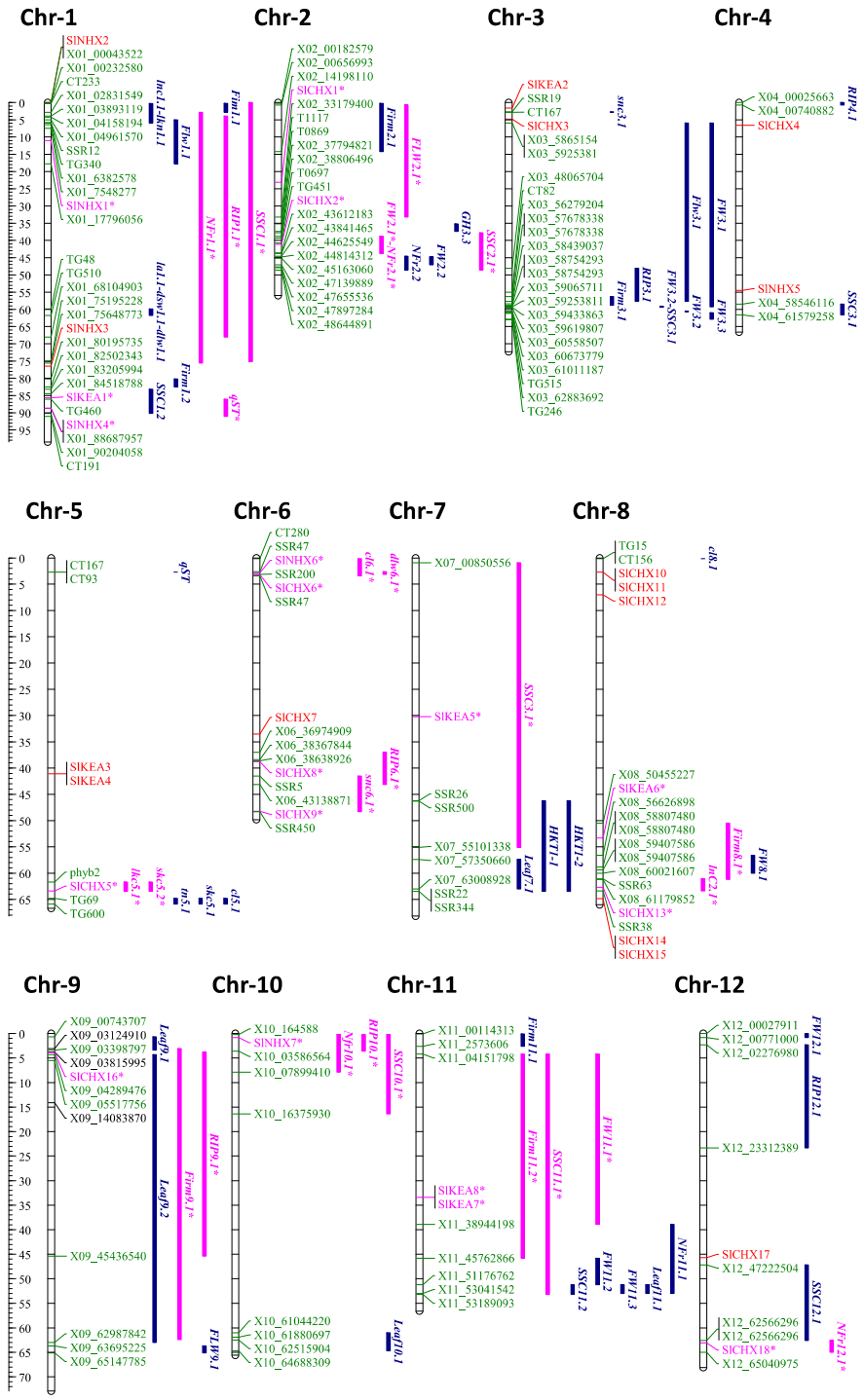

Figure 8. Co-localization of *SlCPA* genes with quantitative trait loci (QTLs) of salt stress-related phenotypes on the chromosomes of tomato. The scale represents the physical position of genes and QTL-linked markers in megabases (Mb). These QTLs were reported previously based on the morpho-biochemical traits under salt stress; i.e., time to flower (Flw), time to ripe (RIP), leaf length (Leaf), leaf area (LA), dry shoot weight (DSW), number of fruits ripen (NFR), fruit weight (FW), fruit firmness (Firm), soluble solid content (SSC), Na^+ concentration in leaves (LNC), K^+ concentration in leaves (LKC), Na^+ concentration in the shoot (SNC), K^+ concentration in shoot (SKC), ratio of K^+/Na^+ in leaves (LKN), Cl^- accumulation in leaves, total Na^+ content (TN), and salt tolerance (ST). *SlCPA* genes, which are not co-localized with any reported QTL, are highlighted with red color and QTLs in blue color, while *SlCPA* genes and QTLs are in pink color with asterisks indicating that these *SlCPA* genes co-localized with QTLs of salt stress-related phenotypes.

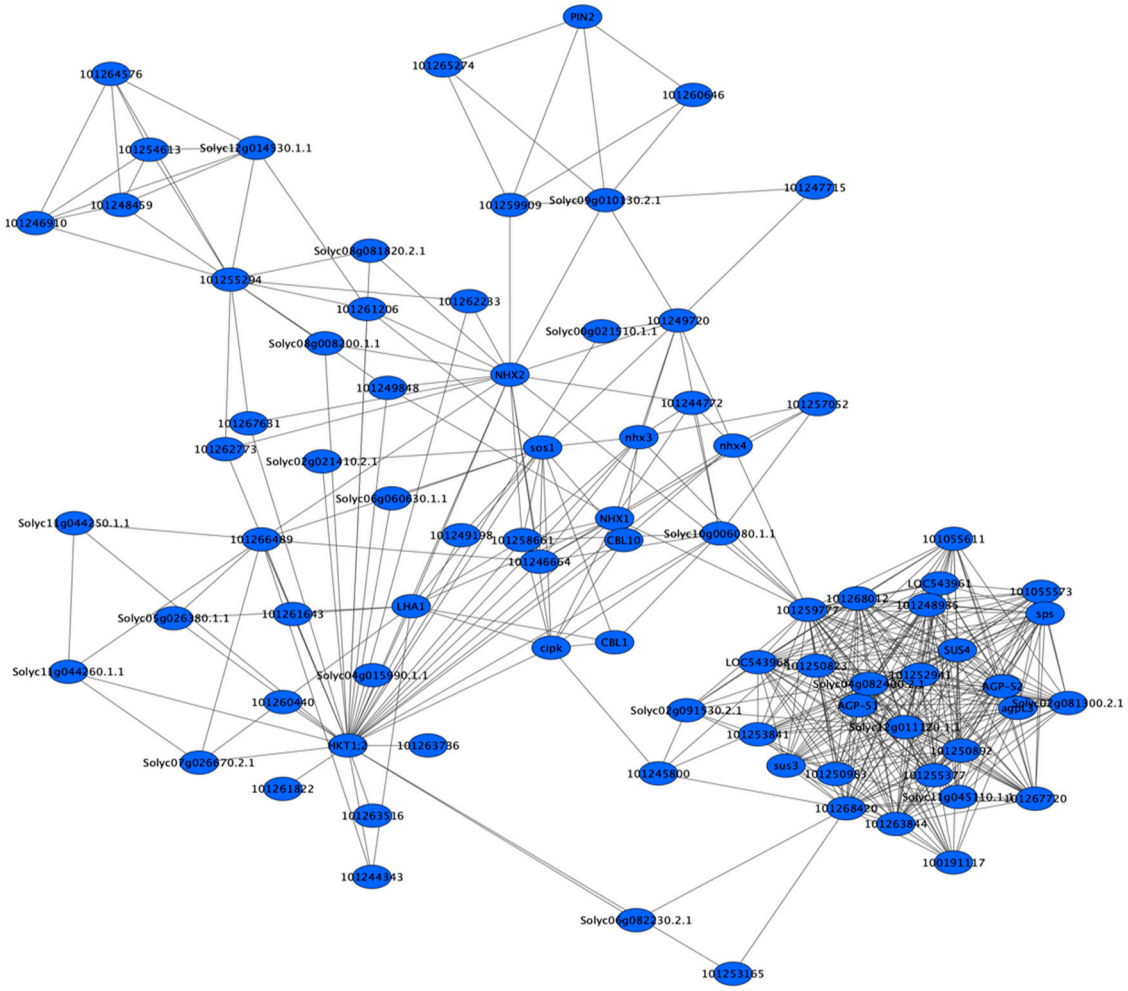

Figure 9. Network interactions of *Sl*CPA proteins. Strong interaction network among *Sl*CPA proteins and other related proteins supports their role in salinity tolerance.

Furthermore, the gene ontology (GO) terms analysis of CPAs in tomato revealed that they are involved in different biological and chemical processes (Figure 10). These functions are more prominently related to transport (34%), homeostatic process (18%), protein targeting (15%), vacuolar transport (15%), response to stress (1.75%), transmembrane transport (2.03%), vesicle-mediated transport (2.03%), and a few others.

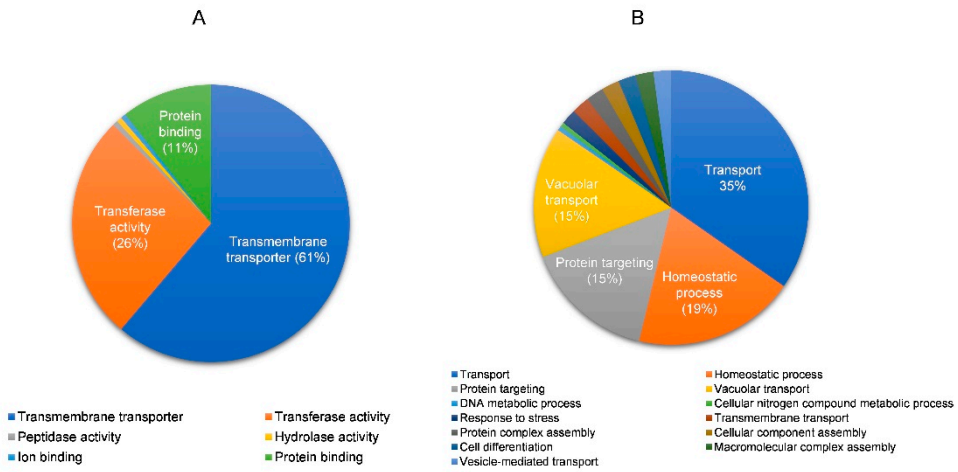

Figure 10. Gene ontology (GO) terms of *SlCPA* genes in tomato. Molecular function (**A**) and biological process (**B**) are shown in pie charts. Different GO terms are represented by different colors (%).

4. Discussion

In plants, ion concentration and pH balance feat are accomplished with a diverse array of transporters, especially the CPA proteins [1] that are localized in the plasma membrane as well as organelle membranes such as endosomes, vacuoles, and chloroplasts [3,7,8]. In this study, we revealed that tomato has 33 CPA members that could be classified into three main subgroups: CHX (18 members), NHX (7 members), and KEA (8 members). The *CPA* genes in tomato were lower in number as compared with the previously reported *CPA* genes in various species such as *Triticum aestivum* (107 genes), *Raphanus sativus* (60 genes), *Brassica rapa* (64 genes), *Zea mays* (33 genes), *Arabidopsis thaliana* (42 genes), *Pyrus communis* (53 genes), *Oryza sativa* (28 genes), *Sorghum bicolor* (28 genes), and *Zea mays* (33 genes) [4]. This might be attributed to the small and compact diploid genome of *Solanum lycopersicum*. Multiple studies investigating other gene families in tomato also revealed that tomato has fewer genes as compared with other plants [41–43]. However, similar to other plant species, the CHX subgroup of SlPCAs has the highest number of genes in tomato compared with KEA and NHX subgroups [4,5]. Furthermore, sub-classification of NHX, KEA, and CHX subgroups into different sub-clusters, such as N1-N2, K1-K2, and C1-C3, has also been observed in other plant species [4]. These observations suggest that CPAs are evolutionary conserved in plants.

The plant CPA1 (NHX) transporters are predicted to have 10–12 transmembrane domains, whereas the CPA2 (CHX and KEA) transporters are predicted to have 8–14 membrane spanning domains with a Pfam00999 domain for the Na^+, K^+/H^+ exchanger [3]. Studies have shown that CPA family members contain a catalytic conserved Na+/H+ exchange domain in the N terminus [44–46] and our analysis of domains revealed that all *Sl*CPA proteins contain the highly conserved N-terminal catalytic NHX domain, suggesting that tomato CPAs may have similar functions as described in other plant species.

CPA genes have been shown to regulate cellular pH and ion homeostasis and are involved in a wide range of physiological events, from vesicle trafficking to development [3,46]. However, some members of the CPAs comprise plasma membrane, vacuolar, and endosomal forms, and they have been identified to play an important role in salinity tolerance [47]. The role of numerous *CPA* genes in *Arabidopsis* and other plant species in salt tolerance has been established in earlier studies [4,46,48]. For example, NHX subgroup members of *CPA* genes have been shown to play a role in abiotic stress tolerance, including salinity stress, in different plant species [3,49–51]. Similarly, members of KEAs subgroup of *Arabidopsis* CPAs (*AtKEA1*, *AtKEA2*, and *AtKEA5*) and soybean CPA (*GmKEAs*) were upregulated during Na^+ and K^+ stresses [52,53]. Furthermore, the CHX subgroup of *CPA* genes of *Arabidopsis*, including *AtCHX13*, *AtCHX17*, *AtCHX21*, and *AtCHX23*, were also reported to play a role in the salinity stress [53–55]. Our results highlighted that members of NHX, CHX, and KEA subgroups, but not all, may play an important role in countering the negative effect of salinity stress in the plant cell (Figures 5 and 6B). Co-localization of *SlCPAs* genes with QTLs of salt stress-related phenotypes further support their role in salinity tolerance. Furthermore, in agreement with this observation, our qRT-PCR confirmed that *SlCHX14*, *SlCHX16*, *SlCHX18*, *SlKEA1*, *SlKEA2*, *SlKEA6*, *SlNHX2*, *SlNHX3*, *SlNHX4*, and *SlNHX6* were several folds up-regulated after salinity stress, supporting their potential role in salt tolerance in tomato. Interestingly, these genes also showed up-regulation in response to heat and drought (Figure 6B). However, similar to the grapevine, wheat, radish, maize, and soybean [12], not all *SlCPAs* responded to salinity stress and other abiotic stresses. Furthermore, *SlCPA* genes responded differentially depending on the intensity and type of stress treatment, suggesting that *Sl*CPAs may work together in response to particular stimuli, and may participate in long-term resistance to abiotic stresses. However, further molecular and biochemical studies are required to validate *Sl*CPAs function in multiple abiotic stresses and understand the underlying molecular mechanism.

Tandem duplication, polyploidy, and segmental duplications primarily contribute to creating new gene families (e.g., MYB, WRKY, and CytP450 gene families) in the evolution of genome and genetic systems [56]. Five segmental duplications were found in the

tomato genome (*SlNHX4/SlNHX7*, *SlKEA1/SlKEA7*, *SlKEA2/SlKEA3*, and *SlCHX2/SlCHX11*, *SlCHX5/SlCHX17*) (Figure 3). Gene duplications occurred in these genes because the identities of the genes flanking both sides of the paralogous tomato *CPA* genes were found to be absolutely conserved and located on duplicated segments on two different chromosomes. Segmental duplication was also predicted as the main driving force for the amplification of the *SlTGL* gene family in tomato. *SlCPAs* carry TATA and CAAT box core cis-elements, light-responsive, environmental stress-related, development-responsive, and phytohormones response elements in their promoters (Figure 5), suggesting that *SlCPAs* are likely to play important roles in response to developmental and environmental cues. Interestingly, these duplicated genes carry different cis-elements in their promoters, suggesting their functional divergence in response to particular stimuli. However, among the duplicated genes, only one gene responded to abiotic stress (Figure 6B). For example, *SlKEA1* responded to all abiotic stresses but not *SlKEA7*; *SlNHX4* responded to all abiotic stresses but not *SlNHX7*, further supporting the functional divergence of duplicated genes.

The network interactions of *Sl*CPA proteins revealed that *Sl*CPAs were predicted to interact with many other proteins such as SOS1, LHA1, CIPK, CBL1, PIN2, HKT1, and HKT2 (Figure 9). SOS1 is the pivotal kinase of the SOS pathway involved in the regulation of ion transport under abiotic stress, especially the salt stress [55]. Similarly, LHA1 is also a member of the plasma membrane H^+ ATPase that plays a critical role in plant adaptation to saline conditions, as it generates proton gradient that actively transports nutrients by H^+-symport [57]. It has been previously observed that HKT1 and HKT2 proteins are expressed during high salinity conditions to neutralize the excess Na^+ ion in xylem tissues [58]. Recent studies have found that CBLs interact with CIPKs to form a CBL–CIPK signaling network that takes part in the transport of ions and participates in multiple abiotic stresses in plants, including drought, heat, cold and salinity [59–61]. These results suggest that *Sl*CPA may belong to larger protein complexes, thus regulating abiotic stress tolerance with their partners. However, further comprehensive studies are required to confirm these interactions.

In brief, our study highlighted the implication of tomato CPAs in abiotic stress adaptation. This study strongly recommends the comprehensive dissection of the biological and cellular function of tomato CPAs, which will eventually lead to a long-term improvement of abiotic stress tolerance in tomato.

5. Conclusions

In this study, a total of 33 members of the *CPA* gene family, comprising *NHX*, *KEA*, and *CHX* subgroups, were identified in the diploid genome of tomato. Tomato *CPAs* are unevenly distributed on their chromosomes, and segmental duplication contributed to the evolution of the *CPAs* family. Cis-elements analysis discovered several plant developments, stress related, hormonal, and light response cis-elements in the promoter of tomato *CPAs*, but each member had peculiar types and numbers. Furthermore, gene expression analysis exhibited that 15 members of the tomato *CPA* family are differentially regulated in response to abiotic stresses. Several tomato *CPA* genes were co-localized with QTLs of salt stress-related phenotypes, which disclosed that tomato *CPAs* play roles in abiotic stress tolerance. Tomato CPAs were predicted to interact with proteins, such as SOS1, LHA1, CIPK, CBL1, PIN2, HKT1, and HKT2, that have been previously described as important players in response to salinity stress. Thus, our study helps to lay the foundation for the functional characterization of the tomato *CPA* gene family by overexpression and knockdown/out using RNAi or CRISPR-Cas9 genome editing. This study also provides a fundamental understanding of tomato *CPAs* in conferring abiotic stress resistance in tomato and will be useful for the long-term improvement of stress tolerance in tomato.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy12020245/s1: Figure S1: Discovered motif logos in *Sl*CPA proteins; Table S1: Physiochemical properties of *SlCPA* genes; Table S2: List of Cis-elements in the promoter of *SlCPA* genes; and Table S3: List of primers used in the study; Table S4: Analysis and annotation of *Sl*CPA

proteins motifs of Figure 2A; Table S5: Description of co-localized genes with QTLs and linked marker position.

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