Pan-Genome-Wide Identification and Transcriptome-Wide Analysis of ZIP Genes in Cucumber

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Article

Pan-Genome-Wide Identification and Transcriptome-Wide Analysis of *ZIP* Genes in Cucumber

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Abstract: The ZIPs (ZRT/IRT-like proteins) play critical roles in the absorption, transport, and intracellular balance of metal ions essential for various physiological processes in plants. However, little is known about the pan-genomic characteristics and properties of *ZIP* genes in cucumber (*Cucumis sativus* L.). In this study, we identified 10 *CsZIP* genes from the pan-genome of 13 *C. sativus* accessions. Among them, only CsZIP10 showed no variation in protein sequence length. We analyzed the gene structure, conserved domains, promoter *cis*-elements, and phylogenetic relationships of these 10 *CsZIP* genes derived from "9930". Based on phylogenetic analysis, the *CsZIP* genes were classified into three branches. Amino acid sequence comparison revealed the presence of conserved histidine residues in the ZIP proteins. Analysis of promoter *cis*-elements showed that most promoters contained elements responsive to plant hormones. Expression profiling in different tissues showed that most *CsZIP* genes were expressed at relatively low levels in *C. sativus* leave, stem, and tendril, and *CsZIP7* and *CsZIP10* were specifically expressed in root, indicating their potential involvement in the absorption and transport of metal ions. Transcriptomic data indicated that these 10 *ZIP* genes displayed responses to both downy mildew and powdery mildew, and *CsZIP1* were significantly downregulated after both salt and heat treatments. In conclusion, this study deepens our understanding of the *ZIP* gene family and enhances our knowledge of the biological functions of *CsZIP* genes in *C. sativus*.

Keywords: pan-genome; *Cucumis sativus*; *CsZIP*

1. Introduction

The homeostasis of metal ions plays a crucial role in the growth and development of plants. Appropriate concentrations of metal ions are required by plants to sustain biochemical reactions and physiological processes within cells. However, an excess or deficiency of metal ions may lead to severe growth issues and physiological disruptions. Therefore, maintaining suitable concentrations of metal ions is vital for the survival of plants. The ZIP gene family performs a significant role in the metal ion homeostasis of plants. This family encodes zinc ion transport proteins responsible for regulating the absorption and distribution of zinc as well as other metal ions. Through the activity of these proteins, plants can respond to changes in environmental metal ion levels and maintain a balanced state of metal ions within cells [1,3]. Most ZIP proteins consist of 309–476 amino acid residues and possess eight putative transmembrane (TM) domains. They exhibit a common membrane topology, with the N- and C-terminal ends located extracytoplasmically [2]. The cytoplasmic loop between TM3 and TM4 contains a histidine-rich domain (HRD), which functions as the metal binding domain involved in metal transport. Additionally, the amphipathic nature of TM4 and TM5 creates cavities that allow for the passage of metal ions [2,3].

Currently, more than 300 members of the ZIP protein family have been identified from plants, and some ZIP members have been functionally characterized. The ZIP gene, *AtIRT1*, was first identified in *Arabidopsis thaliana*. It encodes an iron transporter protein and is mainly expressed in the roots [4]. The previous study has shown that *AtIRT1* also exhibits specific expression in the companion cells of the cortex and plays a role in iron transport in aerial organs [5]. *AtIRT2* is another IRT gene which functions similarly to *AtIRT1* in *A. thaliana*. AtIRT2 is localized to the vacuolar membrane and compartmentalizes Fe into vacuoles to prevent its toxicity in the cytoplasmic compartment [6]. *AtZIP1* and *AtZIP3* are expressed in the roots. AtZIP1 is a vacuolar transporter protein responsible for the reactivation of Mn/Zn from vacuoles to cytoplasm in root cells [7]. AtZIP3 plays a role in the uptake of Zn and Fe from the soil to the plant roots [3].

AtZIP2 mediates Mn/Zn uptake into parenchyma cells in the xylem, thereby facilitating the transport of Mn/Zn towards the shoots [7]. In contrast, AtZIP4 transports zinc ions into cells or between plant tissues [3]. In addition, some ZIP transporter proteins directly participate in the accumulation of Zn in edible plant parts [8,9]. The expression levels of *ZIP* family genes in plants show dynamic changes. For example, under zinc-deficient conditions, some ZIP transporter proteins are expressed at higher levels, but when zinc levels return to normal or high, their expression decreases [10].

In rice (*Oryza sativa* L.), OsIRT1 and OsIRT2 are iron transport proteins that directly absorb Fe^{2+} and Zn^{2+} into root cells [11–14]. When *O. sativa* is exposed to iron deficiency conditions, the expression of *OsIRT1* and *OsIRT2* in the root system is upregulated [15]. OsZIP1 is localized in the endoplasmic reticulum and plasma membrane, and it functions as a metal ion transporter in *O. sativa* under conditions of metal ion excess [16]. Furthermore, studies have shown that overexpression of *OsZIP1* enhances the accumulation of Zn and Fe in roots, shoots, and seeds [13,14]. OsZIP4 and OsZIP5, as plasma membrane-localized Zn transporters, participate in Zn^{2+} uptake in *O. sativa* [17,18]. Overexpression of *OsZIP4* and *OsZIP5* can increase the Zn^{2+} concentration in plant roots [17,18]. OsZIP8 is also a Zn transporter protein in *O. sativa*, participating in the uptake and distribution of Zn^{2+} [19]. Generally speaking, ZIP transport proteins are primarily involved in the absorption and transport of Zn and Fe. However, these proteins are also involved in the transport and uptake of other metal ions, such as Mn and Cu [7].

Additionally, they could also participate in the absorption, transport, and accumulation of various toxic heavy metals, such as cadmium (Cd), in plants [20]. For example, overexpression of *OsIRT1* in *O. sativa* has been found to enhance its resistance to Cd [21]. *ZIP2* and *ZIP3* have been demonstrated the involvement in the uptake and transport of Cd in cabbage, with an increase in their expression levels [22]. In tomato (*Solanum lycopersicum*), the expression of *SlZIP4* is positively correlated with Cd concentration within a certain range [23]. Additionally, *ZIP* genes also participate in the absorption and transport of Cd in mulberry and tobacco [24,25].

Previous studies have reported the genome-wide identification of the *ZIP* gene family in various plant species including *A. thaliana*, peanut, and *Populus trichocarpa* . *Arabidopsis* had 15 ZIP members, peanut 30, and *Populus.trichocarpa* 16, respectively [45–47]. However, there is no report on the *CsZIP* gene family yet in *C. sativus* . Therefore, it is necessary to identify the *CsZIP* gene family.

C. sativus is a popular cash crop due to its unique flavor and refreshing crisp texture [26]. Due to the crucial role of the CsZIP gene family in the absorption and transportation of metal ions, it is imperative to identify and characterize the CsZIP gene family in *C. sativus*. The updated genome database of *C. sativus* provides a better resource for identifying and characterizing *CsZIP* genes. In this study, we used bioinformatics methods to investigate the sequence similarity, gene structure, and expression patterns of the *CsZIP* gene family, with the aim of clarifying the phylogenetic relationship of the *ZIP* gene family, deepening our understanding of *CsZIP* genes, and improving our knowledge of their biological functions.

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2. Materials and Methods

2.1. Identification of ZIP genes in C. sativus

The identification and analysis of *C. sativus* ZIP genes were performed using genome sequence data of *C. sativus* deposited at Cucurbit Genomics Data website (http://cucurbitgenomics.org/organism/20). The hidden Markov model (HMM) profile files of the ZIP conserved domain （PF02535） were downloaded from the Pfam database (http://pfam.xfam.org/). The *ZIP* genes of *C. sativus* were identified from the genome database using HMMER 3.0 with the default parameters and a cutoff value of 0.01. The Pfam (http://pfam.janelia.org)and SMART (http://smart.embl-heidelberg.de/) tools were used to confirm the ZIP conserved domain. ZIP candidates were predicted using the ExPASy Proteomics Server (http://expasy.org/)(Artimo et al. 2012), and their subcellular localization was investigated using Cell-PLoc (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) [27].

2.2. Gene structure and motif analysis

The CDS sequences and genomic of *CsZIP* genes were downloaded from the *C. sativus* genome database (http://cucurbitgenomics.org/organism/20), and the gene structures were visualized using the Gene Structure Display Server online tool (http://gsds.cbi.pku.edu.cn/) [28]. The conserved motifs of CsZIP proteins were then identified with MEME 4.9.1 (http://meme-suite. org/) [29] and visualized with WebLogo (http://weblogo.berkeley.edu/logo.cgi) [30]. The total number of motifs (nmotifs) is 10, the minimum length of motifs (minw) is 6 amino acids, and the maximum length of motifs (maxw) is 10 amino acids.

2.3. Phylogenetic analysis and multiple sequence alignment

The MEGA software (v7.0) was utilized to align the protein sequences of ZIP from *A. thaliana*, *C. sativus*, melon, and *O. sativa* using the ClustalW algorithm. Subsequently, an analysis of the evolutionary relationships among the ZIP proteins was conducted employing the neighbor-joining method with 1000 bootstrap replicates. The resulting phylogenetic trees underwent graphical representation and enhancement through Evolview (https://evolgenius.info//evolview-v2/#login).

2.4. Gene duplication analysis and genome distribution

CsZIP loci were extracted from the *C. sativus* genome database (http://cucurbitgenomics.org/organism/20) and their locations on chromosomes were visualized using MapChart software [31]. From Orthomcl [32], the homology of *ZIP* gene between *C. sativus* and *A. thaliana*, *Cucumis melo* and *O. sativa* were obtained. Circos shows the chromosomal location and collinearity of the *ZIP* gene [33]. All CDS between *Arabidopsis* and *C. sativus* genomes are compared by Clustal W. Then, repeated pairs and homologous pairs are identified based on the alignment result. The Computational Biology Unit (CBU) Ka/Ks calculation tool (http://services.cbu.uib.no/tools/kaks) was used to calculate the non-synonymous (Ka) and synonymous (Ks) nucleotide substitution rates. Then, the formula T =Ks/2r Mya (millions of years) was used to calculate the divergence times of the duplicated genes, where r is equal to 1.5×10^{-8} [34].

2.5. Analysis of promoter regions of CsZIP genes

The 1.5-kb sequences upstream of the initiation codons (ATG) of *CsZIP* genes were downloaded from the cucurbit genomics data website (http://cucurbitgenomics. org/organism/20), and analyzed for *cis*-elements in the promoter region using the online tool PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) [35].

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2.6. Analysis of transcriptome data

The data from different *C. sativus* RNA seq libraries were downloaded from the Cucurbit Genomics Data website (http://cucurbitgenomics.org/organime\/20) and NCBI SAR databases (accession Nos.:PRJNA388584，GSE151055，GSE81234 and GSE11265). The expression profile of *CsZIP* gene was visualized using TBtools software [36].

2.7. Plant materials and growth conditions

In this study, the temporal and spatial expression patterns of *ZIP* genes were analyzed using the *C. sativus* inbred line 'China long' 9930 (North China type). *C. sativus* seedlings were grown in greenhouses at Shandong Agricultural University in China, following standard water management and pest control practices.

2.8. RNA isolation and real-time PCR analysis

To collect samples for expression analysis, the roots, stems, leaves, female and male flowers, ovaries, and tendrils of *C. sativus* plants were collected. These samples were immediately frozen in liquid nitrogen and stored at -80 ℃ for RNA isolation. Total RNAwas extracted with TRIzol (Thermo Fisher Scientific, USA) by following the manufacturer’ sinstructions and then treated with DNase I (Thermo Fisher Scientific, USA) to remove the possible DNA contamination. cDNA synthesiswas carried out with a RevertAid First Strand Synthesis Kit (Thermo Fisher Scientific, USA). Primers were designed for qRT-PCR verification of the select genes with Primer Premier 5.0 (Table S4) and *C. sativus* actin gene was used as an internal control. qRT-PCR for the select genes was performed with an Ultra SYBR Green Mixture qPCRkit (CWBIO, China) on an iCycler iQ™ real-time PCR detection system (Bio-Rad, USA) by following the PCR program of 95° C for 30 s, 40 cycles of 95° C for 15 s and 60° C for 15 s, and finally 72° C for 30 s. Relative expression levels of the select genes were measured using the $2^{-\Delta\Delta Ct}$ method [37].

3. Results

3.1. Identification of CsZIP genes from C. sativus pan-genome

With the release of a graph-based pan-genome [38], we could identify *ZIP* genes in *C. sativus* by analyzing different accession genomes. We obtained the HMM profile files and extracted CsZIP proteins from the *C. sativus* genome database. The candidate genes were further analyzed using NCBI CDD, SMART, and Pfam. As a result, we identified a total of 10 *CsZIP* genes in the 13 *C. sativus* accessions (Table 1). Of these accessions, only 'Cuc80' and 'PI183967' contain nine *CsZIP* genes (Table 2). To avoid confusion, we renamed these genes as *CsZIP1-CsZIP10* based on their chromosomal order (Table 3).

To further investigate the variation of *CsZIP* genes among different *C. sativus* accessions, we counted the length of the identified CsZIP proteins (Table 2). Among the 13 *C. sativus* accessions, only one gene, CsZIP10, had the same protein length across all accessions. The lengths of CsZIP1, CsZIP4, CsZIP6 and CsZIP8 differed in only one accession. CsZIP2, CsZIP3 and CsZIP7 exhibited variations in protein length in two accessions. CsZIP9 showed protein variations in three accessions, while CsZIP5 exhibited protein length differences in multiple accessions. All CsZIP protein sequences can be found in Dataset S2.

3.2. Characterization of CsZIP genes from Chinese long 9930

Subsequent analyses primarily focused on the genes identified in the '9930' genome, as it encompassed all the 10 *CsZIPs* and is the first *C. sativus* genome sequenced and has been updated to the V3 version. The gene name, gene number, gene intron number, chromosome position, gene length, protein length, isoelectric point, relative molecular weight, and subcellular localization prediction of CsZIPs are presented in Table 3. Sequence analysis revealed that the length of CsZIP proteins ranged from 228 to 594 amino acids, with molecular weights ranging from 27.20 to 61.98

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kDa. The longest and most complex gene structure was found in *CsZIP6*, while the shortest gene was *CsZIP8*. *CsZIP4* had the highest relative molecular weight, whereas CsZIP3 the lowest. The isoelectric points of these proteins ranged from 5.58 to 8.53. Furthermore, subcellular localization prediction indicated that most CsZIP proteins were localized to the cell membrane, except CsZIP2 and CsZIP7, which were found in the chloroplasts (Table 3).

Table 1. The origins of the different *C. sativus* accessions.

Accession name	Accession group	Accession name	Accession group
9930	East Asian cultivated accession	Hx117	Indian cultivated accession
XTMC	East Asian cultivated accession	Hx14	Indian cultivated accession
Cu2	East Asian cultivated accession	W4	Indian wild accession
Cuc37	Eurasian cultivated accession	W8	Indian wild accession
Gy14	Eurasian cultivated accession	Cuc64	Indian wild accession
9110gt	Eurasian cultivated accession	PI183967	Indian wild accession
Cuc80	Xishuangbanna cultivated accession		

Table 2. The protein lengths of CsZIPs in different *C. sativus* accessions.

Protein name	9930	XTMC	Cu2	Cuc80	Cuc37	Gy14	9110gt	PI183967	Cuc64	W4	W8	Hx14	Hx117
CsZIP1	249	350	350	-	350	350	350	350	350	350	350	350	
CsZIP2	417	417	417	417	417	441	417	417	417	417	417	408	417
CsZIP3	228	275	275	275	275	306	275	275	306	275	275	275	275
CsZIP4	594	594	639	594	594	594	594	594	594	594	594	594	594
CsZIP5	367	367	367	365	367	367	367	367	369	367	365	369	815
CsZIP6	463	463	463	463	463	407	463	463	463	463	463	463	463
CsZIP7	335	335	335	335	335	335	335	335	335	302	335	335	346
CsZIP8	349	349	349	349	349	349	349	349	349	349	349	349	1096
CsZIP9	334	334	334	334	334	337	334	-	338	334	334	334	337
CsZIP10	354	354	354	354	354	354	354	354	354	354	354	354	354

Table 3. CsZIP family in *C. sativus*.

Gene	Introns	Chromosomal site	Gene length	protein length	Protein MW(kDa)	Isoelectric point	Subcellular location predicted
CsZIP1	2	1	2955	249	27.2	6.88	Cell membrane
CsZIP2	3	2	3491	417	44.5	6.09	Chloroplast
CsZIP3	9	4	9406	228	24.6	7.02	Cell membrane
CsZIP4	4	4	5063	594	62.0	6.27	Cell membrane
CsZIP5	2	4	2777	367	38.8	5.98	Cell membrane
CsZIP6	10	5	21173	463	49.9	5.97	Cell membrane
CsZIP7	1	6	2038	334	35.7	5.73	Chloroplast
CsZIP8	0	6	1490	348	38.0	5.58	Cell membrane
CsZIP9	2	7	7007	334	35.9	6.02	Cell membrane
CsZIP10	2	7	4076	354	37.2	8.53	Cell membrane

3.3. Analysis of phylogenetic relationship and gene structure and protein motif

To analyze the phylogenetic relationship of CsZIP proteins, we constructed the phylogenetic tree of these 10 proteins (Figure 1). Based on the phylogenetic tree, the CsZIP family was divided into three distinct clades. CsZIP2, CsZIP5 and CsZIP10 belonged to the first clade, while CsZIP8 and CsZIP1 occupied the second clade, and the other CsZIPs formed the third clade (Figure 1). The

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number of introns varied among *CsZIP* genes, ranging from 0 to 10: *CsZIP6* has the most introns, and *CsZIP8* has none (Figure 1b). The structures of *CsZIP6*, *CsZIP3* and *CsZIP4* genes were similar, suggesting that they are more closely related (Figure 1).

We further analyzed the conserved motifs of CsZIP family and found that motif type and arrangement were very similar among the members of the first and second clades, in all of which only motif9 was present; but diverse among the members of the third clade, and motif number ranged from one (CsZIP3 and CsZIP4) to seven (CsZIP7) (Figure 2). Motif 2 was present in all the CsZIPs except CsZIP3 indicating that Motif 2 is highly conserved (Figure 2). CsZIP6, CsZIP3 and CsZIP4 are the CsZIP proteins that did not contain Motif 1 (Figure 2). The differences in motifs among CsZIP proteins may contribute to their functional differentiation. The amino acid sequence for each motif is shown in the Figure S1.

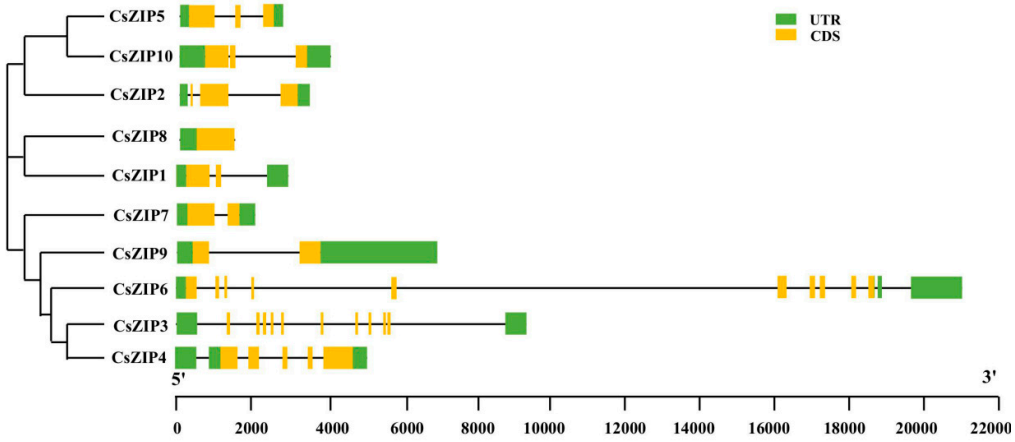

Figure 1. Phylogenetic tree and gene structure of *ZIP* family members in *C. sativus*. The phylogenetic tree was constructed using the neighbor-joining (NJ) method with 1000 bootstrap replicates, based on the alignment of the identified 10 ZIP proteins in *C. sativus*. The gene structures of the identified 10 *ZIP* genes in *C. sativus* were generated using the Gene Structure Display Server v2.0. In the structures, the green box represents the UTR, the yellow box represents the exon, and the black line represents the intron.

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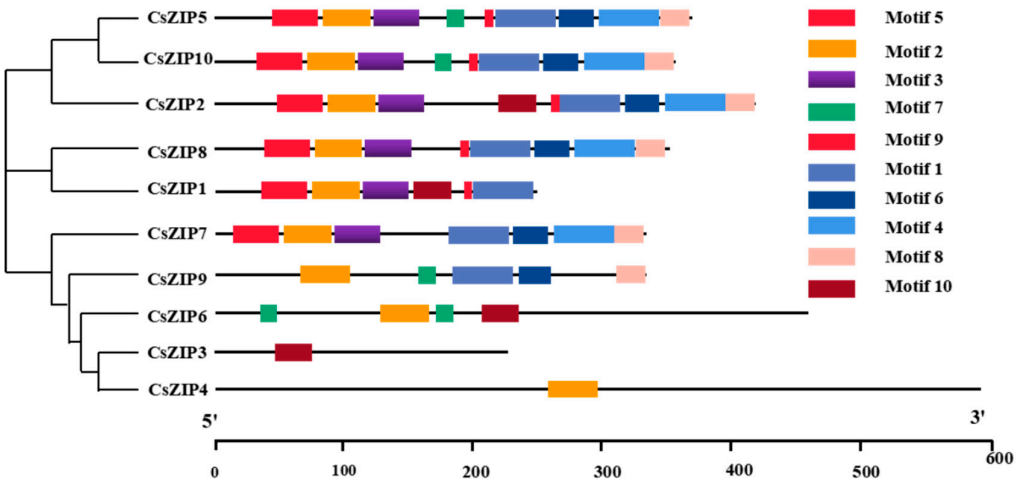

Figure 2. The conserved motifs for *C. sativus* ZIP proteins. The conserved motifs identified by MEME are highlighted with colored boxes.

3.4. Multiple sequence alignment

To analyze the characteristics of ZIP protein homologous sequences in comparison to other species, we conducted a comparative study of ZIP proteins in *Arabidopsis*, *Cucumis melo*, *O. sativa*, and *C. sativus*. The transmembrane domains IV-VII exhibited the highest degree of conservation (Figure 3). Previous studies have reported that transmembrane domains IV and V are amphiphilic and play a crucial role in transport, a conclusion that has been further corroborated by subsequent research [42]. In the mutant *irt1*, mutations in the conserved histidine residues or adjacent polar charged residues within transmembrane domains IV and V were found to abolish their transport function [39]. Notably, the IV regions of the sequence alignment in *Arabidopsis*, *Cucumis melo* , *O. sativa*, and *C. sativus* showed the presence of conserved histidine residues (Figure 3).

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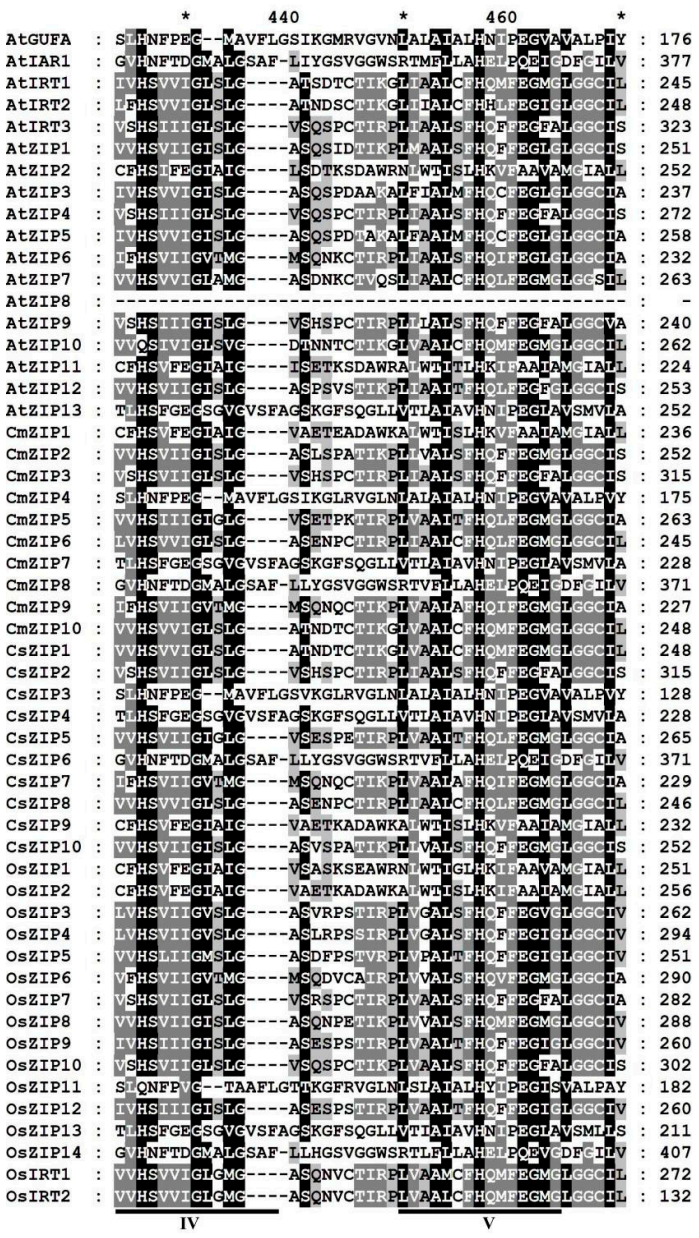

Figure 3. Amino acid sequence alignment for ZIP proteins from *Arabidopsis*, *C. sativus*, *Cucumis melo* and *O. sativa*. The ZIP proteins were aligned using MAFFT v5.3 with the default settings. Completely conserved residues are shaded in black boxes, while highly conserved residues are shaded in gray boxes.

3.5. Phylogenetic analysis among different species

We obtained a comprehensive understanding of the relative relationship of CsZIP proteins by constructing a phylogenetic tree using the amino acid sequences of ZIP proteins from *Arabidopsis*, *C. sativus*, *Cucumis melo*, and *O. sativa* (Figure 4). The phylogenetic analysis revealed three major clades: Group 1, 2 and 3. In Group 1, there were 7 proteins from *Arabidopsis*, 3 from *C. sativus*, 3 from *Cucumis melo*, and 8 from *O. sativa*. Group 2 consisted of 6 proteins from *Arabidopsis*, 3 from *C. sativus*, 3 from *Cucumis melo*, and 3 from *O. sativa*. Group 3 included 5 proteins from *Arabidopsis*, 4 from *C. sativus*, 4 from *Cucumis melo*, and 5 from *O. sativa* (Figure 4). It is worth noting that the number of ZIP proteins varied within each subgroup for *Arabidopsis*, *C. sativus*, *Cucumis melo*, and *O. sativa*. Specifically, half *O. sativa* ZIP proteins were grouped into Group 1, while the distribution among the three clades was

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relatively equal for the other species. Further investigation within the Group 3 revealed a close relationship between CsZIP6, CmZIP8, AtIAR1, and OsZIP14 (Figure 4). For reference, the amino acid sequences of the ZIP proteins can be found in the Dataset S1. This analysis provides valuable insights into the interrelationships of ZIP proteins in different plant species and serves as a foundation for further exploration of their biological functions.

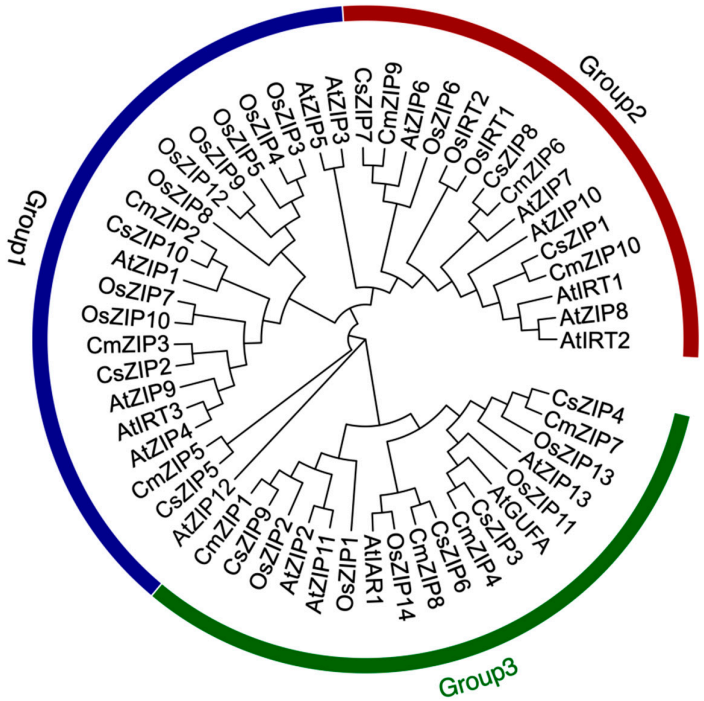

Figure 4. Phylogenetic tree for the ZIP proteins from *Arabidopsis*, *C. sativus*, *Cucumis melo* and *O. sativa*. The unrooted amino acid sequence similarity tree was generated using MEGA 7.0 by neighbor-joining (NJ) method with 1000 bootstrap replicates.

3.6. Chromosome localization and collinearity analysis

The chromosomal distribution analysis of *CsZIP* genes in *C. sativus* revealed an uneven distribution pattern across seven chromosomes (Figure 5). Specifically, three *ZIP* genes (*CsZIP3*, *4* and *5*) were located on chromosome 4, two on each of chromosome 6 and 7, respectively, and one on each of chromosome 1, 2 and 5, respectively, and none on chromosome 3 (Figure 5).

We further conducted a collinearity analysis of the *ZIP* gene family members between *C. sativus* and other plant species, including *Arabidopsis*, *Cucumis melo* , *O. sativa*, and Chinese cabbage (Figure 6). Our results revealed that a total of eight genes were identified to be present in both *C. sativus* and *Arabidopsis*, suggesting a conserved relationship between these two species (Figure 6). In the case of *C. sativus* and *Cucumis melo*, all members of the *ZIP* gene family exhibited homologous alignment, indicating a high level of homology during the long differentiation process between these two closely related species (Figure 6). However, only two genes showed homology between *C. sativus* and *O. sativa*. Similarly, we detected eight homologous genes in both *C. sativus* and Chinese cabbage, similar with the findings between *C. sativus* and *Arabidopsis* (Figure 6). These findings provide valuable insights into the evolutionary relationships and conservation of the *ZIP* gene family among different plant species.

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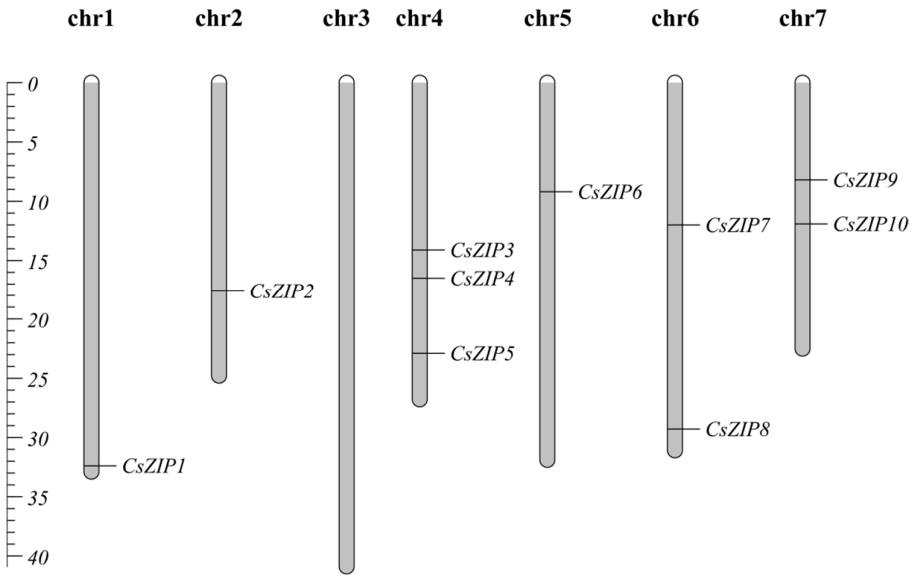
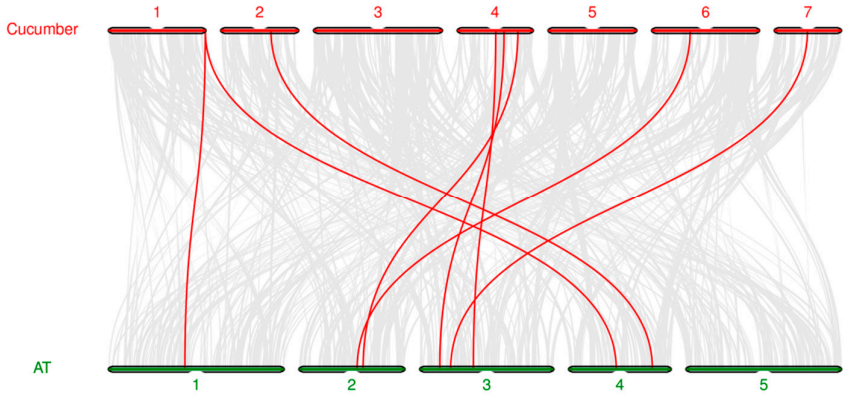


Figure 5. Chromosomal location of *C. sativus ZIP* genes.



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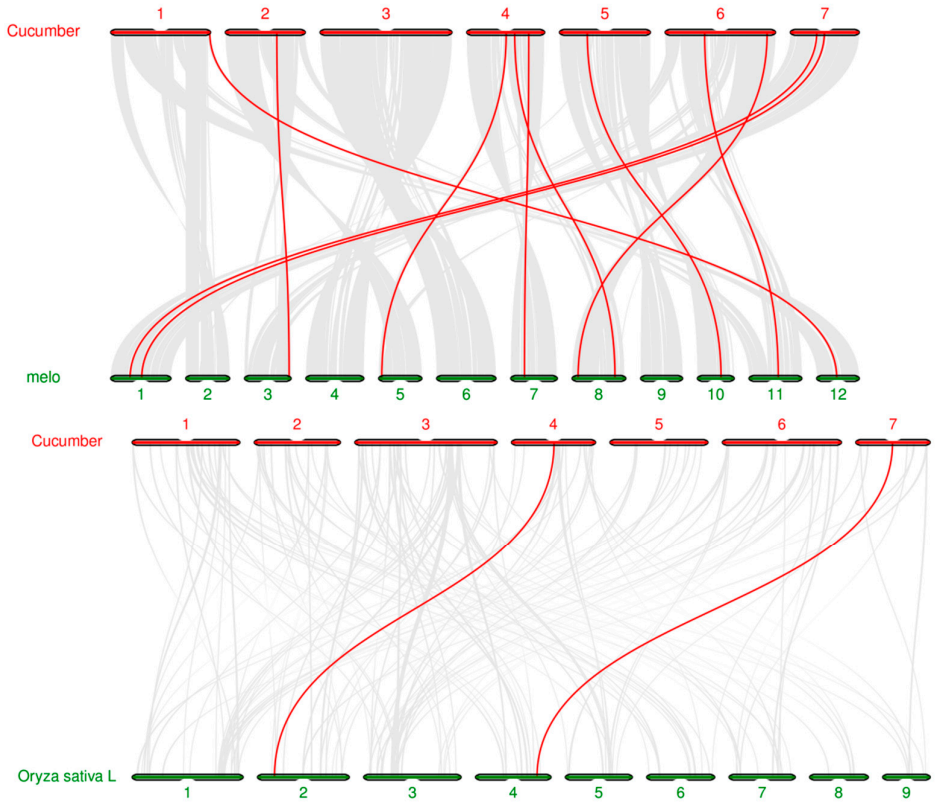

Figure 6. Synteny analysis of *ZIPs* between *C. sativus* and other plant species (*A. thaliana*, *Oryza sativa* and *Cucumis melo*): The collinear blocks are marked by gray lines, while the collinear gene pairs with *ZIP* genes are highlighted by red lines.

3.7. Analysis of cis-acting elements in promoter region of CsZIP genes

The analysis of the 1500 bp sequence upstream of the *ZIP* gene in *C. sativus* revealed the presence of various *cis*-elements (Figure 7). Specifically, the promoter regions of all the genes in this family contained elements related to some of gibberellin, auxin, salicylic acid, jasmonic acid, low temperature, and drought stress. Most of the gene promoters contained hormone-related elements, indicating their potential involvement in hormone signaling pathways. Notably, gibberellin-related elements were found in the promoters of several family members, including *CsZIP1*, *CsZIP2*, *CsZIP4*, *CsZIP6*, *CsZIP7*, *CsZIP8* and *CsZIP10* (Figure 7). This suggests that these genes may play crucial roles in gibberellin-mediated processes. Auxin response elements were predominantly enriched in *CsZIP6*, *CsZIP8* and *CsZIP10* gene promoters (Figure 7). This finding suggests that these specific genes may be involved in the regulation of plant growth and development through auxin signaling pathways.

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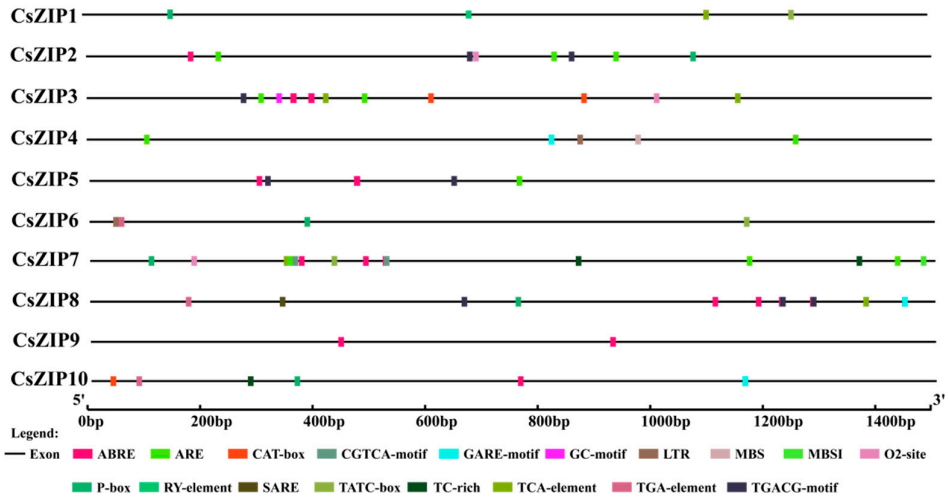

Figure 7. Predicted *cis*-elements in promoter regions of *C. sativus ZIP* genes. The promoter region was defined as a 1.5 kb sequence upstream of the translation initiation codon of the *CsZIP* gene. Identification of *cis*-acting elements using the online tool Plant CARE. Different types of *cis*-acting elements are represented by closed boxes of different colors.

3.8. Expression of CsZIP genes in different tissues

This part aimed to investigate the roles of *ZIP* genes in *C. sativus* development by analyzing *RNA-seq* data from different tissues. The expression patterns of *CsZIP* gene family were examined using data from the Cucurbit Genomics Data website and NCBI SAR database, and heat maps were generated to visualize the expression levels across tissues. The results revealed that the expression of *ZIP* genes in *C. sativus* leaves, stems, and tendrils was relatively lower compared to other tissues (Figure 8a). In contrast, *CsZIP7* and *CsZIP10* showed the highest expression levels in roots, suggesting their possible involvement in regulating ion transport in the underground parts of *C. sativus*. *CsZIP2*, *CsZIP5* and *CsZIP9* were predominantly expressed in flowers, indicating their potential roles in flower differentiation and development (Figure 8a). On the other hand, *CsZIP1*, *CsZIP3*, *CsZIP4*, *CsZIP6* and *CsZIP8* exhibited major expression in the ovary, especially, *CsZIP3*, *CsZIP4* and *CsZIP8* displayed high expression levels in the unfertilized ovary (Figure 8a). These findings suggest that these five *ZIP* genes may have regulatory functions in fruit development.

To further validate the reliability of the *RNA-seq* results, qRT-PCR analysis of *ZIP* gene expression in different tissues was performed. Overall, the qRT-PCR results were consistent with the *RNA-seq* data, confirming the reliability of the datasets (Figure 8b). However, an inconsistency was observed in the expression of *CsZIP4* between male flowers and tendrils, indicating some variations in gene expression in specific tissues (Figure 8b). Therefore, these genes are likely involved in ion transport, flower differentiation and development, and potentially regulate fruit development.

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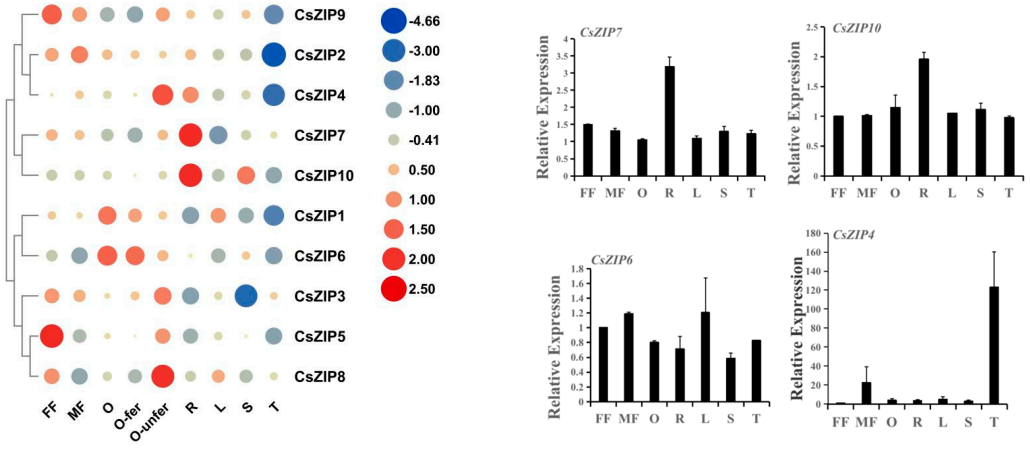

(**a**) (**b**)

Figure 8. Temporal-spatial expression of *C. sativus ZIP* genes. (a) Heatmap displaying the expression profile of *CsZIP* genes in nine different *C. sativus* tissues. The RNA-seq datasets with accession number PRJNA80169 were obtained from the Cucurbit Genomics Data website. The color scale represents Log_2(FPKM) values, where blue and red indicate low and high expression levels, respectively. The FPKM values of *CsZIP* genes can be found in Table S1. (b) Validation of *RNA-seq* results for four *CsZIP* genes using qRT-PCR. The error bars represent the standard error of the mean (n = 3). R: root; S: stem; L: leaf; FF: female flower; MF: male flower; O: unexpanded ovary; O-fer: expanded fertilized ovary; O-unfer: expanded unfertilized ovary; T: tendril.

3.9. Expression profiles of CsZIP genes under abiotic and biotic stresses

Due to the lack of transcriptomic data on metal ion transport, and in order to enhance our understanding of the functional roles within the *ZIP* gene family and predict their potential applications in the future, We investigate the impact of various environmental stresses on the expression of *CsZIP* genes, we assessed their comprehensive expression patterns in response to different stress conditions, including salt, heat, downy mildew (DM, caused by *Pseudoperonospora cubensis*), and powdery mildew (PM, caused by *Podosphaera fusca*), based on publicly available transcriptome data. This analysis enabled us to gain further insights into the potential roles of *CsZIP* genes in mediating plant response to diverse stresses.

Under salt stress, specific expression patterns of *CsZIP* genes were revealed through transcriptome data analysis. *CsZIP4*, *CsZIP5*, *CsZIP6* and *CsZIP8* genes were up-regulated in response to NaCl treatment; In contrast, *CsZIP1*, *CsZIP3* and *CsZIP10* genes showed down-regulation (Figure 9a). It is worth noting that Si treatment has been reported to enhance stress resistance and stimulate plant growth [66]. Interestingly, when plants were solely subjected to Si treatment, most *CsZIP* genes exhibited up-regulation in their expression levels. However, the expression of the *CsZIP1* gene was found to be down-regulated (Figure 9a). Additionally, we observed a similar down-regulation of *CsZIP1* gene expression after exogenous salt and Si treatment. Therefore, it can be concluded that under salt stress conditions, most *CsZIP* genes tend to be up-regulated, while the *CsZIP1* gene specifically demonstrates down-regulation in response to salt stress (Figure 9a).

We also examined the response of *CsZIP* genes to heat stress (Figure 9b). At 3 hours following exposure to high temperatures, the expression levels of *CsZIP2*, *CsZIP3*, *CsZIP4*, *CsZIP5*, *CsZIP6*, *CsZIP7*, *CsZIP8* and *CsZIP9* were up-regulated, whereas *CsZIP1* was down-regulated, and similar patterns emerged at 6 hours after heat stress, suggesting that these genes may play a crucial role in heat tolerance (Figure 9b).

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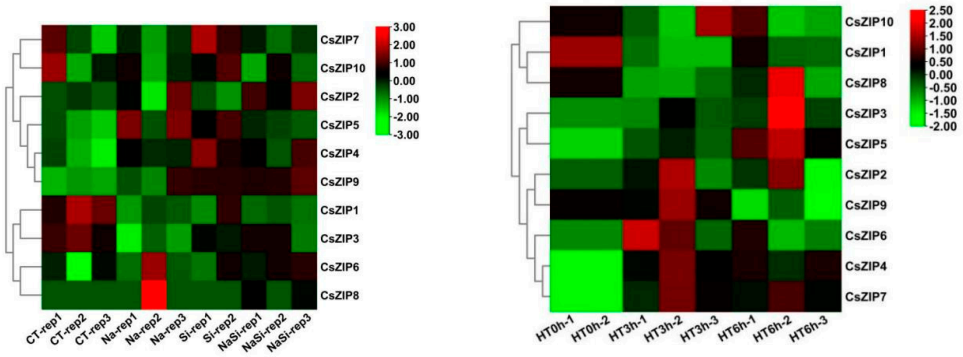
To explore the potential functions of *CsZIPs* in biotic stress resistance, we conducted an analysis of *CsZIP* expression using the *RNA-Seq* database. Following inoculation with powdery mildew (PM), we observed distinct gene expression patterns between the susceptible and resistant *C. sativus* lines (Figure 10a). After inoculation with PM, the expression of *CsZIP3*, *CsZIP4*, *CsZIP7* and *CsZIP9* was down-regulated in both the resistant and susceptible lines. In contrast, the expression of *CsZIP5* was up-regulated (Figure 10a).

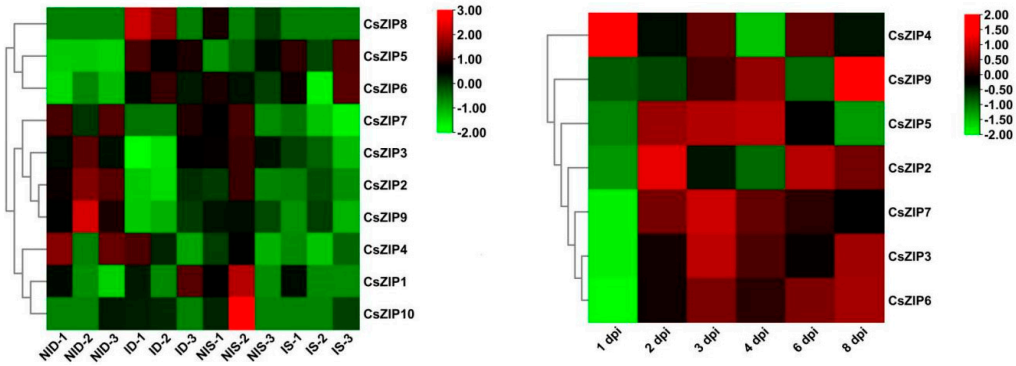
For DM inoculation, *CsZIP2*, *CsZIP3*, *CsZIP5*, *CsZIP6*, *CsZIP7* and *CsZIP9* genes were up-regulated at least at one time point after treatment, while *CsZIP4* genes were down-regulated (Figure 10b).

These findings indicate that *CsZIP* genes are responsive to different environmental stresses and may play diverse roles in mediating plant responses to these stresses. Further research is needed to fully understand the specific functions of each *CsZIP* gene and their contributions to stress resistance in *C. sativus*.



(**a**) (**b**)

Figure 9. Expression of *CsZIP* genes in response to salt and hot stimuli. (a) Heatmap for displaying the expression profile of *CsZIP* genes in response to salt stresses. (b) Heatmap for displaying the expression profile of *CsZIP* genes in response to hot (42°C). RNA-seq datasets with accession numbers of GSE151055 and GSE116265 were downloaded from NCBI SAR database. Color scale represents Log_2(FPKM) values. Green, red, and black color indicates low, high, and no expression, respectively. The FPKM value of *CsZIP* genes under salt and hot treatments are listed in Table S2.



(**a**) (**b**)

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Figure 10. Expression analysis of *CsZIPs* under biotic stresses: The transcriptional levels of *CsZIP* genes after infection with powdery mildew (PM) for 48 h (a) and with downy mildew (DM) for 1–8 days post-inoculation (b) are shown on the heatmaps. The color scale shows increasing expression levels from green to red. ID, PM-inoculated susceptible *C. sativus* line D8 leaves; NID, non-inoculated D8 leaves; IS, PM-inoculated resistant *C. sativus* line SSL508-28 leaves; NIS, non-inoculated SSL508-28 leaves; CT, without inoculation; DPI, days post inoculation. The FPKM value of *CsZIP* genes under powdery mildew and downy mildew are listed in Table S3.

4. Discussion

The ZIP (ZRT/IRT-like protein) gene family is involved in the absorption and transport of metal ions in plants, playing a crucial role in plant growth, development, and response to heavy metal stress [1]. In this study, a comprehensive genome-wide identification of *CsZIPs* was performed, and a total of 10 genes were identified in *C. sativus*. We further investigated their gene structure, phylogenetic relationship, composition of *cis*-regulatory elements in promoter, chromosome localization, collinearity analysis, and expression patterns under different elemental stresses. These findings enhance our understanding of the *ZIP* gene family and provide bases for better elucidating the function and evolutionary relationship of CsZIPs in *C. sativus*.

Previous studies have reported the identification of the *ZIP* gene family in various plant species including *A. thaliana*, peanut, and *Populus.trichocarpa*. *Arabidopsis* had 15 ZIP members, peanut 30, and *P.trichocarpa* 16, respectively [40–42]. However, in this study, only 10 *CsZIP* genes were identified (Figure 1). Of the 13 *C. sativus* germplasm resources analyzed, 'Cuc80' and 'PI183967' contained 9 *CsZIP* genes, whereas the remaining varieties harbored 10 *ZIP* genes (Table 2). Notably, only CsZIP10 exhibited a consistent amino acid sequence length, while the lengths of other CsZIPs varied among the different varieties (Table 2).

Previous studies have predicted and confirmed the subcellular localization of ZIP proteins. Most of ZIP proteins are predicted to be localized in membrane system. For instance, in *O. sativa*, it has been demonstrated that OsZIP1, OsZIP5, OsZIP7 and OsZIP8 are localized in the plasma membrane [17,21,22,51,52]. In peanuts, AhZIP1.2, AhZIP3.2, AhZIP3.5 and AhZIP7.8 are localized in the endomembrane system [42]. In *Arabidopsis*, AtIRT1, AtIRT3, AtZIP1 and AtZIP2 have been confirmed to be localized in the plasma membrane [6,7,53]. In this study, we predicted the subcellular localization of 10 ZIP proteins. The results showed that CsZIP1, CsZIP3, CsZIP4, CsZIP5, CsZIP6, CsZIP8, CsZIP9 and CsZIP10 are predicted to be localized in the cell membrane, while CsZIP2 and CsZIP7 in the chloroplast (Table 3).

In the evolutionary process of plant genomes, the emergence of gene family members often accompanies gene duplication, leading to new functions. For example, most of the *AhZIP* genes experienced gene duplication events except *AhIRT1.1/1.3*, *AhZIP7.2/7.8* and *AhZIP3.5/3.6* [42]. However, unfortunately, we did not detect any gene duplication events in the *CsZIP* genes in *C. sativus*.

The *CsZIP* gene promoters contain many *cis*-acting elements that respond to various hormones and stresses, including gibberellins, methyl jasmonate, abscisic acid, and low temperature. In *S. lycopersicum*, zinc deficiency tolerance is determined by auxin signaling [46]. Furthermore, the response elements of three *ZIP* genes in sunflower are regulated by salicylic acid and methyl jasmonate under iron deficiency [47]. These findings suggest that the expression of *CsZIP* genes can be induced by several plant hormones, such as salicylic acid, MeJA, gibberellins and auxins. Methyl jasmonate acts as a crucial plant hormone in defense against biotic and abiotic stresses by triggering defense mechanisms and regulating growth [48]. Auxin, on the other hand, controls plant development, with tryptophan being a key amino acid in the auxin synthesis pathway. As Zn participates in tryptophan synthesis, *CsZIP* genes might play a significant role in auxin biosynthesis [49]. Being similar to auxin, gibberellins also regulate various plant growth and development processes, including seed germination and fruit development [50]. Salicylic acid has been shown to greatly enhance plant stress resistance [51]. Additionally, several *cis*-acting elements associated with biotic and abiotic stress responses have been identified in the promoter region of *CsZIP* genes (Figure

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7). This suggests that the *CsZIP* gene family may regulate plant growth in hormone signaling pathways to adapt to different environmental stresses.

In general, the expression patterns of genes are correlated with their functions [52]. The expression data of *CsZIPs* in different tissues provide valuable insights into the possible functions of the *ZIP* gene family. Previous studies have confirmed that *ZIP* genes are predominantly expressed in roots, regardless of the plant species, such as *Arabidopsis*, *O. sativa*, or barley, with many genes being induced under deficiencies of environmental zinc or other metallic elements. [7,53–55]. Existing research has indicated that within tomatoes, the *SlZIP* gene family comprises 15 members, with 13 expressing in the roots, exhibiting significantly high expression levels. Among these, 11 *SlZIP* genes are expressed in fruits, albeit with varying expression across different developmental stages, indicating the involvement of *SlZIP* genes in the accumulation of zinc and iron in *S lycopersicum* fruits or their redistribution among different tissues [56]. Similarly, *CsZIP7* and *CsZIP10* exhibit high expression in *C. sativus* roots, while *CsZIP10* has higher expression levels in stems compared to other tissues (Figure 8). This suggests that *CsZIP7* and *CsZIP10* may play functional roles in these two tissues. Furthermore, it was observed that *CsZIP2*, *CsZIP5* and *CsZIP9* show higher expression levels in flowers, while *CsZIP3*, *CsZIP4* and *CsZIP8* exhibit the highest expression levels in fertilized ovaries. *CsZIP1* and *CsZIP6* are also expressed in ovaries (Figure 8). Based on this information, it can be inferred that these *CsZIP* genes may participate in the regulation of fruit development by regulating the accumulation of Zn and Fe or their redistribution among different tissues.

Previous studies have primarily focused on the regulatory roles of *ZIP* genes in plant metal ion uptake and transport. For instance, AtZIP3 is known to play a crucial role in facilitating the absorption of Zn and Fe from the soil into plant roots [8]. On the other hand, AtZIP2 functions by mediating the uptake of Mn/Zn into the parenchyma cells of the xylem, thereby enabling the efficient transportation of Mn/Zn to the aboveground parts of plants [7]. However, in this study, we found that *CsZIP* genes could response to different environmental stresses. We investigated the comprehensive expression patterns of *CsZIP* genes under different environmental stresses, including powdery mildew, downy mildew, salt, and heat. The results showed differential expression of *CsZIP* genes in response to high temperature (Figure 9b), salt, silicon (Figure 9a), powdery mildew (Figure 10a), and downy mildew treatments (Figure 10a). These results provide valuable clues suggesting that *CsZIP* genes may have significant functions in multiple stress conditions, warranting further research and validation of their specific roles.

5. Conclusions

In this study, we performed pan-genome-wide identification of the *ZIP* gene family in *C. sativus*. A total of 10 members were identified. Eleven of the 13 accessions contained all the *CsZIP* genes. The ZIP gene family has three evolutionary branches and contains conserved histidine residues. The *CsZIP* gene promoters contained elements that responded to plant hormone signaling pathways, indicating that plant hormone signals may have an impact on *CsZIP*-mediated biotic and abiotic stresses. The expression patterns of *CsZIPs* in different tissues showed that the *CsZIP* gene family may be related to the growth and development of *C. sativus*. Finally, *CsZIPs* could response to different environmental stresses. In summary, this study helps to improve the current understanding of the *CsZIP* gene family and provides a basis for exploring their functions in *C. sativus*.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org. Figure S1: The conserved motif LOGO of *C. sativus* ZIP proteins; Table S1: The FPKM values of *CsZIP* genes; Table S2: The FPKM value of *CsZIP* genes under salt and hot treatments; Table S3: The FPKM value of *CsZIP* genes under powdery mildew and downy mildew; Table S4: The primer sequences used for the qRT-PCR; Dataset S1: The amino acid sequences of the ZIP proteins. Dataset S2： All CsZIP protein sequences of 13 *C. sativus* accessions.

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