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The CBL and CIPK Gene Family in Cucumber (Cucumis sativus): Genome-wide Analysis and Expression Profiles in Response to Abiotic and Biotic Stresses

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Abstract

The calcineurin B-like proteins (CBLs) act as Ca^{2+} sensors and play crucial roles in plant growth, development, and response to environmental stresses by interacting with CBL-interacting protein kinases (CIPKs). Although *CBL* and *CIPK* gene families have been well studied in many plant species, little is known about them in *Cucumis sativus*. In this study, seven *CBLs* and nineteen *CIPKs* were identified in cucumber. Phylogenetic analysis classified the cucumber *CBL* and *CIPK* into four and five groups, respectively. The gene structure analysis showed that the *CIPKs* can be divided into two groups: intron-poor and intron-rich, indicating that intron acquisition and loss contributed to the evolution of *CIPKs*. Both tandem and segmental duplications have contributed to the evolution and diversification of the *CBLs* and *CIPKs* in cucumber. Expression profile analysis revealed that most *CBLs* and *CIPKs* exhibited tissue-specific expression, suggesting divergent expression during the development. Notably, *CsCIPK14* was not expressed in any tissues. Additionally, the qRT-PCR results revealed that many *CBL* and *CIPK* genes were differentially regulated under various biotic and abiotic stresses, underscoring their significant roles in stress response.

1. Introduction

Ca^{2+} is a secondary messenger that plays vital roles in plant growth, development, and responses to biotic and abiotic stresses (Dong, Wang, et al., 2020, Upadhyay, 2021). Ca^{2+} sensors, e.g., calmodulins (CaMs), calmodulin-like proteins (CMLs), calcium-dependent protein kinases (CPKs), and calcineurin B-like proteins (CBLs), respond to (a)biotic stresses (Aldon, Mbengue, et al., 2018). CBL proteins contain four EF-hand domains and conserved PFPF motifs as calcium binding sites. They function in various signaling pathways by targeting CIPKs (CBL-interacting protein kinases). CIPKs consist of a serine/threonine kinase domain at the N-terminal and an NAF/FISL domain at the C-terminal (Albrecht, Ritz, et al., 2001). The PFPF motifs of CBLs bind to the NAF domains of CIPKs to form CBL-CIPK complexes and release kinase activity.

The gene families CBL and CIPK in plant genomes have resulted from gene expansion due to a series of duplication events. So far, a large number of CBL and CIPK genes have been identified in dozens of plant species, including Arabidopsis (Luan, Kudla, et al., 2002), rice (Gu, Ma, et al., 2008, Kanwar, Sanyal, et al., 2014), soybean (Jiao, Zhang, et al., 2024, Zhu, Chen, et al., 2016), maize (Chen, Gu, et al., 2011), wheat (Sun, Wang, et al., 2015), canola (Zhang, Yang, et al., 2014), cotton (Zhang, Yang, et al., 2014), pepper (Ma, Gai, et al., 2019), and *Medicago truncatula* (Du, Yang, et al., 2021, Zhang, Ren, et al., 2022). These CBLs and CIPKs are involved in plant growth and development (Cheong, Pandey, et al., 2007, Tang, Liu, et al., 2012), such as pollination (Yang, Kong, et al., 2008), seed germination(Yang, Kong, et al., 2008), and root development (Tripathi, Parasuraman, et al., 2009). Additionally, CBLs and CIPKs participate in the signal transduction of several plant hormones, e.g., jasmonic acid (JA) (Ma, Li, et al., 2021, Panda, Busatto, et al., 2019), gibberellins (GA) (An, Zhang, et al., 2023), and abscisic acid (ABA) (Chen, Wang, et al., 2013, Garcia-Mas, Benjak, et al., Lyzenga, Liu, et al., 2013).

The Cucurbitaceae, also known as cucurbits or the gourd family includes important vegetable crops such as cucumber (*Cucumis sativus*), watermelon (*Citrullus lanatus*), melon (*Cucumis melo*), wax gourd (*Benincasa hispida*), and pumpkin (*Cucurbita moschata*) that play significant roles in agricultural production. The published whole genome sequences make it possible to characterize and analyze numerous gene families, such as *WRKY* (Ling, Jiang, et al., 2011, Yang, Li, et al., 2018), *GASA* (Zhang, Hu, et al., 2022), *TIFY* (He, Cheng, et al., 2022), *FKBP* (Yang, Li, et al., 2023), and *HSF* (Zhou, Zhang, et al., 2013). However, the research of CBLs and CIPKs was not intensively analyzed in the gourd family. We characterized *CBL* and *CIPK* genes in 9 Cucurbitaceae species by analyzing the genome sequences. To further understand the *CBL* and *CIPK* genes in cucumber, we conducted a comprehensive genome-wide analysis, including chromosomal localizations, genomic distributions, motif composition of proteins, and phylogenetic relationships. Additionally, we analyzed the tissue-specific expression and expression divergence of cucumber CBLs and CIPKs in response to various stresses to explore the functions of the CBL and CIPK transcriptions in cucumber. These findings provide valuable information that enables further study of the evolution and function of the CBL and CIPK genes in Cucurbitaceae species.

2. Materials and Methods

2.1. Identification of putative CBL and CIPK proteins in 9 Cucurbitaceae species

To identify the *CBL* and *CIPK* genes, we downloaded the annotated genome sequences of 9 Cucurbitaceae species from the Cucurbit Genomics Database (CuGenDB) (Zheng, Wu, et al., 2019). The sequence of 10 CBL and 26 CIPK proteins in A*rabidopsis thaliana* were downloaded from TAIR (https://www.arabidopsis.org/) and used as queries searching against the annotation databases of the 9 genomes by local blast with e-value < 1e − 5 and identity > 90% (Zheng, Wu, et al., 2019). The HMM profile of the NAF domain (PF03822) was downloaded from the Pfam protein family database (http://pfam.sanger.ac.uk/) (Zheng, Wu, et al., 2019), and the putative CIPK proteins in 9 genomes were explored using HMMER 3.0 (http://hmmer.org/) with the default parameters. The predicted CBL sequences were manually checked with four conserved EF-hand motifs. All the identified CBL and CIPK protein sequences were used for further analyses. Length of sequences, molecular weight (MW), and isoelectric point (pI) of the related proteins were calculated using an online tool from the Expasy website (https://web.expasy.org/compute_pi/).

2.2. Classification and phylogenetic analysis of the cucumber CBL and CIPK gene families

Based on the *AtCBL* and *AtCIPK* classification in *Arabidopsis* and DNA-binding domain alignments using Clustal X 2.1 with default settings (Zheng, Wu, et al., 2019), the *CsCBL* and *CsCIPK* genes were classified into different groups. Multiple sequence alignments of the CsCBL and CsCIPK domains were performed using MUSCLE (Edgar, 2004). The phylogenetic trees were constructed using IQ-TREE with the maximum-likelihood (ML) method(Lam-Tung, Schmidt, et al., 2015). The reliability of phylogenetic trees

was tested using Bootstrap with 1,000 replicates. The results were submitted to the iTOL online website (https://itol.embl.de) to display the phylogenetic trees (Letunic and Bork, 2021).

2.3. Chromosomal locations and synteny analysis of CBLs and CIPKs in cucumber

The chromosomal locations of *CsCBL*s and *CsCIPK*s were retrieved from the annotated genome information's general feature format (GFF) file. The map of the location image was drafted with the Circos program in TBtools software (GitHub—CJ-Chen/TBtools: GUI/CommandLine Tool Box for biologists to utilize NGS data.) (Chen, Chen, et al., 2020). Tandem and segmental duplications in cucumber genomes were analyzed using the MCScanX software (Wang, Tang, et al., 2012). The Ka/Ks ratios of tandem and segmental duplicated gene pairs were calculated using the KaKs_Calculator (Zhang, Li, et al., 2006). To show the interspecies syntenic relationship, a comparative syntenic map of *C. sativus*, *C. lanatus*, *C. moschata*, *A. thaliana*, and *O. sativa* was generated using the Dual Systeny Plotter software in TBtools. The synteny results were visualized in TBtools.

2.4. Phylogenetic tree, gene structure, and motif composition investigation of cucumber CBL and CIPK proteins

The phylogenetic trees of cucumber *CBLs* and *CIPKs* were constructed using MEGA 11 (Tamura, Stecher, et al., 2021) with the ML method. The reliability of phylogenetic trees was tested using Bootstrap with 1,000 replications. To examine gene structure, the General Feature Format (GFF) file of cucumber was utilized with the TBtools software. The analysis of *CBL* and *CIPK* gene structures was carried out using the MEME Suite (Bailey, Boden, et al., 2009). The conserved motifs were identified using the following parameters: minimum motif width of 6, maximum motif width of 50, and a maximum of 10 motifs.

2.5. RNA-Seq analysis of cucumber transcriptome data

The transcriptome datasets were retrieved from the NCBI SRA database. The SRA data were then converted to fastq format using fasterq-dump (V3.0.5). We assessed the quality of the fastq data using FastQC software (Wingett and Andrews, 2018) and eliminated low-quality sequences using Trimmomatic software (Version 0.39) (Bolger, Lohse, et al., 2014). Gene expression levels were quantified using Kallisto (Bray, Pimentel, et al., 2016) in TPM units (transcripts per kilobase of exon model per million mapped reads) (Mortazavi, Williams, et al., 2008, Trapnell, Williams, et al., 2010). The TPM values were log2 transformed, and a heatmap was generated using the pheatmap package in TBtools. Differential gene expression analysis based on the counts data of CsCBL and CsCIPK genes was conducted using DESeq2 software (Love, Huber, et al., 2014).

2.6. Expression profiling of cucumber CBLs and CIPKs in different tissues

The Illumina RNA-seq data for 10 tissues was downloaded from the NCBI SRA database under the accession number PRJNA80169 (Li, Zhang, et al., 2011). This data created a tissue-specific expression heatmap for the CsCBL and CsCIPK genes. Additionally, transcriptome data for root development (PRJNA271595) (Li, Zhang, et al., 2011) was obtained to generate a heatmap using TBtools software following the abovementioned methods.

2.7. Expression analyses of cucumber CBLs and CIPKs under various stresses

The transcriptome data under various treatments were analyzed, including GA treatment (PRJNA376073) (Zhang, Zhao, et al., 2017), low temperature (PRJNA438923), high temperature (PRJNA634519) (Chen, Chen, et al., 2020), salt and silicon (PRJNA477930) (Zhu, Yin, et al., 2019), waterlogging (PRJNA678740) (Keska, Szczesniak, et al., 2021), root-knot nematode (PRJNA419665) (Wang, Cheng, et al., 2018), powdery mildew (PRJNA321023) (Xu, Xu, et al., 2017), downy mildew (PRJNA285071) (Burkhardt and Day, 2016), and *Fisarium wilt* (PRJNA472169) (Dong, Wang, et al., 2020). The data was downloaded from the SRA database and analyzed using the abovementioned methods. The expression heatmaps were created using TBtools software.

2.8. Plant material and stress treatment

Cucumber seeds of the Chunqiu variant were used as the plant material. The cultivation conditions were maintained at 22°C during the day and 18°C at night, with a relative humidity of 80% to promote optimal incubation growth. The plants at the three-leaf stage were treated with 100 μM ABA, 20% PEG-6000, or 200 mM NaCl for 3 or 6 hours, respectively. Cucumber plants at the three-leaf stage were also subjected to heat stress at 42°C. Samples were collected at 0, 3, and 6 hours after these treatments. For cold stress, the cultivators of plants at the three-leaf stage were incubated at 6°C, with samples collected after 0, 2, 6, and 12 hours.

2.9. RNA isolation, cDNA synthesis, and quantitative real-time PCR analysis

Total RNA was extracted from the samples using the FreeZol Reagent (Vazyme, China) according to the manufacturer's instructions. cDNA was synthesized with the HiScript Q RT SuperMix for qPCR (provided by + gDNA Wiper) using 1000 ng of RNA as the template. For fluorescence quantitative analysis, the ChamQ Blue Universal SYBR qPCR Master Mix (Vazyme, China) was utilized. Primers were synthesized by Shanghai Shenggong Company using an online primer design tool (see Table S3). The Cs-Actin gene (Csa6G484600) was used as the reference gene. The reaction system had a total volume of 10 μL, comprising 2 μL of cDNA, 5 μL of SYBR qPCR Master Mix, 0.4 μL of 10 μM forward and reverse primers, and 2.6 μL of distilled deionized water. qRT-PCR analysis was conducted using the Bio-Rad CFX Connect™ real-time fluorescence quantitative PCR instrument. The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with three independent biological replicates.

3. Results

3.1. Identification of CBL and CIPK genes in 9 genomes

Based on the conserved domains, the *CBL* and *CIPK* gene families of *Cucumis sativus* (7 *CBL*s and 19 *CIPK*s), *Citrullus lanatus* (7 *CBL*s and 20 *CIPK*s), *Cucumis melo* (6 *CBL*s and 20 *CIPK*s), *Cucurbita moschata* (9 *CBL*s and 31 *CIPK*s), *Cucurbita pepo* (10 *CBL*s and 32 *CIPK*s), *Cucurbita argyrosperma* (10 *CBL*s and 31 *CIPK*s), *Benincasa hispida* (7 *CBL*s and 20 *CIPK*s), *Lagenaria siceraria* (5 *CBL*s and 19 *CIPK*s), and *Cucurbita maxima* (8 *CBL*s and 31 *CIPK*s), were examined. The complete sequences of *CsCBL*s and *CsCIPK*s in *C. sativus* were thoroughly analyzed. The CsCBL proteins varied from 212 aa for CsCBL5 to 245 aa for CsCBL1. The CsCIPKs ranged from 270 aa for CsCIPK12 to 639 aa for CsCIPK11. The molecular weights (MW) of the CsCBLs ranged from 24451.81 Da to 28304.48 Da, and their isoelectric points (pI) were between 4.57 and 5.21 (Table 1), indicating high similarity among CsCBLs. Additionally, the MWs of the CsCIPKs varied from 30968.56 Da to 72868.03 Da, and their pIs fell between 5.66 and 9.31 (Table 1). The isoelectric points (pIs) of all the CsCBLs were less than 7, while 84% (16/19) of CsCIPKs had a pI greater than 7. The variations in lengths, MWs, and pI values suggest that the functions of CBLs and CIPKs are highly complex and may involve multiple processes. The sequences of the 69 CBL and 223 CIPK proteins in 9 Cucurbitaceae species were noted in supplementary documents (Doc S1 and Doc S2).

Table 1
CBL and CIPK family genes detected in cucumber.

Name	Gene ID	Chr	aa	CDS	Exons	pI	MW (Da)
CsCBL1	CsaV3_2G025590.1	2	245	9	9	4.73	28304.5
CsCBL2	CsaV3_3G002200.1	3	213	9	9	4.57	24533.9
CsCBL3	CsaV3_3G003870.1	3	223	8	8	4.73	25655.2
CsCBL4	CsaV3_3G008040.1	3	219	8	8	5.21	24741.3
CsCBL5	CsaV3_3G019930.1	3	212	9	9	4.76	24531
CsCBL6	CsaV3_3G049770.1	3	213	8	8	4.69	24451.8
CsCBL7	CsaV3_6G048120.1	6	226	9	10	4.73	25882.3
CsCIPK1	CsaV3_1G028820.1	1	430	1	1	9.07	48505
CsCIPK2	CsaV3_1G030280.1	1	356	15	15	5.66	40558.4
CsCIPK3	CsaV3_1G033260.1	1	463	2	2	9.21	52400.1
CsCIPK4	CsaV3_1G036820.1	1	464	16	16	9.1	52053.8
CsCIPK5	CsaV3_2G003670.1	2	433	1	2	9.1	48687.2
CsCIPK6	CsaV3_2G013250.1	2	430	1	1	9.13	47876.3
CsCIPK7	CsaV3_2G030480.1	2	446	14	14	7.56	50850.6
CsCIPK8	CsaV3_3G007670.1	3	441	14	14	8.34	50076.2
CsCIPK9	CsaV3_3G012720.1	3	433	1	1	9.31	48595.3
CsCIPK10	CsaV3_3G015590.1	3	442	12	12	5.87	49579.4
CsCIPK11	CsaV3_4G017300.1	4	639	20	20	7.26	72868
CsCIPK12	CsaV3_4G027060.1	4	270	1	1	7.81	30968.6
CsCIPK13	CsaV3_4G036630.1	4	443	1	1	8.6	50289
CsCIPK14	CsaV3_6G000230.1	6	436	12	12	6.05	49378.7
CsCIPK15	CsaV3_6G000240.1	6	431	12	12	8.33	49029.3
CsCIPK16	CsaV3_6G019060.1	6	408	1	4	8.37	46143.7
CsCIPK17	CsaV3_6G019070.1	6	424	1	1	8.74	48144.5
CsCIPK18	CsaV3_6G050960.1	6	467	1	1	8	53011.1

Notes: Chr, Chromosome; aa, number of amino acids; CDS, number of coding sequences; Exons, number of exons; pI, isoelectric point; MW, molecular weight.

Name	Gene ID	Chr	aa	CDS	Exons	pI	MW (Da)
CsCIPK19	CsaV3_6G051970.1	6	454	15	15	9	51169.1

Notes: Chr, Chromosome; aa, number of amino acids; CDS, number of coding sequences; Exons, number of exons; pI, isoelectric point; MW, molecular weight.

3.2. Chromosomal locations and gene duplication events of CsCBLs and CsCIPKs

Based on the available location data, all the *CsCBL*s and *CsCIPK*s were mapped on the chromosomes (Fig. 1), revealing a comprehensive distribution across chromosomes 1 to 7 (Table 1). Chromosome 3 exhibited the most concentrated group with the largest number of 5 *CsCBL*s, followed by Chromosomes 2 and 6, each with only one *CsCBL* gene. Additionally, 19 *CsCIPK* genes were unevenly distributed among the five chromosomes, excluding chromosomes 5 and 7. Chromosome 6 contained the largest number of 6 *CsCIPK*s, while chromosome 1 had 4 *CsCIPK*s. Furthermore, chromosomes 2, 3, and 4 each had three *CsCIPK*s. Notably, no *CsCBL*s or *CsCIPK*s were found on chromosomes 5 and 7. These findings indicate a random distribution of the *CsCBL*s and *CsCIPK*s among the different chromosomes in cucumber.

Furthermore, a syntenic analysis was conducted to observe the duplication events in *CsCBL* and *CsCIPK* gene families. We identified only one segmental duplication event (*CsCBL*2/6) in the *CsCBL* genes, indicating that the *CsCBL*s were not mainly generated from duplication events. In the *CsCIPK* family, we found 3 pairs of segmental duplications (*CsCIPK*1/13, *CsCIPK*3/18, *CsCIPK*6/9) and 2 pairs of tandem duplications (*CsCIPK*14/15, *CsCIPK*16/17), suggesting that both segmental and tandem duplication events contributed to the expansion and evolution of *CsCIPK*s. The analysis of duplications indicated that some gene pairs in the intron-poor clade likely arose from segmental duplication events.

The Ka (nonsynonymous) and Ks (synonymous) values were calculated to verify whether the gene duplication was under evolutionary selection pressure. The results showed that the Ka/Ks values of the duplicated genes ranged from 0.07 to 0.20 (Table S1), indicating that these genes had undergone purified selection during the evolutionary process.

3.3. Phylogenetic analysis of the CBL and CIPK gene families in Cucumis sativus

To study the evolutionary relationships among predicted CBL and CIPK proteins in *Cucumis sativus* and other plant species, we collected potential family members from *Arabidopsis thaliana* and 9 Cucurbitaceae species, which include *Cucumis sativus*, *Citrullus lanatus*, *Cucumis melo*, *Cucurbita moschata*, *Cucurbita pepo*, *Cucurbita argyrosperma*, *Benincasa hispida*, *Lagenaria siceraria*, and *Cucurbita maxima*. Using the full-length sequences of the 79 predicted CBL and 249 predicted CIPK proteins, a maximum likelihood (ML) phylogenetic tree was constructed for each (Fig. 2). CBLs varied from 5 in *Lagenaria siceraria* to 10 in *Arabidopsis thaliana*, *Cucurbita pepo*, and *Cucurbita argyrosperma*.

In contrast, CIPKs ranged from 19 in *Cucumis sativus* and *Lagenaria siceraria* to 32 in *Cucurbita pepo*. The results revealed that all the CBLs and CIPKs were categorized into four and five groups, respectively. Among the seven CsCBLs (CBLs in *Cucumis sativus*), CsCBL4 and CsCBL5 were placed in Group Ⅰ, CsCBL1 in Group Ⅱ, CsCBL7 and CsCBL3 in Group III, and CsCBL2 and CsCBL6 in Group IV (Fig. 2a). Moreover, all the CsCBLs had counterparts in the other species, signifying the conservation of the CBL genes. The original phylogenetic trees of CBL proteins in ten species can be found in Fig. S1.

The CsCIPK proteins are clustered into 5 groups (Fig. 2b). CsCIPK2, CsCIPK4, CsCIPK7, CsCIPK8, CsCIPK10, CsCIPK11, CsCIPK14, CsCIPK15, and CsCIPK19 are in group Ⅰ, CsCIPK13 is in group Ⅱ, CsCIPK1 and CsCIPK5 are in group Ⅲ, CsCIPK3, CsCIPK12, and CsCIPK16 are in group Ⅳ, CsCIPK6, CsCIPK9, CsCIPK17 and CsCIPK18 are in group Ⅴ. Additionally, the CsCIPK proteins in groups Ⅰ, Ⅲ, Ⅳ, and Ⅴ are clustered together as intron-poor CIPK proteins. While the CsCIPK proteins in group Ⅱ are intron-rich CIPKs. The result suggests that the acquisition and loss of introns have played a significant role in the evolution of the CIPK gene family. The original phylogenetic tree of CIPK proteins in ten species can be found in Fig. S2.

3.4. Gene structure and domain analysis of CsCBL and CsCIPK proteins

Multiple sequence alignment results indicated that the sequences of CsCBLs are highly conserved (Fig. S3). All CsCBLs contain four EF-hands, similar to CBLs in Arabidopsis. The conserved acidic amino acids Glu (E) and Asp (D) in the EF-hands may play a significant role in binding Ca^{2+} (Sanchez-Barrena, Fujii, et al., 2007). Our research identified a conserved N-terminal myristoylation site (MGXXXSK/T) in four CsCBL proteins (CsCBL2, CsCBL4, CsCBL5, and CsCBL6) as well, which may be involved in membrane targeting of CBL-CIPK complexes and protein stability. Additionally, all seven CsCBLs have a typical palmitoylation site at the N-terminal, essential for ABA responses (Fig. 3S). Notably, CsCBL2, CsCBL4, CsCBL5, and CsCBL6 exhibit a conserved glycine (G) followed by a cysteine (C) residue as a structural feature (MGCXXSK/T), indicating that dual fatty acyl modification might play a role in plasma membrane targeting of CBL-CIPK complexes (Batistic, Sorek, et al., 2008). Furthermore, all CsCBL proteins, except CsCBL1, contain a highly conserved PFPF motif at the C-terminal, potentially responsible for the phosphorylation of CBLs by CIPKs. As shown in Fig. S4, CsCIPK proteins, except CsCIPK2, CsCIPK12 and CsCIPK16 have an ATP-binding site and an activation loop at the N-terminal. Our analysis revealed the presence of NAF and PPI motifs in the conserved C-terminal of all CsCIPKs, which are known to mediate interaction with CsCBLs.

To analyze the similarity and diversity of motif compositions among different CsCBLs and CsCIPKs, all identified CBL and CIPK proteins were used to predict putative motifs using the online MEME program. Five conserved motifs, except for motif 6, were found in all seven CsCBLs. Motif 6 in the N-terminal of CsCBL2, CsCBL6, CsCBL4 and CsCBL5 contained a sequence of MGCXXS/T (Fig. 3b and Fig. S5a). Seven to nine introns were found in CsCBLs, suggesting relatively conserved structures (Fig. 3c). In CsCIPKs, the numbers of introns varied from 0 to 19, dividing them into two major groups: intron-rich and

intron-poor. CsCIPK2, CsCIPK4, CsCIPK7, CsCIPK8, CsCIPK10, CsCIPK11, CsCIPK14, CsCIPK15 and CsCIPK19 clustered in the intron-rich group, while CsCIPK1, CsCIPK3, CsCIPK5, CsCIPK6, CsCIPK9, CsCIPK12, CsCIPK13, CsCIPK16, CsCIPK17 and CsCIPK18 formed the intron-poor group. Additionally, 13 motifs were identified in CsCIPKs. Among them, motif 8 contained a conserved NAF domain, and motif 9 contained a conserved PPI domain (Fig. 3).

3.5. Synteny analysis and the evolution of CsCBLs and CsCIPKs

A comparison of the gene arrangement in *C. sativus* with that of *A. thaliana*, *O. sativa*, *C. maxima*, and *C. lanatus* was conducted to demonstrate the evolution of CBL and CIPK genes (Fig. 4). We found that 7 *CsCBL*s in *C. sativus* were similar to *CBL*s in *A. thaliana* (8), *O. sativa* (2), *C. maxima* (14), and *C. lanatus* (10). Similarly, 26, 13, 42, and 26 *CIPK*s in *A. thaliana*, *O. sativa*, *C. maxima*, and *C. lanatus*, respectively, showed similarity with 19 *CsCIPK*s in *C. sativus*. It's important to note that some *CsCBL*s and *CsCIPK*s had more than one similar gene pair in the other species. For example, CsaV3_3G049770.1 (*CsCBL6*) on chromosome 6 was associated with AT4G17615.3 and AT5G47100.1 in *Arabidopsis*.

The analysis revealed that some *CsCBL*s and *CsCIPK*s did not have syntenic gene pairs in other species. Figure 4 shows two syntenic gene pairs, such as CsaV3_2G030480.1 (*CsCIPK7*)/CmaCh05G005610.1 and CsaV3_2G030480.1 (*CsCIPK7*)/CmaCh12G001760.1, which were found between *C. sativus* and *C. maxima*. However, no syntenic gene pairs related to *CsCIPK7* were found in *C. sativus-A. thaliana* and *C. sativus-O. sativa*. These results suggest that the *CBL*s and *CIPK*s between *C. sativus* and *C. maxima* have a higher homology. Interestingly, CsCIPK15 (CsaV3_6G000240.1) did not have any orthologous pairs with genes in other species, indicating that it might have originated from its tandem repeated gene *CsCIPK14* (CsaV3_6G000230.1). The syntenic analysis showed that CsCBLs except for CsCBL7 had no orthologous gene pairs in *O. sativa*, indicating that CsCBLs except for CsCBL7 formed after the divergence of dicotyledonous and monocotyledonous plants (Table S2). The syntenic analysis suggests that the acquisition and loss of orthologous gene pairs may play significant roles in the evolution and divergence of species.

3.6. Tissue-Specific Expression of the CBL and CIPK genes in cucumber

For the analysis of expression patterns of *CsCBLs* and *CsCIPKs* in different tissues, we examined the expression of *CsCBL*s and *CsCIPK*s in 10 tissues: leaf, tendril (base part), tendril, unexpanded ovary, root, fertilized expanded ovary, unfertilized expanded ovary, male flower, female flower, and roots. Figure 5 shows that some Cs*CBL*s and Cs*CIPK*s had similar expression levels across the ten tissues, while others showed tissue-specific expression patterns. This suggests that CsCBLs and CsCIPKs function differently in various tissues during growth and development. *CsCIPK1*, *CsCIPK11*, *CsCIPK12*, and *CsCBL3* were highly expressed in all tissues, while *CsCIPK14* was not expressed in any tissues. This suggests that the loss of function may be associated with gene duplication events in the evolution of gene families.

Notably, *CsCBL3, CsCIPK1*, *CsCIPK11* and *CsCIPK13* showed higher expressions, while *CsCBL4*, *CsCBL5*, *CsCBL6*, *CsCIPK9* and *CsCIPK15* were not expressed in male flowers, indicating that these gene expression differences may have a role in male flower development. Additionally, *CsCBL5* and *CsCBL6* showed higher expression levels in female flowers than in male flowers, suggesting their involvement in fruit formation. Furthermore, *CsCIPK13* was specifically expressed in roots, indicating its role in root development. We analyzed the transcriptome data to understand the root's expression patterns further. *CsCBL3*, *CsCIPK1*, *CsCIPK4* and *CsCIPK11* exhibited high expression levels, while *CsCIPK3* and *CsCIPK9* showed low expression levels in different root zones. Notably, certain genes exhibited tissue-specific expression in different root zones. For instance, *CsCIPK5* and *CsCIPK11* demonstrated significantly higher expression in the differentiation zone (Fig. 5b), indicating their potential involvement in root development.

3.7. Responses of Cucumber CBL and CIPK genes to GA

To analyze the expression pattern of *CsCBL*s and *CsCIPK*s under GA treatment, the transcriptome sequencing data (PRJNA376073) was utilized to draw a heatmap (Fig. 6). The results revealed that *CsCIPK9* and *CsCIPK14* showed no expression, while *CsCIPK1* and *CsCIPK5* exhibited high expression levels. These genes were not influenced by GA treatment. However, *CsCBL6* and *CsCIPK3* showed a significant increase in expression after 12 h of GA treatment.

3.8. Expression of CsCBL and CsCIPK genes under abiotic stresses

To investigate the involvement of *CsCBL* and *CsCIPK* in response to abiotic stress, we examined the expression profiles of their genes under salt and silicon stress (PRJNA477930), low-temperature stress (PRJNA438923), high-temperature stress (PRJNA634519), and waterlogging stress (PRJNA678740). Our analysis revealed that the expression levels of *CsCIPK1*, *CsCIPK6*, and *CsCIPK15* increased under salt and silicon stresses, while *CsCIPK5* and *CsCIPK12* were only up-regulated under silicon stress (Fig. 7a). Additionally, under low-temperature stress for 12 h, the expressions of *CsCIPK5* and *CsCIPK9* were notably inscreased, whereas *CsCIPK1* and *CsCIPK6* transcription levels reduced compared to the control. However, no effects were observed on *CsCBL*s and *CsCIPK*s with low-temperature treatment for 2 h and 6 h (Fig. 7b). During high-temperature conditions for 6 h, four genes (*CsCIPK3*, *CsCIPK5*, *CsCIPK13*, and *CsCIPK15*) were significantly down-regulated, while *CsCBL4* was up-regulated (Fig. 7c). Under waterlogging stress, the transcription levels of seven genes (*CsCBL5*, *CsCBL6*, *CsCIPK1*, *CsCIPK9*, *CsCIPK10*, *CsCIPK11*, *CsCIPK12*, *CsCIPK15*, and *CsCIPK19*) decreased in both resistant and sensitive materials. However, the expression levels of *CsCBL4*, *CsCIPK8*, and *CsCIPK13* were significantly increased in both resistant and sensitive materials. *CsCIPK5* and *CsCIPK18* were only downregulated in the sensitive material, while *CsCIPK8* was only downregulated in the resistant plants. *CsCIPK16* and *CsCBL1* were only up-regulated in the sensitive material (Fig. 7d).

3.9. Expression of CsCBL and CsCIPK genes under biotic stresses

To analyze the expression patterns of *CsCBL*s and *CsCIPK*s under biotic stresses, the published transcriptome sequencing data of root-knot nematodes (PRJNA419665), powdery mildew (PRJNA321023), downy mildew (PRJNA285071), and *Fusarium wilt* (PRJNA472169), were utilized to create expression heatmaps. Compared to the control, the expression level of *CsCIPK15* was increased in both the sensitive and resistant varieties under the root-knot nematode stress. *CsCIPK13* was up-regulated in the sensitive plants under the root-knot nematode treatment (Fig. 8a). When subjected to powdery mildew treatment, *CsCIPK17* and *CsCBL2* were down-regulated only in the sensitive material, while*CsCIPK1* was down-regulated only in the resistant material. Both sensitive and resistant varieties displayed downregulation of *CsCBL1*. Furthermore, the expression levels of *CsCIPK3* and *CsCIPK18* were increased only in the sensitive and resistant varieties, respectively (Fig. 8b). With the onset of downy mildew infection, 4 genes were downregulated in resistant and sensitive varieties: *CsCBL1*, *CsCIPK3*, *CsCIPK8*, and *CsCIPK16*. However, *CsCIPK5*, *CsCIPK10*, and *CsCIPK6* were

downregulated only in resistant varieties, and *CsCIPK11* was downregulated only in sensitive varieties. Conversely, the transcription levels of *CsCIPK15* and *CsCIPK19* were increased in both sensitive and resistant varieties. When experiencing downy mildew stress, *CsCIPK1* and *CsCBL2* were up-regulated only in resistant varieties, while *CsCIPK17*, *CsCBL4*, and *CsCBL6* were up-regulated only in sensitive varieties (Fig. 8c). In comparison to the control, the expression levels of *CsCBL6*, *CsCIPK3*, and *CsCIPK12* were increased, and the expression levels of *CsCIPK9* and *CsCIPK19* were reduced under *Fusarium wilt* stress (Fig. 8d).

3.10. Relative expression analysis of CsCBL and CsCIPK genes under abiotic stresses and ABA treatment

To evaluate the relative expression levels of the *CsCBL* and *CsCIPK* genes under various abiotic stresses and hormone treatments, such as NaCl, PEG, high temperature, low temperature, and ABA, we selected one *CsCBL* gene and seven *CsCIPK* genes that were identified as responsive to these stresses in RNA-seq analysis. The results from qRT-PCR expression analysis revealed that these genes displayed differential expression in response to abiotic stresses and ABA treatment. Under 20% NaCl, *CsCBL4*, *CsCIPK5*, *CsCIPK11*, and *CsCIPK13* were downregulated at 3 h and upregulated at 6 h. In contrast, *CsCIPK1* was upregulated at 3 h and downregulated at 6 h. Additionally, *CsCIPK16* was downregulated at all time points (Fig. 9a). As shown in Fig. 9b, the expression levels of *CsCBL4*, *CsCIPK1*, *CsCIPK5*, and *CsCIPK15* decreased at all time points. In contrast, the expression level of *CsCIPK13* increased at 3 h and then decreased at 6 h. Furthermore, the expression of *CsCIPK16* decreased at 3 h but increased at 6 h. In response to the treatment with 200 mmol/L NaCl (Fig. 9c), five selected genes—except for *CsCIPK11* and *CsCIPK16*—showed increased expression at 3 h, followed by a decrease after 6 h of treatment. *CsCIPK16* was downregulated at all time points, while CsCIPK11 was upregulated at all time

points. During cold stress, *CsCIPK5* displayed an increasing trend, whereas *CsCIPK1* showed a decreasing trend. These findings align with the RNA-seq results. *CsCIPK11* and *CsCIPK16* had the highest expression levels at 6 h, and *CsCBL4* reached an expression peak at 2 h (Fig. 9d). After exposure to hot stress, *CsCIPK1*, *CsCIPK11*, *CsCIPK13*, and *CsCIPK15* exhibited a significant decreasing trend, while *CsCIPK5* showed an increasing trend. Notably, the expression levels of *CsCBL4* and *CsCIPK16* were highly upregulated at the 3-hour mark, then decreased by the 6-hour mark (Fig. 9e). These results were also confirmed by RNA-seq analysis during the hot treatment. Together, these findings indicate that the CBL and CIPK genes are involved in the response to abiotic stresses and in ABA signaling pathways.

4. Discussion

As Ca^{2+} sensors, CBLs, and CIPKs are involved in responses to biotic and abiotic stresses. With the publication of plant genome sequences, *CBL-CIPK* gene families have been identified in various plants, but little is known about Cucurbitaceae species. Using bioinformatic methods, our study identified 69 CBL and 223 CIPK proteins in 9 Cucurbitaceae species. Additionally, we conducted further analysis using 7 *CsCBL*s and 19 *CsCIPK*s in cucumber. The cucumber has fewer *CBL* and *CIPK* genes compared to many other species. For instance, Arabidopsis has 10 *AtCBL*s and 26 *AtCIPK*s (Kolukisaoglu, Weinl, et al., 2004), rice has 10 *OsCBL*s and 33 *OsCIPK*s (Kanwar, Sanyal, et al., 2014), grapevine has 8 *VvCBL*s and 20 *VvCIPK*s (Xi, Liu, et al., 2017), and canola has 7 *BnaCBL*s and 23 *BnaCIPK*s (Zhang, Yang, et al., 2014). The variation in gene numbers between different species may be due to gene duplication events and the loss of functional genes during evolution (Jain, Tyagi, et al., 2006). According to the gene balance hypothesis, the ratio of CBLs and CIPKs should be maintained at N□N to prevent stoichiometric differences (Zhang, Li, et al., 2020). Duplication events with gene loss have altered the pattern of gene family numbers. Our study revealed that the cucumber has a similar gene number ratio (719□) of *CBL*□*CIPK* as peach (8:18) (Qiu, Pan, et al., 2022) and grape (820□) (Xi, Liu, et al., 2017), suggesting that *CBL* genes may have undergone significant gene loss events after duplication events.

The analysis of gene locations showed that the *CsCBL*s and *CsCIPK*s are unevenly distributed on the chromosomes, which could be linked to genome evolution and genetic variation. Intra-species collinearity analysis revealed both segmental and tandem duplications in the *CsCIPK* family and only one segmental duplication event in the *CsCBL* family. This suggests that segmental and tandem duplications have expanded gene families. The phenomenon can also be observed in numerous other plant gene families (Yang, Li, et al., 2023, Yang, Koo, et al., 2012, Zhu, Chen, et al., 2016). A previous study suggested that positive selection plays a role in collecting and spreading new mutations, while negative selection helps remove harmful mutations and prevents the emergence of new functions (Starr, Jameson, et al., 2003). The Ka/Ks ratios of the duplication events among the *CsCBL*s and *CsCIPK*s showed that they were all under negative selection, indicating that the *CsCBL*s and *CsCIPK*s are functionally conserved.

The seven *CsCBL*s were found to have four conserved EF-hands, which combine with *CIPK*s. Similarly to the *CBL*s in rice (*Oryza sativa*) (Gu, Ma, et al., 2008), four CsCBL proteins (CsCBL2, CsCBL4, CsCBL5, and CsCBL6) have an N-terminal myristoylation site. This site may contribute to Ca^{2+} signal transduction, membrane targeting, and protein-protein interactions. In foxtail millet, the mutation of the N-

myristoylation site of CBL changed the location of the CBL-CIPK complex from the plasma membrane to the cytoplasm. It disrupted its function in salt stress tolerance as well (Zhang, Linghu, et al., 2017). Compared with the CBLs in Arabidopsis, all seven CsCBLs have a typical palmitoylation site at the N-terminal, which may contribute to abiotic stress responses (Hrabak, Chan, et al., 2003). The conserved gene structure of *CBL* genes indicates that they may have a conserved function in transforming Ca^{2+} signals (Schlicker, Domingues, et al., 2006).

Analysis of sequence alignment revealed that all the CsCIPKs contain an NAF motif and a PPI domain. A conserved NAF motif is crucial for the interaction of CIPKs with CBLs. When CBLs interact with the NAF motif located at the C-terminal of CIPKs, they activate the CIPKs to participate in Ca^{2+} signal transduction. Moreover, all CsCIPKs, except for CsCIPK2, CsCIPK12, and CsCIPK16, possess an ATP-binding domain that may have specialized functions.

The analysis of phylogenetic relationships revealed that the CsCBLs and CsCIPKs were divided into four and five groups. This finding was consistent with previous studies in grape (Xi, Liu, et al., 2017) and pepper (Ma, Gai, et al., 2019). The number of introns and the organization of exons and introns may have played a role in the evolution of gene families. The analysis of gene structure showed that the *CsCBL*s had 7 to 9 introns, indicating that the structures and functions of *CsCBL*s were conserved. Interestingly, the gene structures divided the *CIPK*s into two groups: an intron-rich group (group I) and an intron-poor group (groups II, III, IV, and V). The results were consistent with previous studies in Populus (Yu, Xia, et al., 2007) and quinoa (Xiaolin, Baoqiang, et al., 2022). It was observed that the intron-poor genes emerged after the intron-rich genes, possibly due to intron reinsertion during the reverse transcription process. The loss of introns occurred faster than the gain during duplication events, suggesting that the intron-rich *CIPK*s might have higher similarity with the original genes. The phylogenetic analysis showed that the intron-poor groups were clustered into one clade. This indicates that the intron-poor genes shared a higher sequence similarity and might have evolved from the intron-rich members. These results propose that intron deletion events may have contributed to the evolution of the *CIPK* family (Zhu, Chen, et al., 2016). Furthermore, several studies indicate that the expansion of intron-poor *CIPK*s may contribute to the response to abiotic stresses (Ye, Xia, et al., 2013, Zhu, Chen, et al., 2016). It has been observed that most *CIPK*s, particularly those in the intron-poor clade, are stress-responsive genes (Zhu, Chen, et al., 2016, Zhu, Fan, et al., 2022). For instance, *CaCIPK3*, an intron-poor gene in pepper, is involved in various stress responses such as drought and salt (Ma, Li, et al., 2021). Our research discovered that all intron-poor *CsCIPK*s, except *CsCIPK6*, were either upregulated or downregulated under waterlogging (Fig. 7d). This suggests that the intron-poor *CIPK*s play a role in the response to waterlogging stress. The collinearity analysis of the *CBL* and *CIPK* families in cucumber and four other species (Fig. 4) revealed fewer orthologous gene pairs between *C. sativus* and *O. sativa* than between *C. sativus* and *C. maxima*. This suggests that the *CBL* and *CIPK* families have accumulated more mutations over evolution and share fewer conserved fragments. As species evolved, the differences in the *CBL* and *CIPK* families increased, which may have led to the development of new functions (Paixão, Suárez, et al., 2021).

The diversity and specificity of the expression patterns suggest that the CBLs, CIPKs, or CBL-CIPK complexes may play roles in growth and development, as well as responses to (a)biotic stresses. Previous studies reported that *AtCIPK19* is a key regulator of pollen tube tip growth and is specifically exhibited in pollen grains and pollen tubes (Zhou, Lan, et al., 2015). A similar expression pattern was also observed in its ortholog gene *CsCIPK18* which was highly expressed in male flower, suggesting that the ortholog genes in different species might share the same function. *AtCIPK25*, by interacting with *AtCBL4* and *AtCBL5*, displayed strong expression in the flower and root (Kumar Meena, Kumar Vishwakarma, et al., 2018). *SlCIPK2* was specifically expressed in tomato flower organs, indicating its participation in flower development (Yuasa, Ishibashi, et al., 2012). This study utilized published transcriptome data to analyze the differential expression patterns of cucumber *CBL*s and *CIPK*s. *CsCIPK1*, *CsCIPK11*, *CsCIPK12*, and *CsCBL3* showed high expression in 10 tissues, while *CsCIPK9* had low expression. Additionally, some genes exhibited tissue-specific expression patterns. For instance, *CsCIPK1* displayed higher expression in female and male flowers, suggesting a potential role in flower formation. *CsCIPK13* was highly expressed in the root, suggesting a potential role in root development. To better understand the functions of CBLs and CIPKs in root growth and development, we examined the expression levels of *CsCBL*s and *CsCIPK*s at different stages of root development. The results indicated that *CsCBL3*, *CsCIPK1*, and *CsCIPK11* were highly expressed in all three zones, suggesting their significant role in root development. Furthermore, *CsCIPK1*, *CsCIPK5*, *CsCIPK11*, *CsCIPK13*, and *CsCIPK17* showed high expression in the root differentiation zone, indicating their potential involvement in root growth.

Many *CBL* and *CIPK* genes have been found to play important roles in signal transduction and biosynthesis. For example, AtCBL1 interacts with AtCIPK15 to regulate *ABI* genes in ABA signaling (Guo, Xiong, et al., 2002). AtCIPK26 positively regulates ABA signaling by phosphorylating ABI5 (Lyzenga, Liu, et al., 2013). In apples, SINA1/2 integrating with MdCIPK20 dynamically regulates GA signaling and participates in GA-inhibited anthocyanin biosynthesis (An, Zhang, et al., 2023). In this study, *CsCBL6* and *CsCIPK3* were found to be down-regulated when treated with GA, indicating their potential involvement in GA signaling. Except for *CsCIPK2* and *CsCIPK14*, 24 *CsCBL* and *CsCIPK* genes were shown to be regulated under both biotic and abiotic stresses, highlighting their extensive role in stress responses. *CsCBL6* and *CsCIPK1* exhibited differential expression under various stress conditions, indicating their active participation in stress responses. In the Arabidopsis root, the AtCBL4 (SOS3)-AtCIPK24 (SOS2) complex was found to export Na^+ to the extracellular space to maintain cell ion balance and enhance salt tolerance (Shi, Ishitani, et al., 2000, Yang, Chen, et al., 2009). However, our results showed that the similar genes *CsCBL5* (homologous to *AtCBL4*) and *CsCIPK2* (homologous to *AtCIPK24*) did not display the same expression pattern under salt stress treatment. This suggests that these homologous genes may not function similarly during species evolution (Du, Yang, et al., 2021). The duplicated gene pairs, such as *CsCBL2/6, CsCIPK1/13, CsCIPK3/18, CsCIPK6/9, CsCIPK14/15* and *CsCIPK16/17*, showed an imbalanced expression pattern in different tissues as well as in response to various stresses, suggesting that the duplicated genes might undergo a divergent evolutionary process. Interestingly, *CsCIPK14* showed no expression in any tissue or under biotic and abiotic stresses, suggesting a potential loss of function related to gene duplication events in the evolutionary process of gene families.

Previous study showed that overexpression of *AtCIPK6* gene in Arabidopsis increased plant tolerance to salt and drought stresses (Chen, Wang, et al., 2013). The similar expression pattern was also found in its orthologous gene *CsCIPK1*, which was highly expressed in response to salt and drought stresses at 3 h. This finding indicated that *CsCIPK1* might have the similar function in salinity and drought tolerance. Notably, the expression levels of *CsCIPK1* and *CsCIPK15* increased in drought and salt stress treatments, but reduced under ABA treatment, suggesting that they positively participated in salt and drought stress related to response in ABA signal pathway (Fig. 9a, 9b, 9c). Additionally, the transcripts of *CsCIPK5*, *CsCIPK11*, *CsCIPK13* and *CsCIPK16* were highly accumulated under cold stress, predicating their positive role in response to cold tolerance (Fig. 9d). The divergency of expression levels in CsCBLs and CsCIPKs revealed that they might play various regulatory roles in response to different stress treatments.

5. CONCLUSION

The CBLs, by targeting the CIPKs, have been implicated in plant development and responses to multiple (a)biotic stresses. We identified 69 CBL and 223 CIPK proteins in 9 Cucurbitaceae species. To better understand the CBL and CIPK genes in cucumber, we used 7 CBLs and 19 CIPKs in cucumber for further study. All these genes were unevenly anchored on 5 chromosomes, except chromosomes 5 and 7. The phylogenetic analyses showed that the CBL and CIPK families were divided into four and five groups, respectively. The syntenic analysis revealed that the CBL and CIPK gene families underwent gene duplication events in cucumber. The expression heatmaps showed that the cucumber CBL and CIPK genes, except for CsCIPK2 and CsCIPK14, were downregulated or upregulated under biotic and abiotic stresses. CsCBL6 and CsCIPK1 actively participated in various stress responses, suggesting they might be selected as key candidate genes for further study. In conclusion, this research lays a foundation for further studying the function of CBLs and CIPKs in cucumber, enabling us to screen candidate genes for breeding new cucumber varieties with high yield and various stress resistances.

Abbreviations

TFs: transcription factors; RNA-seq, RNA sequencing; HMM, hidden Markov model; MW, molecular weight; pI, isoelectric point; aa, amino acid; CBL, calcineurin B-like; CIPK, CBL interacting protein kinase; *C. sativus*, *Cucumis sativus*; ML, maximum-likelihood; TPM, transcripts per kilobase of exon model per million mapped reads; GA, gibberellins; qRT-PCR, quantitative real-time polymerase chain reaction.

Declarations

Author contributions

N.W., Z.S., and E.K. designed the research and finished writing the article; N.W. and Y.F. conducted the experiments; Y.L. and E.K. contributed to research data analysis. All authors have read and agreed to the published version of the manuscript.

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Data availability

The original data presented in the study are publicly available, which can be found at the NCBI database and CuGenDB database: https://www.ncbi.nlm.nih.gov/bioproject/80169; https://www.ncbi.nlm.nih.gov/bioproject/271595; https://www.ncbi.nlm.nih.gov/bioproject/376073; https://www.ncbi.nlm.nih.gov/bioproject/438923; https://www.ncbi.nlm.nih.gov/bioproject/634519; https://www.ncbi.nlm.nih.gov/bioproject/477930; https://www.ncbi.nlm.nih.gov/bioproject/678740; https://www.ncbi.nlm.nih.gov/bioproject/419665; https://www.ncbi.nlm.nih.gov/bioproject/321023; https://www.ncbi.nlm.nih.gov/bioproject/285071; https://www.ncbi.nlm.nih.gov/bioproject/472169; http://cucurbitgenomics.org/.

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Declaration of competing interest

The authors declare no conflicts of interest.

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Figures

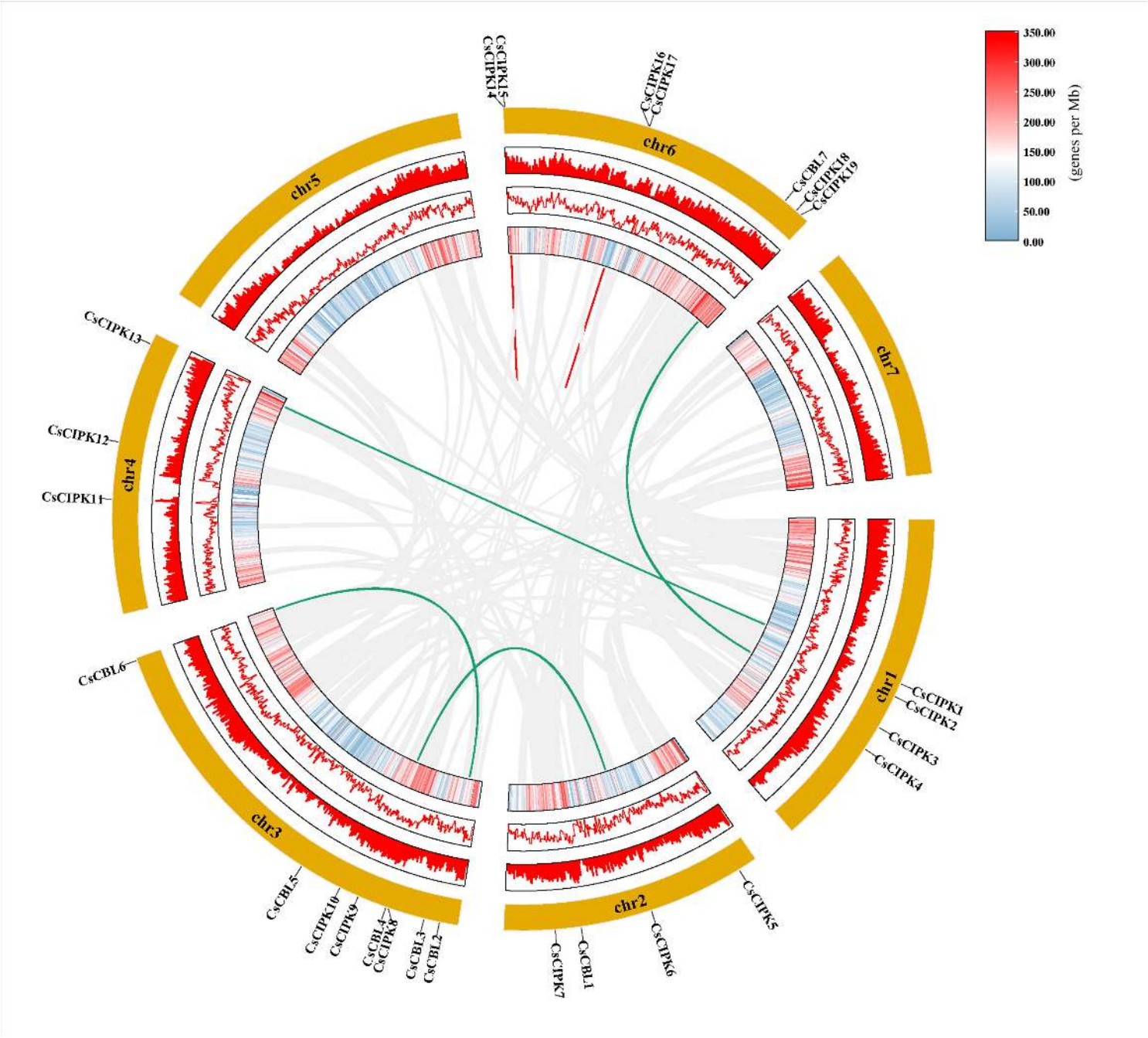


Figure 1

Chromosome locations and syntenic relationships of *CBL*s and *CIPK*s in cucumber. The gray lines indicate the segmentally duplicated pairs in cucumber. The green lines indicate segmental duplications of *CBL*s and *CIPK*s. The red lines indicate the tandem duplications of *CBL*s and *CIPK*s in cucumber. The gene density bar is shown in the upper-right corner.

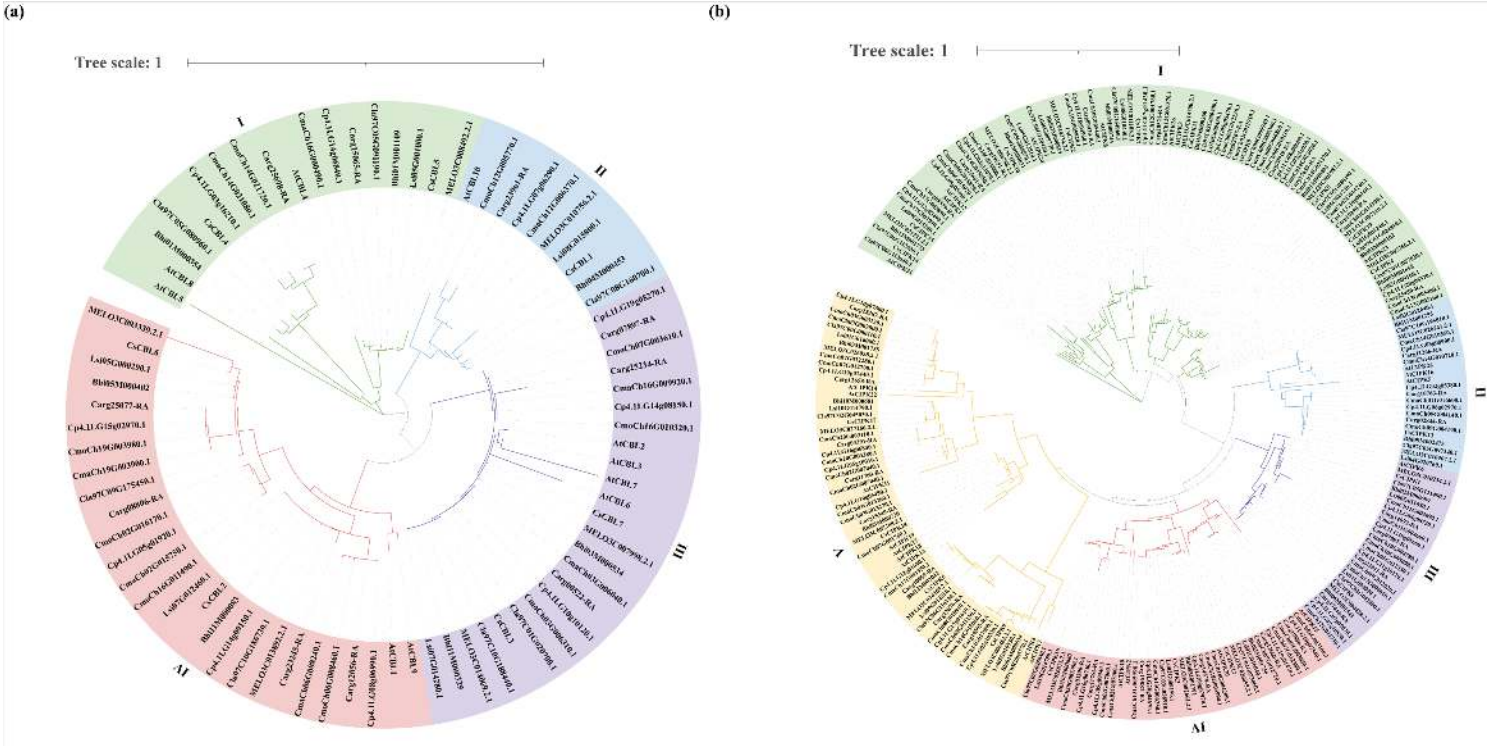


Figure 2

Phylogenetic relationships of CBLs (a) and CIPKs (b) of Arabidopsis and 9 Cucurbitaceae species. Multiple sequence alignments were carried out using MUSCLE. The phylogenetic trees were constructed using TBtools, using the maximum likelihood (ML) method with 1000 bootstrap replicates. Different groups are highlighted with different colors, respectively.

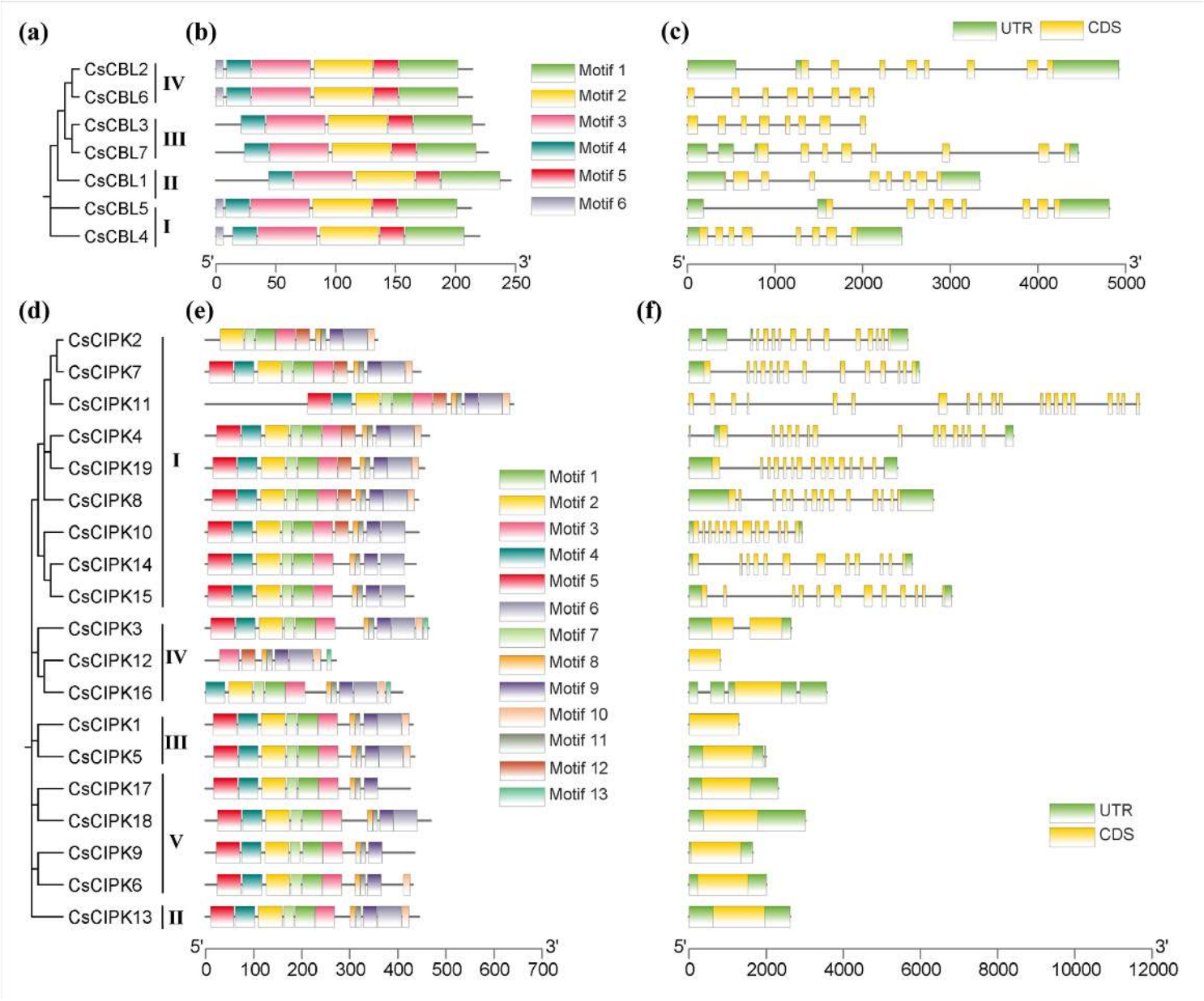


Figure 3

Phylogenetic trees, motif locations, and gene structures of CBLs and CIPKs in cucumber. (a) Phylogenetic trees of CBLs. (b) Motif composition of CBLs, with 6 conserved motifs marked in different colors. (c) Gene structure of CBLs, with black lines indicating introns, yellow boxes indicating exon regions (CDS), and green boxes indicating untranslated regions (UTR). (d) Phylogenetic trees of CIPKs. (e) Motif composition of CIPKs, with 13 conserved motifs marked in different colors. (f) Gene structure of CIPKs, with black lines indicating introns, yellow boxes indicating CDS, and green boxes indicating UTR.

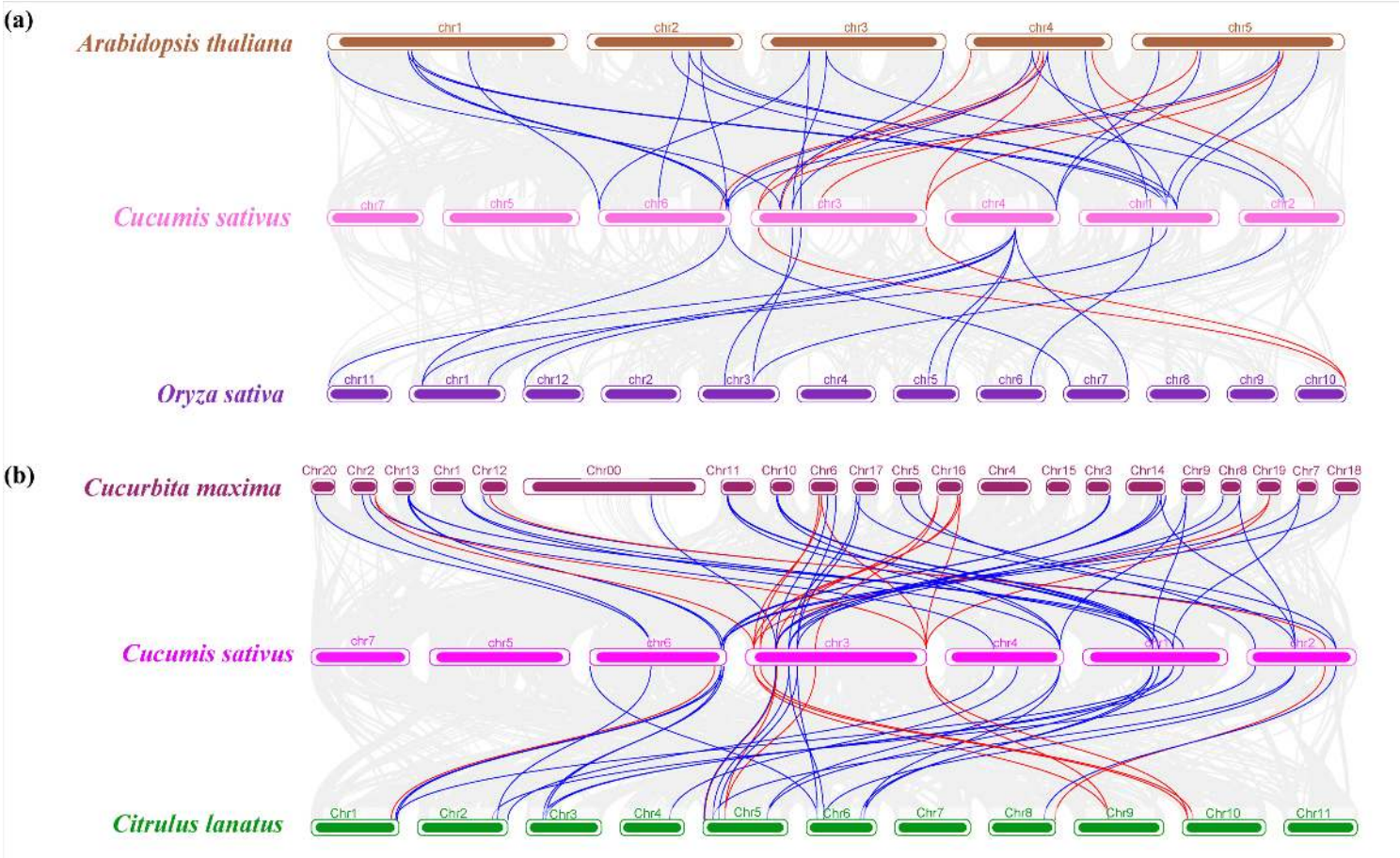


Figure 4

Synteny analysis of the *CBL* and *CIPK* gene families. Blue lines depict syntenic *CIPK*gene pairs across different species, while red lines represent syntenic *CBL*gene pairs for different species. Gray lines indicate collinear blocks for plant genomes.

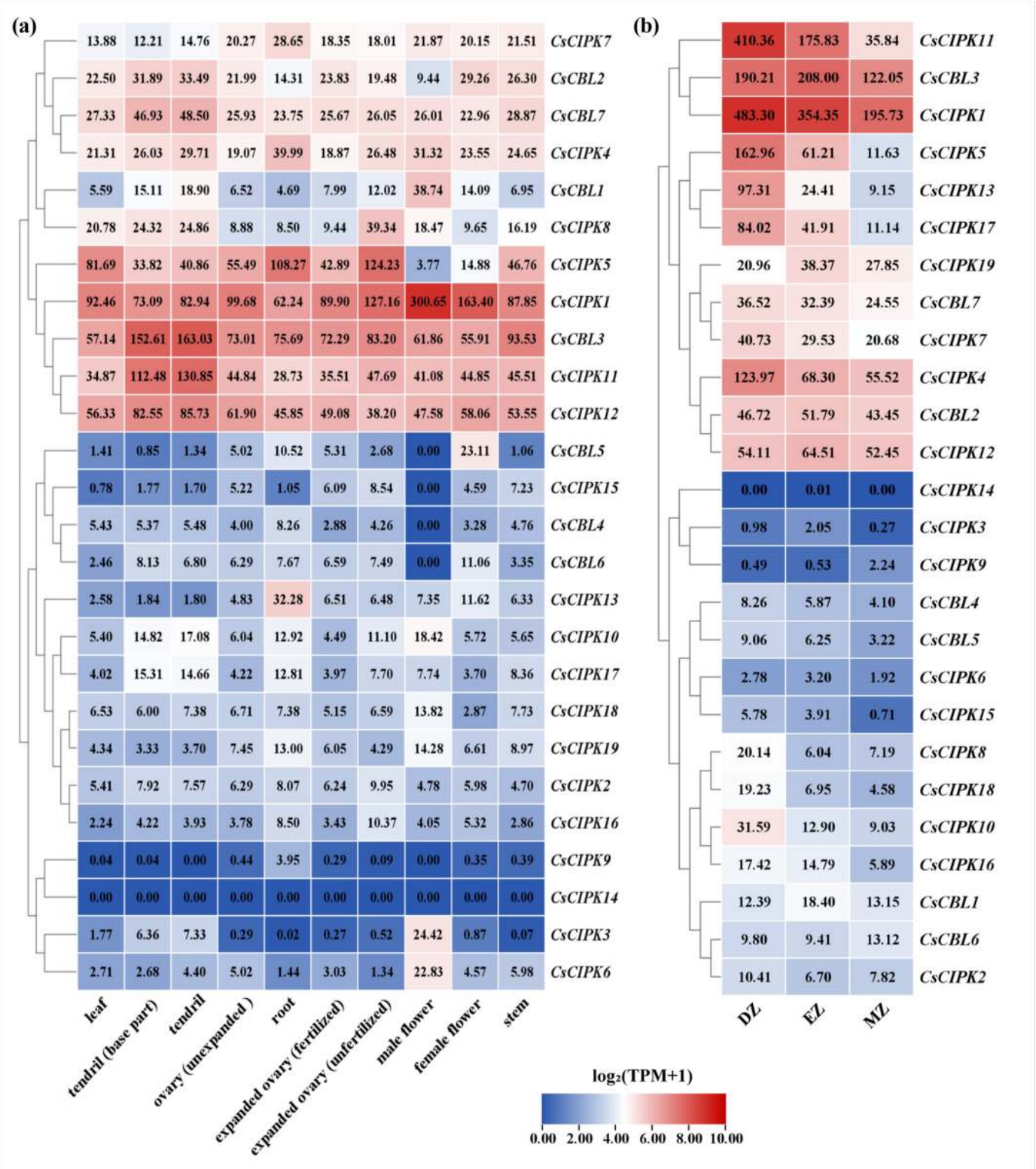


Figure 5

Expression profiles of *CsCBL*s and *CsCIPK*s in various tissues and developmental stages. (a) Heatmap showing the expression of *CsCBL*s and *CsCIPK*s in 10 different tissues. (b) Expression profiling of root development. DZ, differentiation zone; EZ, elongation zone, MZ, meristematic zone. The color bar indicates the expression level, calculated using TPM values. The values in the boxes represent the original TPM values.

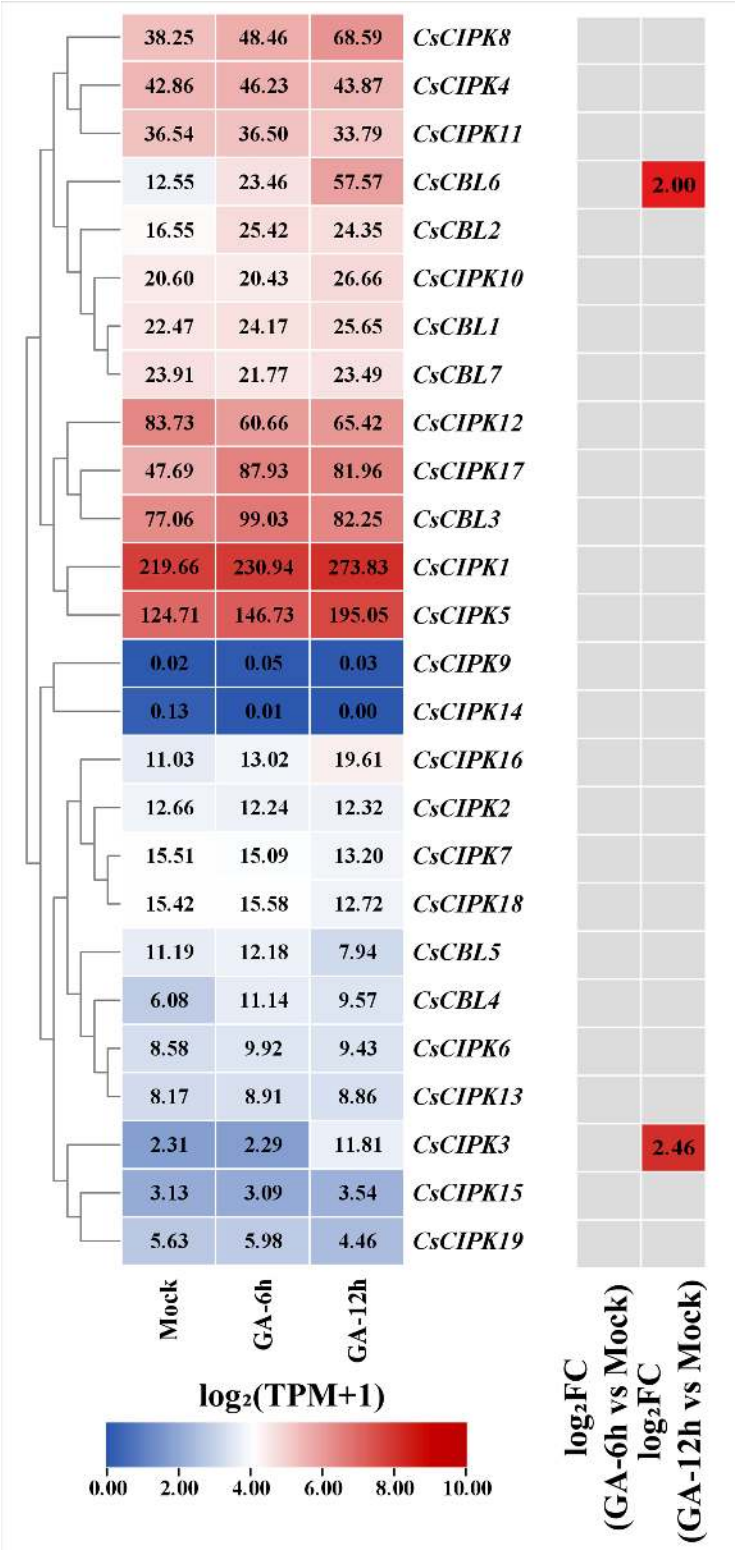


Figure 6

An expression heatmap shows the activity of *CsCBL*s and *CsCIPK*s when treated with GA. The data in the left boxes represents the original TPM values, while the data in the right boxes shows the log_2FC(fold of change) values. The up-regulated values are highlighted in red.

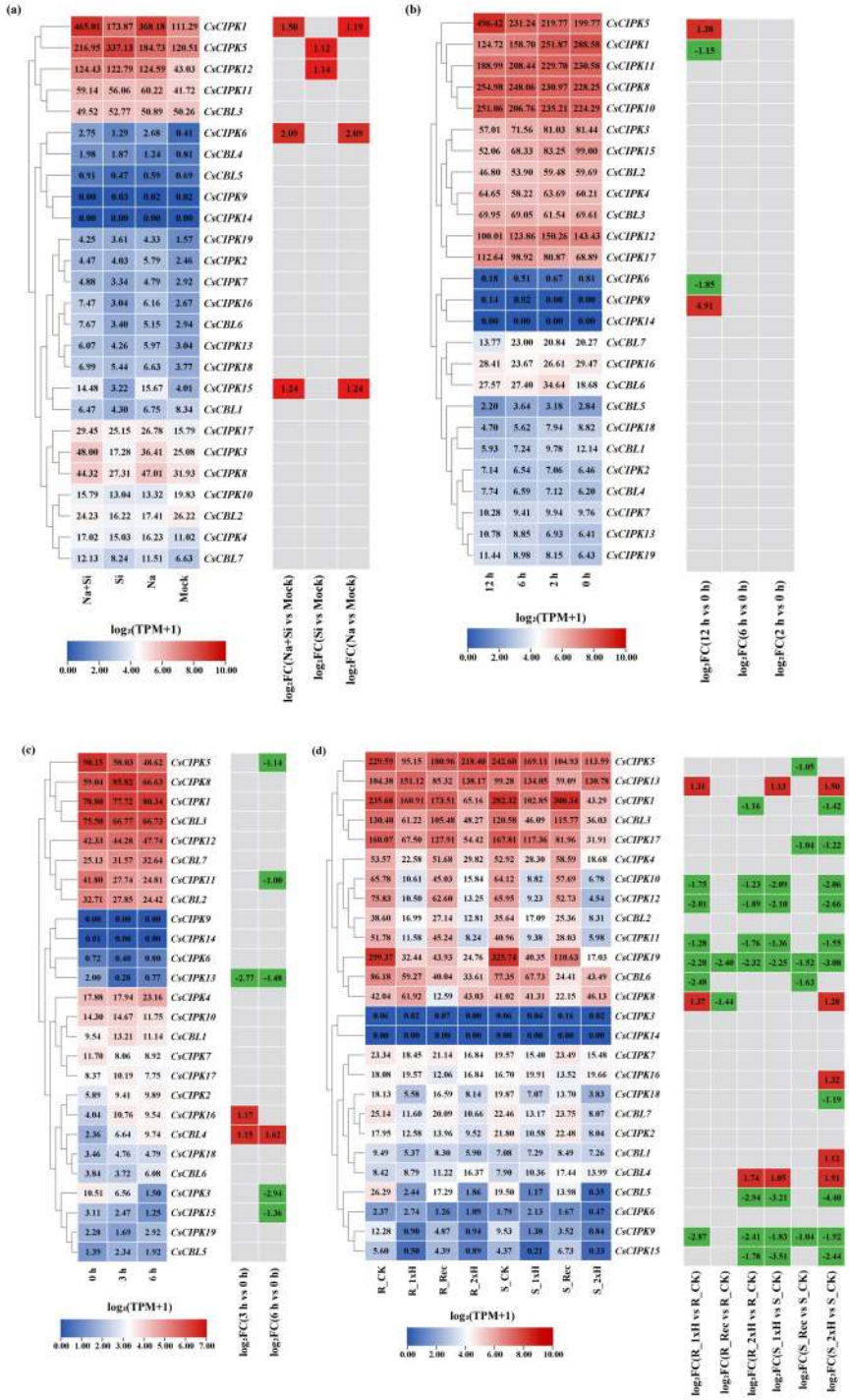


Figure 7

Expression heat maps show the activity of *CsCBL*s and *CsCIPK*s when subjected to abiotic stresses. (a) Expression patterns of *CsCBL*s and *CsCIPK*s under salt stress. Na: salt stress; Si: silicon stress; Na + Si: salt + silicon stress. (b) Expression patterns of CsCBLs and CsCIPKs under low-temperature (6 °C) stress. (c) Expression patterns of CsCBLs and CsCIPKs under high temperature (42 °C) stress. (d) Expression patterns of cucumber CBL and CIPK genes in response to waterlogging stress. S: sensitive

plant; R: resistant plant; CK: the control; 1xH: non-primed plants waterlogged for 7 days only once; Rec: plants after 7 days of waterlogging and 14 days of recovery; 2xH: primed plants waterlogged for 7 days, recovered for 14 days, then waterlogged again. In each Fig., the left boxes show the original TPM values, and the right boxes display log_2FC(fold of change) values, with up-regulated values highlighted in red and down-regulated values in green.

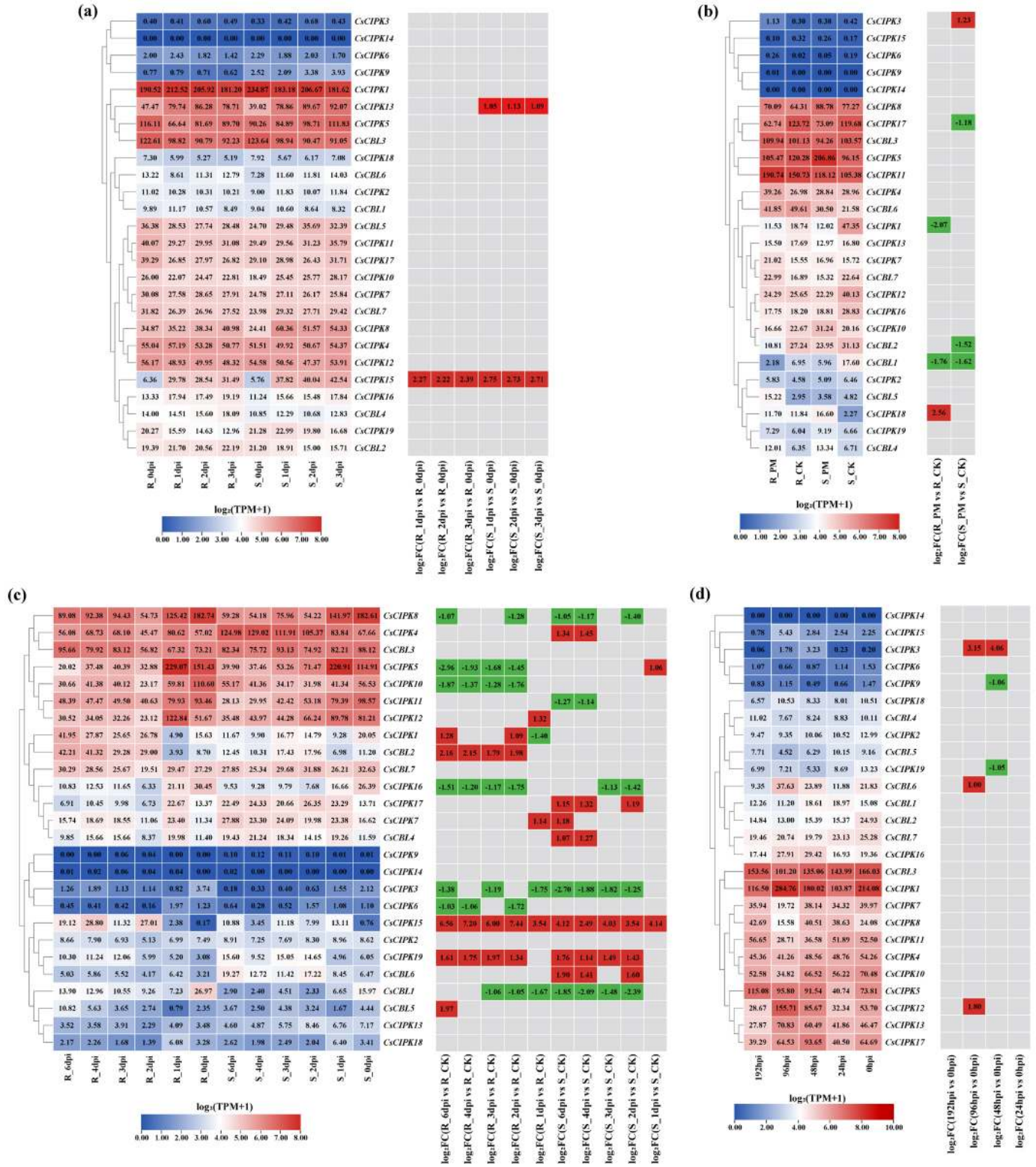


Figure 8

The expression profiles of CsCBLs and CsCIPKs under several biotic stresses. The expression patterns of genes in response to root-knot nematode (a), powdery mildew (b), downy mildew (c), and Fusarium wilt (d) are displayed. S: sensitive plant; R: resistant plant; dpi: days after inoculation; PM: powdery mildew inoculation; hpi: hours after inoculation. In each Fig., the left boxes show the original TPM values and the right boxes display log_2FC (fold of change) values, with up-regulated values highlighted in red and down-regulated values in green.

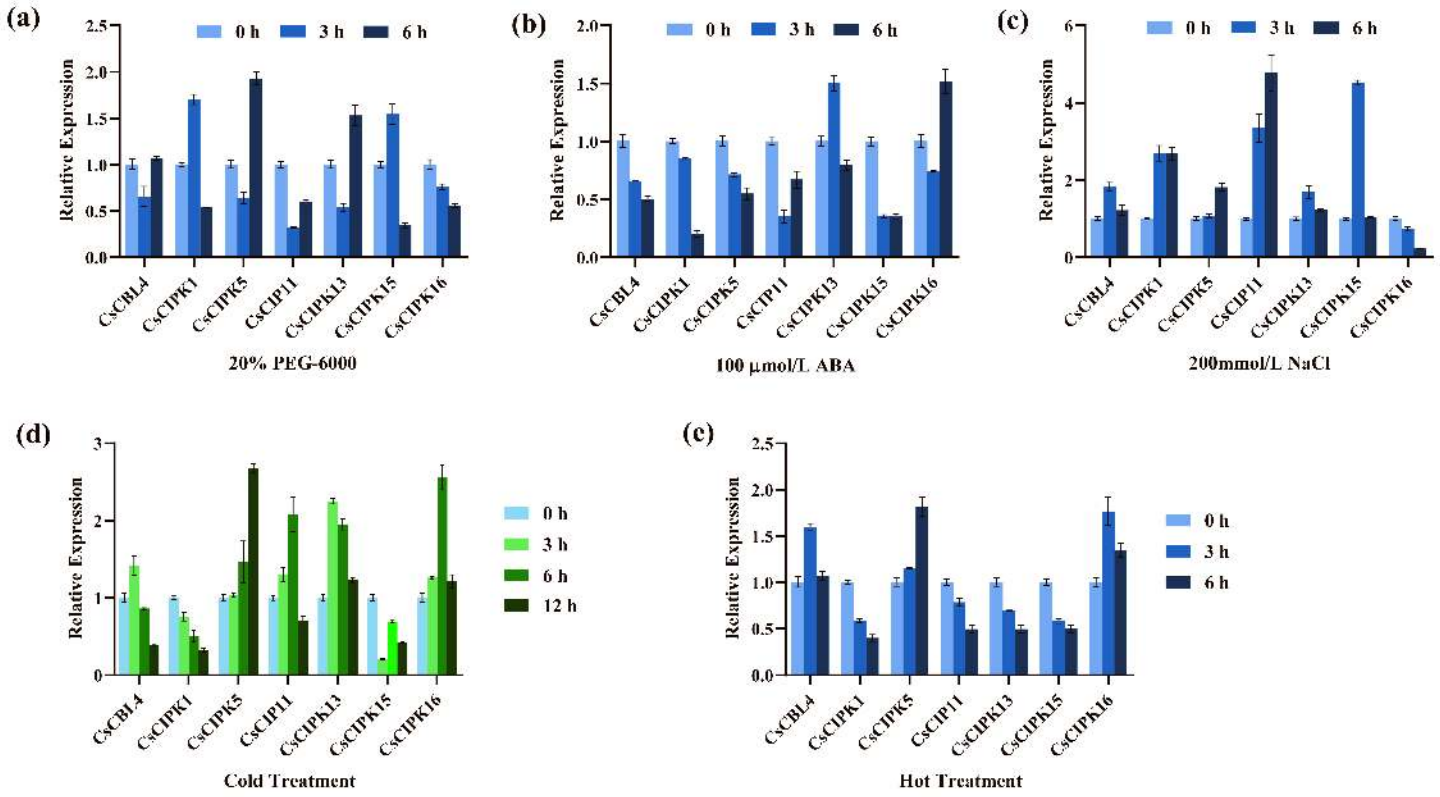


Figure 9

The qRT-PCR analysis of one *CsCBL* and six *CsCIPK* genes under 20% PEG-6000 (a), 100 μmol/L ABA (b), 200 mmol/L NaCl (c), cold stress (d), or heat stress(e). Data are represented as mean±SEM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- DocS1.Thewholelengthsequencesofthe69CBLproteinsin9Cucurbitaceaespecies.fasta
- DocS2.Thewholelengthsequencesofthe223CIPKproteinsin9Cucurbitaceaespecies.fasta
- TableS1.KaKsvaluesofthegenepairsinCsCBLandCsCIPKgenefamilies.xlsx
- TableS2.OnetooneorthologousrelationshipsofCBLandCIPKgenefamilies.xlsx
- TableS3.TheprimersofselectedCBLandCIPKgenesforqRTPCR.xlsx
- FigS1.Theoriginalphylogenetictreeof CBLproteinsin10species.tif

- FigS2.TheoriginalphylogenetictreeofCIPKproteinsin10species..tif
- FigS3.ThemultiplealignmentsofCsCBLproteins.tif
- FigS4.ThemultiplealignmentsofCsCIPKproteins.tif
- FigS5.TheconservedmotifsinCsCBLsandCsCIPKs..tif
- Supplementarydata.docx