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Evolution and comparison of the expression of *TCP* genes in the benincaseae and cucurbitae tribes

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TCP genes are plant-specific transcription factors that play essential roles in plant growth, development, metabolism, and responses to biotic and abiotic stresses. However, the roles of *TCP* genes in Cucurbitaceae species remain unknown. In this study, 111 and 119 *TCP* genes were identified in the Benincaseae (*C. melo*, *C. sativus*, *C. lanatus* and *L. siceraria*) and Cucurbitae (*C. maxima*, *C. moschata* and *C. pepo*) tribes, respectively, and were analyzed. Segmental duplication, tandem duplication, and whole-genome duplication (WGD) were identified as the major driving factors in the expansion of *TCP* genes in Cucurbitaceae species, with the majority of *TCP* genes undergoing purifying selection. Using the melon genome as a reference, an integrated map containing 29 loci across nine chromosomes was constructed, 28 of which were shared by seven Cucurbitaceae species. Gene structure analysis revealed that their function was conserved. The result of promoter sequence analysis indicated that *TCP* genes have many phytohormone-related *cis*-regulatory elements. GO term enrichment analysis showed that *TCP* genes were the major regulators of many downstream transcriptional networks and primarily functioned in the nucleus. Transcriptome analysis of different tissues and developmental stages of the Cucurbitae tribe revealed tissue-specific spatial and temporal expression patterns of *TCP* genes, suggesting that *TCP* genes play an important role in the growth and development of Cucurbitaceae. Gene expression profiling demonstrated that *TCP* genes are involved in the responses of plants to abiotic and biotic stresses. In conclusion, this is the first systematic analysis of *TCP* genes in Cucurbitaceae, which provides deeper insights into their evolutionary dynamics and functional properties, which may be crucial for the genetic improvement of Cucurbitaceae.

Keywords *TCP*, Gene family, Cucurbitaceae, Duplication, Stress

Transcription factors (TFs), also known as transacting factors, contain two functional structural domains involved in DNA binding or the activation/repression of gene expression in response to stimuli, which forms the genetic basis of phenotypic evolution^{1–3}. TFs play important roles in the growth, metabolism, and responses to environmental stresses in higher plants, and their variation (expansion and diversification) plays a central role in the evolution of key phenotypes^{4,5}. Identifying and deciphering the molecular functions of DNA-binding domains, transcriptional regulatory domains, oligomerization sites, and nuclear localization signals of key TFs enhances our understanding of signal transduction pathways and plant responses to stress. TF families regulate specific target genes involved in various processes that are crucial for plant development^{6,7}. TFs can be categorized into various families, including WRKYs (WRKYGQ), SPLs (SQUAMOSA promoter binding protein-like), NACs (NAM, ATAF, and CUC), AP 2/ERF (Apetala 2/ethylene response factor), and TCPs (TEOSINTE BRANCHED 1/CYCLO BRANCHIDEA/ PCF) based on their structural domain. The *TCP* TF family is plant-specific^{8,9}.

TCP genes are named after the four initially characterized members: TB1 (TEOSINTE BRANCHED 1) in maize, CYC (Cycloidea) in snapdragons, and PCF 1/2 (proliferating cell nuclear antigenic factors 1 and 2)¹⁰. *TCP* TFs contain a basic helix-loop-helix (bHLH) structure comprising approximately 59 amino acids at the N-terminus that form a conserved structural domain that is essential for DNA binding and is involved in protein-protein interactions and protein localization^{11,12}. *TCP* genes can be categorized into two classes: the PCF class (*TCP-P* class) and *TCP-C* class, referred to as Class I and Class II, respectively¹⁰. In addition to the different binding sequences, four amino acids that are present in the Class II structural domain are absent from the Class I structural domain. Class I can bind to the GTGGNCCC sequences and Class II binds to the GGNCCAC

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sequences¹³. Class II *TCPs* have a unique R structural domain and ECE motif, which allow them to be subdivided further into CIN and CYC/TB1 subclasses, which may be involved in protein-protein interactions^{14,15}.

TCP genes are involved in several plant growth and development processes, such as seed germination, leaf morphogenesis, flower development, lateral branch formation, circadian rhythms, hormone biosynthesis and signal transduction^{9,11,16–19}. *AtTCP14* can activate seed embryo growth and break dormancy in seeds¹⁶. Activation of *AtTCP16* regulation of microspore development using transgenic technology, affects pollen development²⁰. *SITCP12/15/18* play an important role in the regulation of fruit development and ripening in tomatoes²¹. Overexpression of *GhCYC2* leads to the acquisition of a ray-flower-like morphology in disk flowers, which is involved in defining the complex inflorescence structure of Asteraceae²². *TCP* genes are involved in the regulation of plant secondary metabolism, which plays an essential role in responses to environmental stresses, such as, salt, drought, and low temperature stress^{23–25}. The overexpression of *OsPCF2* in rice using transgenic technology can activate responses to salt stress and PEG-induced drought stress²⁶. Similarly, *PeTCP10* overexpression increases drought resistance in bamboo²⁶. Overexpression of *DgTCP1* could increase cold resistance in chrysanthemums¹². In addition, overexpression of *miR319* decreases the expression of Class II *TCP* TFs, thereby increasing salt tolerance in grasses¹¹. Furthermore, *GbTCP3/4/7* are involved in flavonoid biosynthesis in *Ginkgo biloba*²⁷.

Cucurbitaceae is one of the most genetically diverse plant families, and most of its members are vital vegetable or medicinal plants and are widely distributed globally²⁸. Members of the Cucurbitaceae family include many popular vegetables and fruits, such as melon (*C. melo*), cucumber (*C. sativus*), watermelon (*C. lanatus*), bottle gourd (*L. siceraria*), zucchini (*C. pepo*), and pumpkin (*C. maxima* or *C. moschata*)²⁹. Cucurbitaceae is commonly used as model for understanding the molecular regulation of plant tendrils and the biosynthesis of bitter compounds^{30,31}. In recent years, genome sequencing and bioinformatics, whole genome sequencing, and data sharing have developed rapidly. The first cucurbit species to be sequenced was cucumber, and with the generation of chromosome conformation capture technology and novel computational methods, the genomes of more cucurbit species have been sequenced and updated, such as *Cucumis melo*, *Cucumis sativus*, *Citrullus lanatus*, *Lagenaria siceraria*, *Cucurbita pepo*, *Cucurbita moschata*, and *Cucurbita maxima*^{32–39}. The assembly and updating of high-quality genomes enable researchers to explore gene family members and determine their biological functions.

Considering the critical role of *TCP* genes in different biological processes, *TCP* homolog genes have been explored in various plants. *TCP* genes have been identified in many species, including 24 *AtTCP* genes in *Arabidopsis*. Moreover, Parapunova et al. detected 30 *SITCP* genes in tomatoes, and 27 *CITCP* and 27 *CsTCP* genes in watermelon and cucumber, respectively^{11,40,41}. Moreover, 66 *TCP* genes in *Triticum aestivum*, and 74 *TCP* genes in *Gossypium raimondii* were identified^{42,43}. *TCP* genes emerged early in the history of land plants and expanded rapidly during the evolution of higher plants⁹. *TCP* genes in the same class or evolutionary branch generally show highly conserved gene/protein structures and expression profiles in terrestrial plants, suggesting a common origin and functional conservation. *CmTCP1* is reportedly involved in development of tendrils from lateral shoots in melon⁴⁰. *TEN* encodes a *TCP* transcription factor conserved within the cucurbits, and regulates tendril-less phenotype⁹. Interaction network predictions have indicated that this gene family tends to form protein complexes that are involved in different biological processes, such as phytohormone pathways, the cell cycle, and environmental responses⁹.

Due to their wide variation among species, the evolutionary relationships of *TCP* genes among cucurbit species are not well characterized. In this study, to improve our understanding of the evolutionary history of the *TCP* genes in Cucurbitaceae species, we used seven genomic and multiple transcriptomic datasets to reconstruct the evolutionary relationships between *TCP* genes. A total of 230 *TCP* genes were identified in cucurbit species, and their chromosomal distribution, gene structure, motif and evolutionary relationships, and promoter *cis*-regulator elements were analyzed. We found that whole-genome duplications (WGDs) play crucial roles in increasing biodiversity and function in both growth process and responses to environmental stress²⁹. To study the duplication events of inter- and intraspecific *TCP* genes in cucurbit species using gene covariance analysis, we calculated synonymous substitution (Ks) and non-synonymous substitution rates (Ka) and their ratios (Ka/Ks) to determine the time of differentiation of *TCP* gene pairs and the main forces promoting differentiation in cucurbit species. In addition, we analyzed transcriptomic datasets of *TCP* genes in Cucurbita species from different tissues and in response to various environmental stresses, and we observed that *TCP* genes were involved in plant growth, development, and responses to biotic and abiotic stresses. This study reviews the identification of *TCP* genes in Cucurbitaceae and their molecular function analysis, laying a foundation for the molecular regulation of major traits in cucurbit plants, quality improvement and plant breeding.

Materials and methods

Identification of *TCP* genes in cucurbitaceae

In order to explore the evolutionary relationships of *TCP* genes in cucurbit crops, the whole genome sequences of seven representative Cucurbitaceae species (*Cucumis melo*, *Cucumis sativus*, *Citrullus lanatus*, *Lagenaria siceraria*, *Cucurbita pepo*, *Cucurbita moschata*, and *Cucurbita maxima*) were obtained (<http://cucurbitgenomics.org/>). Based on the *TCP* protein sequences of the model plants (PF03634) (<http://pfam.sanger.ac.uk/>), the HMMER software with default parameters was used to establish a profile HMM with an *E*-value cutoff of $1e^{-10}$ to search the gene libraries of the seven Cucurbitaceae species and obtain candidate *TCP* genes. The candidate gene sequences were subjected to multiple homology comparison, redundant gene sequences were removed, and structural domains were detected using the online tools CDD (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and SMART (<http://smart.embl-heidelberg.de/>) to obtain *TCP* genes in Cucurbitaceae. Basic information on *TCP* genes (e.g., amino acid number, molecular weight, instability index, fat index, and the grand

average of hydropathicity [GRAVY] values) was obtained from cucurbit crops, using the ExPASy Proteomics Server online tool in standard mode (<http://expasy.org>).

Collinearity analysis and chromosomal location of TCP genes

To analyze the type and relative order of genes conserved among different species that diverged from a uniform ancestral type, seven Cucurbitaceae species (*C. melo*, *C. sativus*, *C. lanatus*, *L. siceraria*, *C. maxima*, *C. moschata* and *C. pepo*) were subjected to covariance analysis using TBtools software⁴⁴. To measure collinearity among these species of Cucurbitaceae and between melon and other Cucurbitaceae species (*C. sativus*, *C. lanatus*, *L. siceraria*, *C. maxima*, *C. moschata* and *C. pepo*), Circos and collinearity plots were constructed using the default parameters of the TBtools multiple synteny plots. In addition, tandem and segmental duplication events among *TCP* genes were detected using TBtools and MCScanX with the *e*-value set to 1.0×10^{-10} ^{44,45}. Using Mapchat software under standard model conditions, the *TCP* gene location information for the seven species (*Cucumis melo* L. cv. DHL92, *Cucumis sativus* L. var. *sativus* var. 9930, *Citrullus lanatus* subsp. *vulgaris* cv. 97103, *Lagenaria siceraria* var. *USVL1VR-Ls*, *Cucurbita pepo* subsp. *pepo*, *Cucurbita moschata* var. *Rifu*, *Cucurbita maxima* var. *Rimu*), obtained from the Cucurbitaceae Genome Website (<http://cucurbitgenomics.org>), was visualized.

Phylogeny tree construction and multiple sequence alignment analysis

Multiple sequence comparisons of the full-length TCP protein sequences from *Cucumis melo*, *Cucumis sativus*, *Citrullus lanatus*, *Lagenaria siceraria*, *Cucurbita pepo*, *Cucurbita moschata* and *Cucurbita maxima* were performed in MEGA 11 using ClustalW with default pairwise settings. A phylogenetic tree was constructed using the Maximum Likelihood (ML) method in the MEGA 11 software with 1000 bootstrap replicates. Evolutionary trees were generated using the iTOL (<https://itol.embl.de/>) website⁴⁶. TCP genes were categorized into groups based on the TCP conserved structural domains belonging to Cucurbitaceae species. TCP conserved structural domains were analyzed by amino acid sequence comparison using DNAMAN software, under standard conditions.

Gene structure analysis of TCP genes

TCP gene sequences were extracted from the location information of genes in the genome, and the *TCP* gene structures in Cucurbitaceae species were analyzed using the Gene Structure Display Server (GSDS) (<https://gsds.gao-lab.org/index.php>) online tool⁴⁷. The Basic-Helix I-Loop-Helix II structures of the *TCP* genes were analyzed in MEGA 11 using ClustalW.

TCP gene pairs and divergence time Estimation

TCP gene collinearity analysis and visualization were performed using the Tbtools software⁴⁴. Briefly, collinearity, CTL and .gff files were generated using the One Step MCScanX-Super Fast module, gene pair files were generated from the covariance files using the file merge module of MCScanX, *TCP* gene pairs were generated using the Text Block Extraction and Filtering module. The protein sequences and open reading frames (ORFs) of the *TCP* gene pairs were compared using ClustalW in the MEGA 11 software. The CODEML program was used to calculate the synonymous substitution rate (Ks) and non-synonymous substitution rates (Ka) of PAL2NAL (<http://www.bork.embl.de/pal2nal/>).

Gene ontology and promoter cis-regulator element analysis

GO (Gene Ontology) enrichment analysis of *TCP* genes in the Benincaseae tribe (*C. melo*, *C. sativus*, *C. lanatus* and *L. siceraria*) and Cucurbitae tribe (*C. maxima*, *C. moschata* and *C. pepo*) was performed using the agriGo online tool (<http://systemsbiology.cau.edu.cn/agriGOv2/>) with default parameters, and the terms are presented using a Venn plot (<https://jvenn.toulouse.inrae.fr/app/example.html>). Sequence similarity was used to predict function, sequence alignment was used to predict homology, *E*-values and bit scores were used to filter low-quality homology matches, GO annotation terms associated with proteins involved in well-known biological processes were used to classify function, and GO terms with *P* values ≤ 0.05 were considered highly representative. Sequences within 2000 bp upstream of the ATG promoter in *TCP* genes were examined and searched for in the Cucurbitaceae genome. *Cis*-regulator elements were identified using the PlantCARE online tool (<http://bioinformatics.psb.ugent.be/webtools/plantcare>), and *cis*-regulator elements were statistically analyzed and visualized using heat maps constructed in R.

Transcript expression analysis of TCP TFs

Using published RNA-seq data, the expression patterns of *CmTCP* genes were analyzed in fruit, root, female flower, male flower and leaf in melon⁴⁸. Expression patterns of *CsTCP* genes were analyzed in the root, stem, leaf, tendril base, tendril, female flower, male flower, ovary, fertilized ovary and unfertilized ovary in cucumber⁴⁹. Expression profiling of *CITCP* genes was conducted in multiple watermelon tissues, including root, leaf, FF10 (Fruit flesh 10 days after pollination), FF18, FF26, FF34, FR10 (Fruit rind at 10 days after pollination), FR18, FR26 and FR34 in watermelon^{23,29}. The expression patterns of *LsTCP* genes were analyzed in root, flower, leaf, fruit and stem in bottle gourd⁵⁰. Expression profiling of *TCP* genes was conducted in *C. maxima* and *C. moschata* tissues, including leaf, root, fruit and stem³⁸. The expression patterns of *CpTCP* genes were analyzed in leaf, fruit at 5d (days after pollination), fruit at 10d, fruit at 15d, fruit at 20d, fruit at 40d, seed at 5d, seed at 10d, seed at 15d, seed at 20d, and seed at 40d in *C. pepo*^{38,39}. *TCP* gene expression data analysis was performed using the free online platform OmicShare Tools (<https://www.omicshare.com/tools>). Dynamic mountain range maps were used to visualize gene tissue expression specificity, with the height of the mountain range indicating the number of genes, and the degree of smooth extension indicating gene expression.

Transcriptome data of Cucurbitaceae *TCP* gene expression in response to different environmental stressors were obtained from publicly available transcriptome databases, downloaded from the Cucurbit Expression Atlas

(<http://cucurbitgenomics.org/rnaseq/home>), and analyzed to identify genome-wide differentially expressed genes (DEGs) before and after cold^{14,51}, salt^{51,52}, PM (Powdery mildew)^{53–55}, and DM (Downy mildew)^{56,57} stress. The FDR (or *P*-value) and Log₂ (Fold-change) values published in the literature were used to identify DEGs. If the gene ID was inconsistent with that of the reference genome, the *TCP* gene sequence was aligned to the reference genome to determine the gene ID. The ComplexHeatmap showing the expression of *TCP* genes was plotted using the R language software. The transcript abundance of *TCP* genes was calculated as the number of exon model reads per kilobase per million mapped reads (RPKM). For transcriptome analyses of *TCP* genes in response to abiotic and biotic stresses, a threshold of FDR values (or *P*-value) ≤ 0.05 and $|\text{Log}_2(\text{Fold-change})| \geq 1$ were used to define DEGs.

Results

Identification and basic information of *TCP* genes in Cucurbitaceae species

TCP genes were identified in seven cucurbit genomes using HMMER, including *C. melo*, *C. sativus*, *C. lanatus*, *L. siceraria*, *C. pepo*, *C. moschata* and *C. maxima* (Supplemental Table 1). A total of 230 *TCP* genes were identified and predicted using the CDD and SMART online tools, which verified the existence of the following numbers of unique *TCP* domains in each species: 29 in *C. melo*, 27 in *C. sativus*, 27 in *C. lanatus*, 28 in *L. siceraria*, 42 in *C. maxima*, 42 in *C. moschata*, and 35 in *C. pepo* (Supplemental Table 1). All identified *TCP* genes were renamed according to their locations on the chromosome (Supplemental Fig. 1 and Supplemental Table 2). The *TCP* genes were widely distributed throughout the genome, but were not uniformly distributed between chromosomes. (Supplemental Fig. 1). Basic information on the *TCP* genes is summarized in Table 1. The results showed that the lengths of the *TCP* proteins ranged from 61 (CmTCP4) to 631 (CpTCP25) amino acid residues. The molecular weights of *TCP* proteins ranged from 7.11 (CmTCP4) to 69.83 (CpTCP25) kDa, and the PIs ranged from 5.27 (LsTCP26) to 10.07 (CmoTCP11) in Cucurbitaceae species (Supplemental Table 2). The theoretical PI was < 7 for acidic proteins and > 7 for basic proteins, there were four acidic proteins in *C. melo*, seven in *C. sativus*, six in *C. lanatus*, nine in *L. siceraria*, ten in *C. maxima*, six in *C. moschata*, and four in *C. pepo* (Supplemental Table 2). The instability index was less than 40 for stable proteins, and most *TCP* genes encode stable proteins, except for two *TCP* genes in *C. melo*, two in *C. sativus*, one in *C. lanatus*, three in *C. maxima*, and one in *C. moschata*. The higher the aliphatic index, the better the stability of the protein, and the more suitable they are to function in different environments. GRAVY score was negative for hydrophilic proteins, and all *TCP* genes encoded hydrophilic proteins (Supplemental Table 2).

Gene structure and collinearity analysis of *TCP* genes

To further validate the 230 *TCP* genes found in the Cucurbitaceae species, we performed multiple sequence alignments on the *TCP* domains of all *TCP* genes to comprehend the phylogenetic relationships of the *TCP* genes in each species. *TCP* domain comparison and phylogenetic analysis showed that *TCP* proteins were divided into two classes: Class I (blue) and Class II (red and orange), Class II was further divided into the subclasses CYC/TB 1 and CIN (Supplemental Fig. 2). *TCP* genes belong to a protein superfamily, which distinguishes them from other gene families. Although most *TCP* genes have an integral basic helix-loop-helix structure, a partial absence of the basic structural sequence was present in CsTCP1, LsTCP5/6, CmaTCP24, and CpTCP14, which was also found in other species. The results of the gene structure analysis showed that most *TCP* genes in the Benincaseae tribe contained one or two CDS regions, and a few contained three CDS regions, including CsTCP4/18/27 and LsTCP3/4/13/24. Although most *TCP* genes in the Cucurbitae tribe contained one or two CDS regions, others contained more CDS regions; for example, CmaTCP41 and CpTCP21 have seven CDS regions, CmoTCP33 had six, and CpTCP7 have five (Supplemental Fig. 2).

To further explore the relationships among the *TCP* genes across different species, duplication events in the seven species (*C. melo*, *C. sativus*, *C. lanatus*, *L. siceraria*, *C. pepo*, *C. moschata*, and *C. maxima*) were investigated (Fig. 1). The results revealed 55 segmental duplication events in the Benincaseae (*C. melo*, *C. sativus*, *C. lanatus*, and *L. siceraria*) (Fig. 1A and Supplemental Table 3). Furthermore, 15, 13, 12, and 15 collinear gene pairs were identified in *C. melo*, *C. sativus*, *C. lanatus*, and *L. siceraria*, respectively (Fig. 1A and Supplemental Table 3). More segmental duplication events were identified in the Cucurbitae tribe (*C. pepo*, *C. moschata* and *C. maxima*), and 36, 38, and 26 collinear gene pairs were observed in *C. maxima*, *C. moschata* and *C. pepo*, most of which were observed between sub-genomes (Fig. 1B and Supplemental Table 3). Our findings suggest that segmental duplication plays a crucial role in the expansion of *TCP* genes.

Species	No. of Genes	No. of Chr.	No. of amino acids	Molecular weight	Theoretical PI	Total number of atoms	Instability index	Aliphatic index	GRAVY
<i>Cucumis melo</i> L. cv. DHL92	29	9	61–600	7115.16–65469.2	5.62–10.06	1003–9044	32.81–69.82	52.62–87.87	-0.709
<i>Cucumis sativus</i> L. var. <i>sativus</i> var. 9930	27	5	174–449	18912.21–48972.23	5.57–9.92	2625–6811	30.25–73.29	56.62–75.82	-0.743
<i>Citrullus lanatus</i> subsp. <i>vulgaris</i> cv. 97,103	27	9	182–517	20185.62–53272.36	5.42–9.92	2801–7296	37.49–79.31	55.65–87.7	-0.667
<i>Lagenaria siceraria</i> var. USVL1VR-Ls	28	10	171–593	18805.01–66231.24	5.27–9.92	2618–9080	40.22–75.62	55.83–75.82	-0.609
<i>Cucurbita maxima</i> var. <i>Rimu</i>	42	15	148–562	16357.49–61742.57	5.6–9.91	2277–8497	37.53–72.94	49.83–85.39	-0.762
<i>Cucurbita moschata</i> var. <i>Rifu</i>	42	15	156–567	17666.23–62692.86	5.97–10.07	2491–8615	39.3–76.57	52.78–87.05	-0.931
<i>Cucurbita pepo</i> subsp. <i>pepo</i>	35	15	127–631	13460.35–69832.92	5.43–9.91	1900–9606	40.7–77.47	50.17–86.65	-1.072

Table 1. Numbers and characteristic properties of *TCP* genes in Cucurbitaceae species.

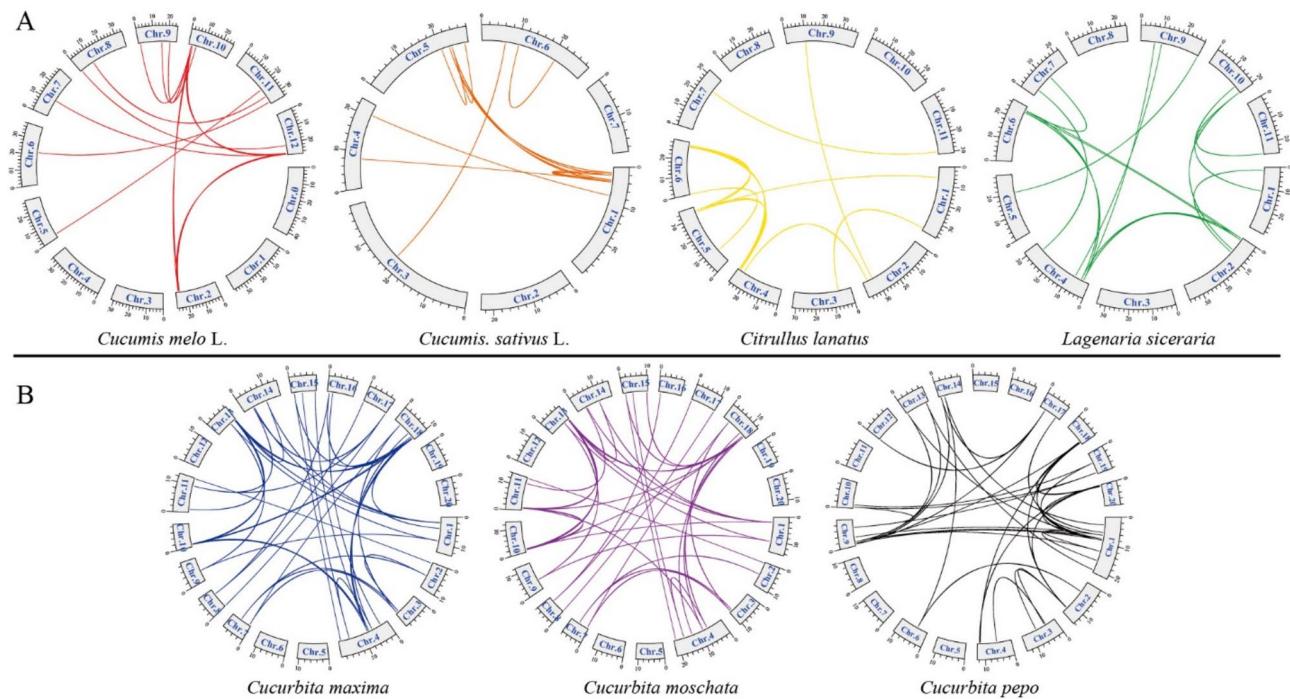


Fig. 1. Collinearity analysis of segmental repeats of *TCP* genes in seven representative species. The red lines represent 15 segmental duplicates of 29 *CmTCP* genes. The orange lines represent 13 segmental duplicates of 27 *CsTCP* genes. The yellow lines represent 12 segmental duplicates of 27 *CITCP* genes. The green lines represent 15 segmental duplicates of 28 *LsTCP* genes. The blue lines represent 36 segmental duplicates of 42 *CmaTCP* genes. The purple lines represent 38 segmental duplicates of 42 *CmoTCP* genes. The black lines represent 26 segmental duplicates of 35 *CpTCP* genes. The detailed information is listed in Supplemental table 3.

Construction of the *TCP* gene integration map

Melon, cucumber, and watermelon are horticultural crops that are grown worldwide. Using the melon genome as a reference, collinearity analysis revealed a similar number of collinear regions in both genomes (*C. sativus* and *C. lanatus*) (Supplemental Table 4). Watermelon and bottle gourds were closely related, and collinearity analysis revealed approximately 53 collinear gene pairs between melon and bottle gourds (Supplemental Table 4). More collinear genes were detected in the Cucurbita tribe when using melon as the reference genome. In total, 81, 77, and 61 collinear gene pairs were detected in *C. maxima*, *C. moschata*, and *C. pepo*, respectively (Supplemental Table 4).

Cucurbit species have 20 chromosomes, 19 of which can be divided into two sub-genomes, A and B, except for chromosome 4, which consists of two sub-genome A chromosomes and one sub-genome B chromosome³⁸. The number of *TCP* genes retained in both subgenomes was similar, which was consistent with similar evolutionary proportions of genes lost or gained in both subgenomes after polyploidization in cucurbit species (Fig. 2). Modern structural studies of chromosomes in Cucurbitaceae species indicate that their genome is an ancestral Cucurbitaceae karyotype consisting of 12 protochromosomes³⁷. To investigate the conserved loci of *TCP* genes on chromosomes, we constructed an integrated genetic map containing 29 *TCP* loci, using the melon genome as a reference (Fig. 2). Most of the loci were common to all species, except for the eighth locus at the end of chromosome 6 (Fig. 2). Interestingly, some loci were shared by different genes of the same species; for example, the first locus contained one *CmTCP*, three *CsTCP*, three *CITCP*, three *LsTCP*, four *CmaTCP*, three *CmoTCP*, and four *CpTCP* (Supplemental Fig. 3).

Analysis of phyletic evolution and duplication events in *TCP* genes

To explore the evolutionary relationships among the 230 *TCP* proteins in Cucurbitaceae, we constructed an ML evolutionary tree based on *TCP* structural domain comparisons using MEGA11. The 230 *TCP* proteins were categorized into Class I and Class II, and no species-specific branching was observed, suggesting that the classification and function of the *TCP* genes were conserved (Fig. 3). The number of proteins in Class II (129 members) was slightly larger than that in Class I (101 members), and Class II was further divided into CIN and CY/TB 1 branches, with the CIN branch (76 members) being larger than the CY/TB 1 branch (53 members). All investigated cucurbits had CIN branch *TCP* genes, and *TCP* genes in the CYC branch were also present in the seven cucurbit species, but at a lower frequency than the other branch (Fig. 3). In our phylogenetic tree, the *TCP* proteins from each species were clustered in a single sub-branch, indicating that they expanded after diverging from a common ancestor. For example, *TCP* proteins from melons were clustered in Class I with 13 members and in the CIN branch with 10 members (Fig. 3). Phylogenetic analyses suggested that each gene classification in

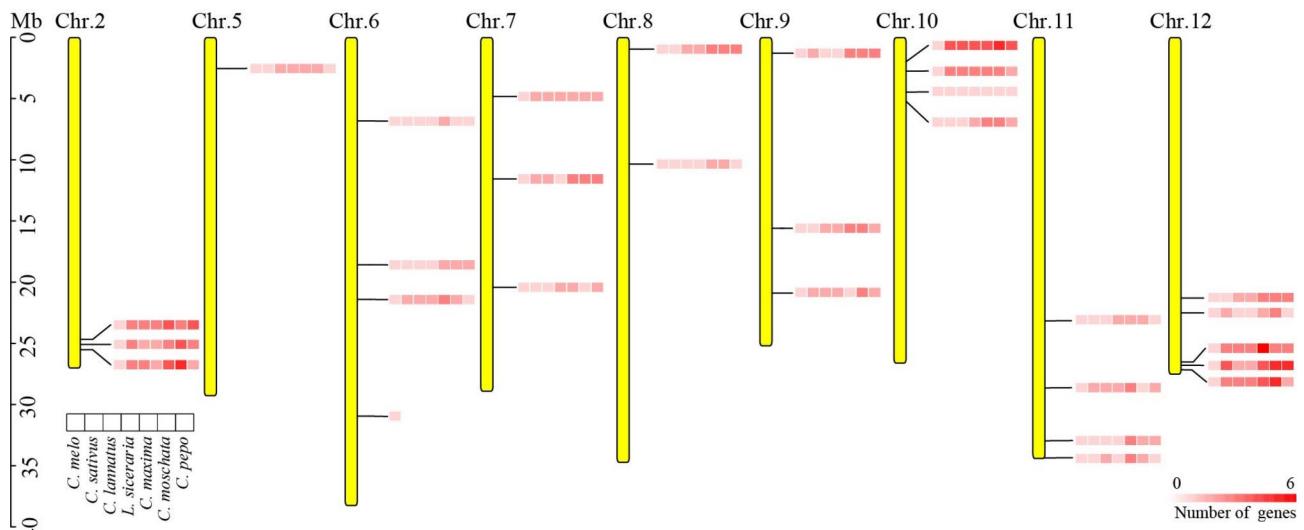


Fig. 2. Comprehensive map of *TCP* loci in Cucurbitaceae species. *TCP* genes were localized on 12 chromosomes in melons. The different species are represented by different squares, in the following order: *C. melo*, *C. sativus*, *C. lanatus*, *L. siceraria*, *C. maxima*, *C. moschata*, and *C. pepo*. The color of the squares indicates the number of genes.

the family was conservatively distributed during cucurbit evolution, and that *TCP* genes in the Class I and CIN branches might have been ancient.

To further study the duplication events among *TCP* genes, we analyzed the collinearity of *TCP* genes at the genomic level using the TBtools software. Among the 230 *TCP* genes, 379 collinearity events were found between melons and other cucurbit crops, suggesting duplication events experienced by the *TCP* genes. To understand the divergence of the gene pairs, we calculated the synonymous substitution rate (Ks/Ka), with most members of the same subfamily experiencing duplicate events (Supplemental Tables 5 and 6). Combined with the results of the phylogenetic tree, these findings suggest a more distant evolutionary relationship, which makes discovering divergence more challenging. Ka/Ks indicates the method of gene selection based on experience. Ka/Ks < 1, indicates that the gene underwent purifying selection, indicating that the selection process neutralized the mutation to maintain protein stability. In contrast, Ka/Ks > 1, suggests that the gene underwent positive selection, indicating that the gene has undergone a large mutation that ultimately leads to a change in the encoded protein. Our identified *TCP* gene pairs had Ka/Ks values ranging from 0.05 to 0.44, therefore all of these genes underwent purifying selection, suggesting that the *TCP* genes have experienced little to no severe mutational disruption (Supplemental Table 5).

GO enrichment and Cis-regulator element analysis of *TCP* genes

To fully explore *TCP* gene function, GO enrichment analysis was performed for the 230 *TCP* genes in Cucurbitaceae species. A total of 114 GO terms (*P*-value < 0.05) were identified, of which 95 belonged to biological process (BP), 10 belonged to cellular components (CC), and nine belonged to molecular function (MF). Regarding the BP-related terms, five GO terms were enriched in the Benincaseae tribe, including ‘regulation of cellular metabolic processes’ (GO:0031323), ‘regulation of metabolic processes’ (GO:0019222), ‘regulation of cellular processes’ (GO:0050794), ‘regulation of biological processes’ (GO:0050789), and ‘biological regulation’ (GO:0065007) (Fig. 4 and Supplemental Table 7). Moreover, 72 GO terms were co-enriched in Cucurbitaceae tribe, except for ‘organic cyclic compound binding’ (GO:0097159). The findings are consistent with the fact that *TCP* genes are a major regulator and suggest that *TCP* genes are involved in many downstream transcriptional networks and functions primarily in the nucleus of Cucurbitaceae species. Several other terms, such as ‘nucleic acid-templated transcription’ (GO:0097659) and ‘response to endogenous stimulus’ (GO:0009719) were enriched, which are consistent with the proposed function of *TCP* genes (Fig. 4 and Supplemental Table 7).

Regions that are 2000 bp upstream of the *C. melo*, *C. sativus*, *C. lanatus*, *L. siceraria*, *C. maxima*, *C. moschata*, and *C. pepo* *TCP* genes were extracted to identify cis-regulatory elements and explore promoter region functions. In total, 619, 607, 508, 537, 1084, 873, and 849 cis-regulatory elements were identified in *C. melo*, *C. sativus*, *C. lanatus*, *L. siceraria*, *C. maxima*, *C. moschata*, and *C. pepo*, respectively. The cis-regulators included phytohormone responsive elements (ABA, auxin, gibberellin, MeJA, and SA responsive elements), stress responsive elements (defense and stress, drought, light, and wound responsive elements) and growth and development elements (anaerobic induction, circadian control, and meristem expression) (Fig. 5). Light-responsive elements were the most prevalent functional elements of *TCP* gene promoters in each species, indicating that light plays a critical role in regulating *TCP* gene function throughout plant growth and development (Supplemental Fig. 4). The second and third predominant cis-regulator elements were ABA-responsive- and MeJA-responsive elements, which were also widely distributed in *TCP* gene promoter regions of Cucurbitaceae species (Supplemental Fig. 4). These results suggest that these elements may control the actions of the two phytohormones. Furthermore, we

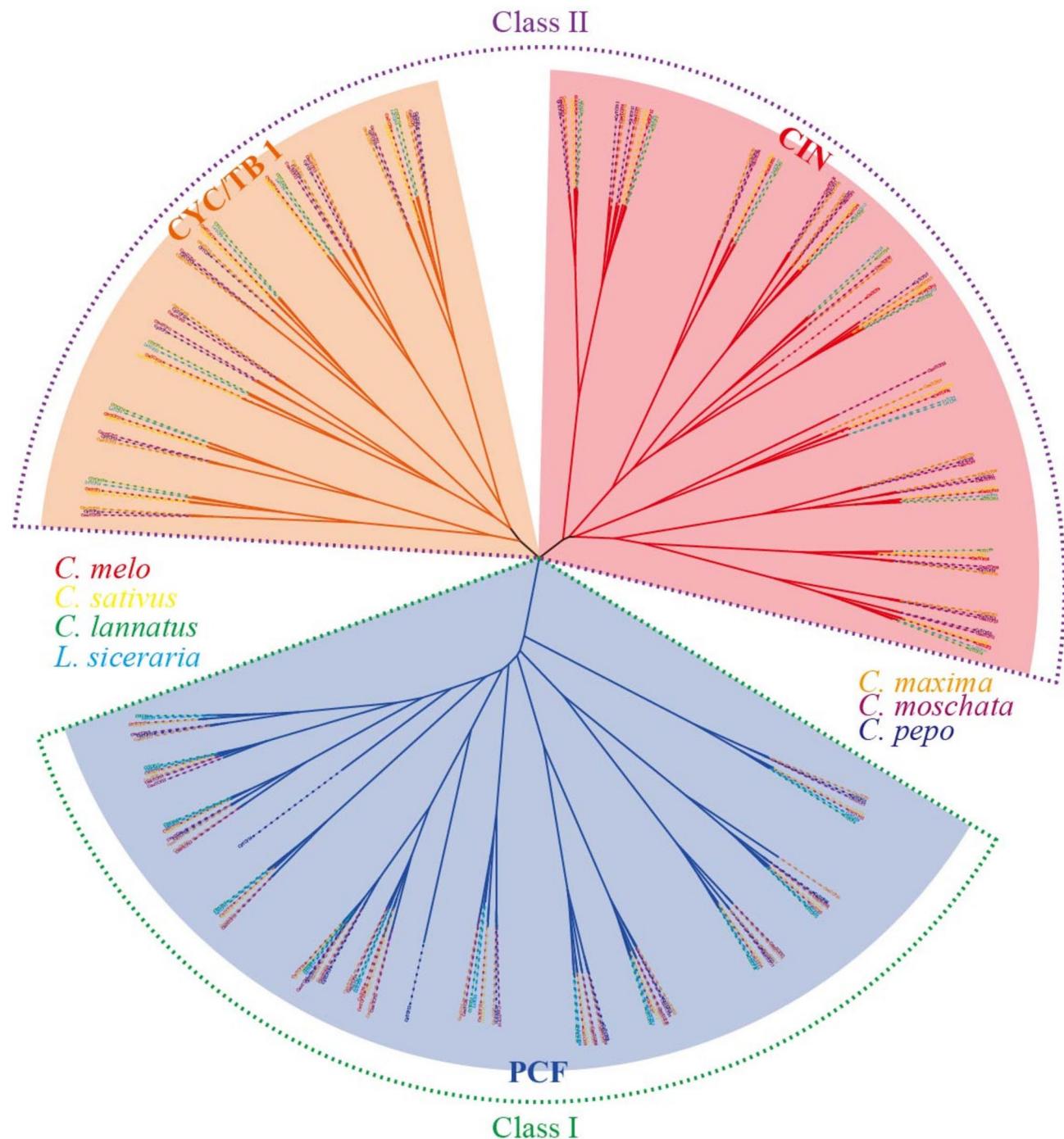


Fig. 3. Phylogenetic tree of the TCP gene family was constructed based on the amino acid sequences of the TCP structural domains in Cucurbitaceae species. Maximum likelihood (ML) tree of the 230 TCP proteins categorized into Class I and II. Class II was subdivided into the clades CIN and CYC. Colored lines represent different classes, and colored dashed lines represent the TCP genes in different species.

identified components involved in meristem expression and anaerobic induction, which supports the critical function of *TCP* genes in preserving meristem homeostasis (Fig. 5).

Expression profiles of TCP genes in different tissues

To better understand the expression patterns of *TCP* genes, their expression patterns in different tissues from the seven cucurbit species were analyzed using published RNA-seq data. The expression of 29 *CmTCP* genes in the fruit, roots, female flowers, male flowers, and leaves of melons were analyzed (Fig. 6). In addition, *TCP* genes expression in different tissues of other cucurbit species was analyzed. The dynamic volcano plots were constructed to visualize tissue-specific expression of *TCP* genes in cucurbit species, with the height of each

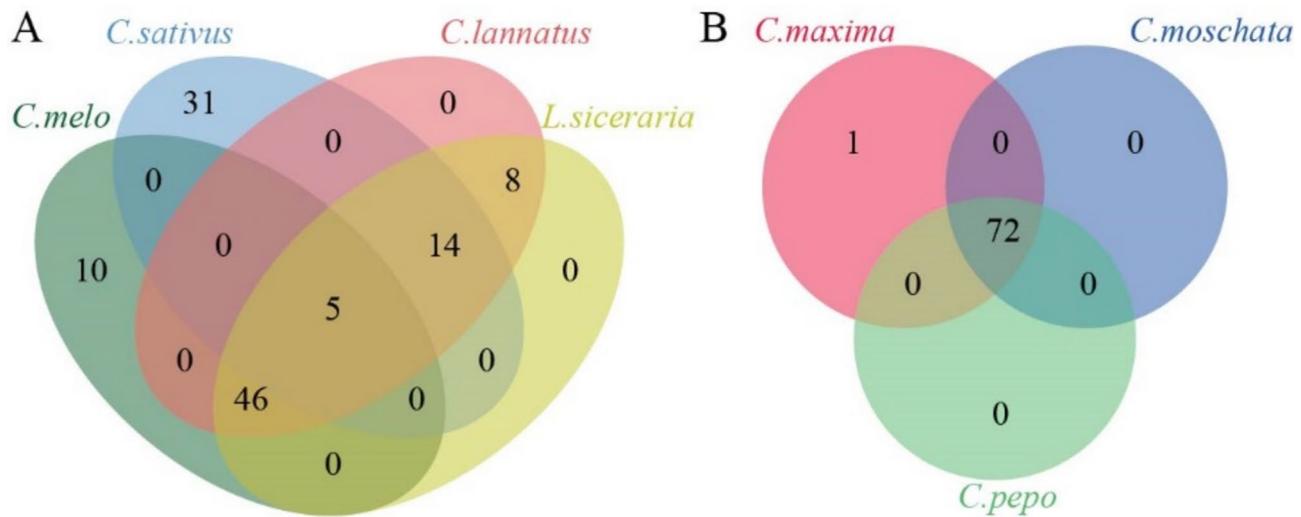


Fig. 4. Enriched GO (gene ontology) terms associated with TCP genes in the Benincaseae (A) and Cucurbitae tribes (B). GO terms with a *P*-value ≤ 0.05 were considered to be enriched. The detailed information is listed in Supplemental table 7.

peak indicating the number of genes, and the degree of smoothing indicating the amount of gene expression (Fig. 6). The result showed that the expression profiles of *TCP* genes were different among tissues, suggesting that *TCP* genes have different functions in different plant development stages. In all cucurbit species, *TCP* genes showed moderate expression levels in the leaves, suggesting that *TCP* genes play a role in plant growth (Fig. 6). *CmTCP*, *CsTCP*, and *LsTCP* were highly expressed in flowers, indicating the potential significance of these *TCP* genes in driving tissue differentiation. *CmaTCP*, *CmoTCP*, and *CpTCP* were also highly expressed in the stems, indicating that these *TCP* genes play an important role in plant development. The results suggest that different *TCP* genes play different roles in plant growth and development.

Activation of *TCP* gene transcription in response to abiotic and biotic stressors

To determine whether *TCP* genes are involved in abiotic stress, we analyzed the expression of *CmTCP* and *CsTCP* after cold stress in melons and watermelons. As shown in Fig. 7A, *TCP* genes exhibited diverse expression patterns after cold stress, which varied among the cucurbit species. After cold stress, *TCP21* expression was significantly upregulated in melons, and the expression of *TCP21*, which has the same gene ID, was significantly upregulated in watermelons (Fig. 7A). These results suggest that *TCP21* may be involved in the regulating responses to cold stress in cucurbit species. To further understand the role of *TCP* genes in adaptations to environmental stress, we analyzed the expression of *TCP* genes in the leaves, leaf mesophyll, and leaf veins following salt stress. Compared to that under normal conditions, the expression of *CsTCP6/7/13/20/26* was significantly increased after salt stress treatment, and the expression of 12 *CmoTCP* genes (*CmoTCP4/5/7/13/21/27/28/29/34/36/38/41*) was significantly altered (Fig. 7B). After salt stress, *CmoTCP2/10* expression was significantly upregulated in the leaf mesophyll, as well as *CmaTCP2* expression (Fig. 7B). After salt stress, *CmoTCP31* and *CmaTCP2/15/32* expression was significantly upregulated in the leaf veins (Fig. 7B). These results indicate that *TCP* genes may be involved in the response to salt stress in cucurbit species.

To predict the possible functions of *TCP* genes in environmental adaptation, we investigated the transcriptional profiles of *TCP* genes under biotic stress conditions, including powdery and downy mildew stress. *CmTCP29* expression was downregulated after powdery mildew infection in resistant (MR-1) and susceptible melons (top mark) (Fig. 8A). Notably *CmTCP18* expression was significantly elevated after powdery mildew infection with MR-1. Compared with that under normal conditions, the expression of seven *CmTCP* genes (*CmTCP3/9/13/18/21/28/29*) was upregulated at 48 h post-infection (hpi) (Fig. 8A). After powdery mildew infection, *CsTCP13/19* expression was significantly upregulated in SSL508-28 cells, whilst *CsTCP14* expression was significantly upregulated on D8 (Fig. 8A). *CsTCP1/25* expression showed a gradual increase after downy mildew infection, whereas *CsTCP23/31* and *CsTCP18* expression showed a gradual decrease in resistant (PI197088) and susceptible melons (Vlaspik), respectively (Fig. 8B). In addition, we analyzed *TCP* genes expression in the early stages of *Pseudoperonospora cubensis* infection (0/6/24 hpi) and found that *CsTCP1/5/12/15/16/19* expression increased gradually, whilst *CsTCP13/24/25* expression decreased gradually (Fig. 8B). Overall, the findings indicate that the transcript levels of *TCP* genes differ under different stresses, suggesting that *TCP* genes play an important role in response to abiotic and biotic stresses in cucurbit species.

Discussion

The plant-specific TF *TCP* plays various roles in processes related to plant growth, development, and stress responses. With the extensive sequencing of plant genomes, genome-wide identification of *TCP* genes has been performed in a variety of plants, including rice, maize, *Arabidopsis*, tomatoes, and potatoes^{41,58–60}. Across

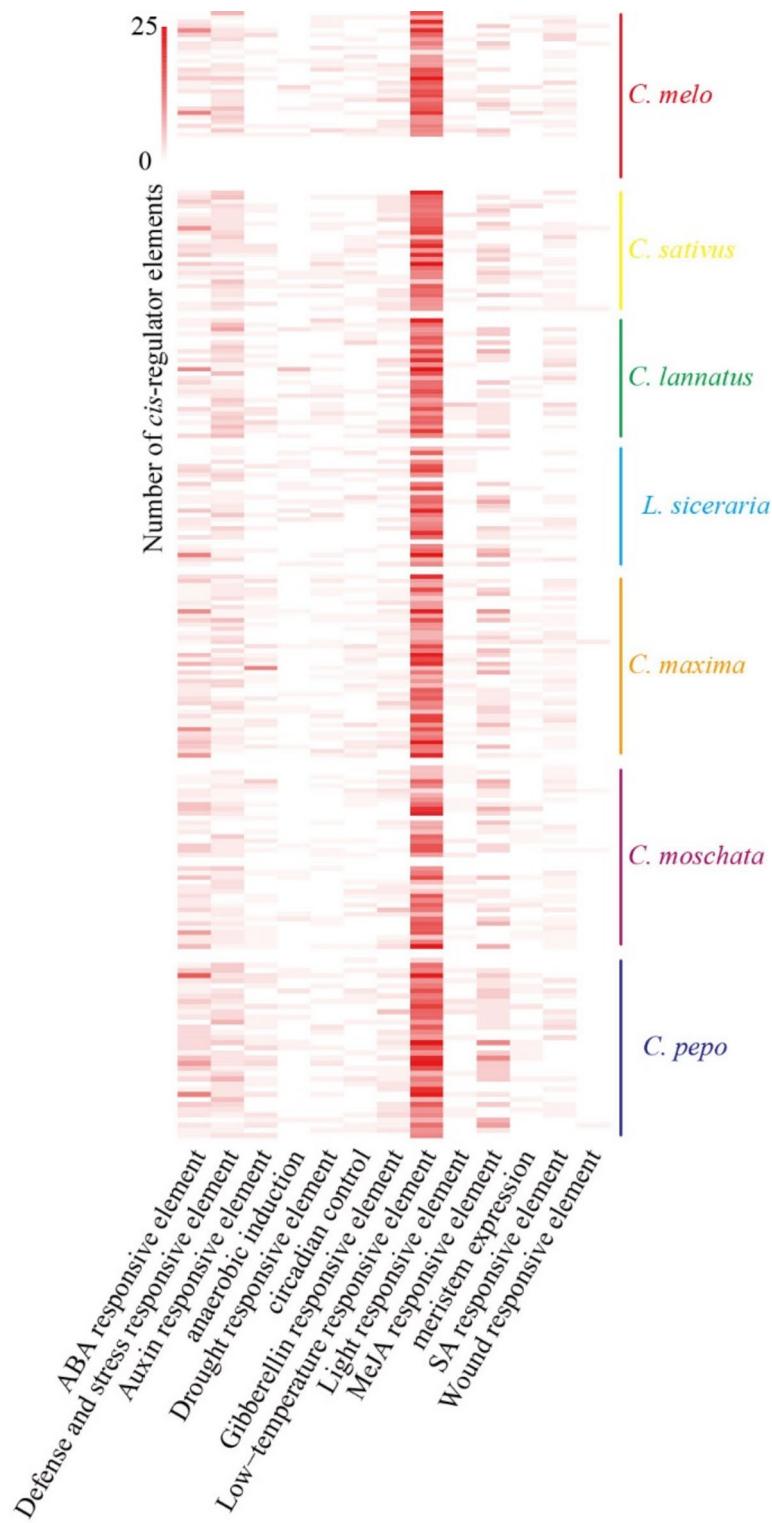


Fig. 5. Cis-regulator elements in the promoter regions of TCP genes. The detailed information is listed in Supplemental Figure 4. The colored bar shows the number of cis-regulator elements. The horizontal axis of the heatmap indicates the class of cis-regulator elements. The ordinate represents TCP genes of different species, from smallest to largest.

species, *TCP* gene sequences are generally conserved, with most members belonging to two classes^{37,42}. Although *TCP* gene analyses have been conducted for individual Cucurbitaceae species, there is a lack of comprehensive summaries²⁴. In this study, *TCP* genes from seven species of Cucurbitaceae were recharacterized and analyzed, and 230 members were identified, of which 101 *TCP* genes belonged to Class I and 129 members belonged to

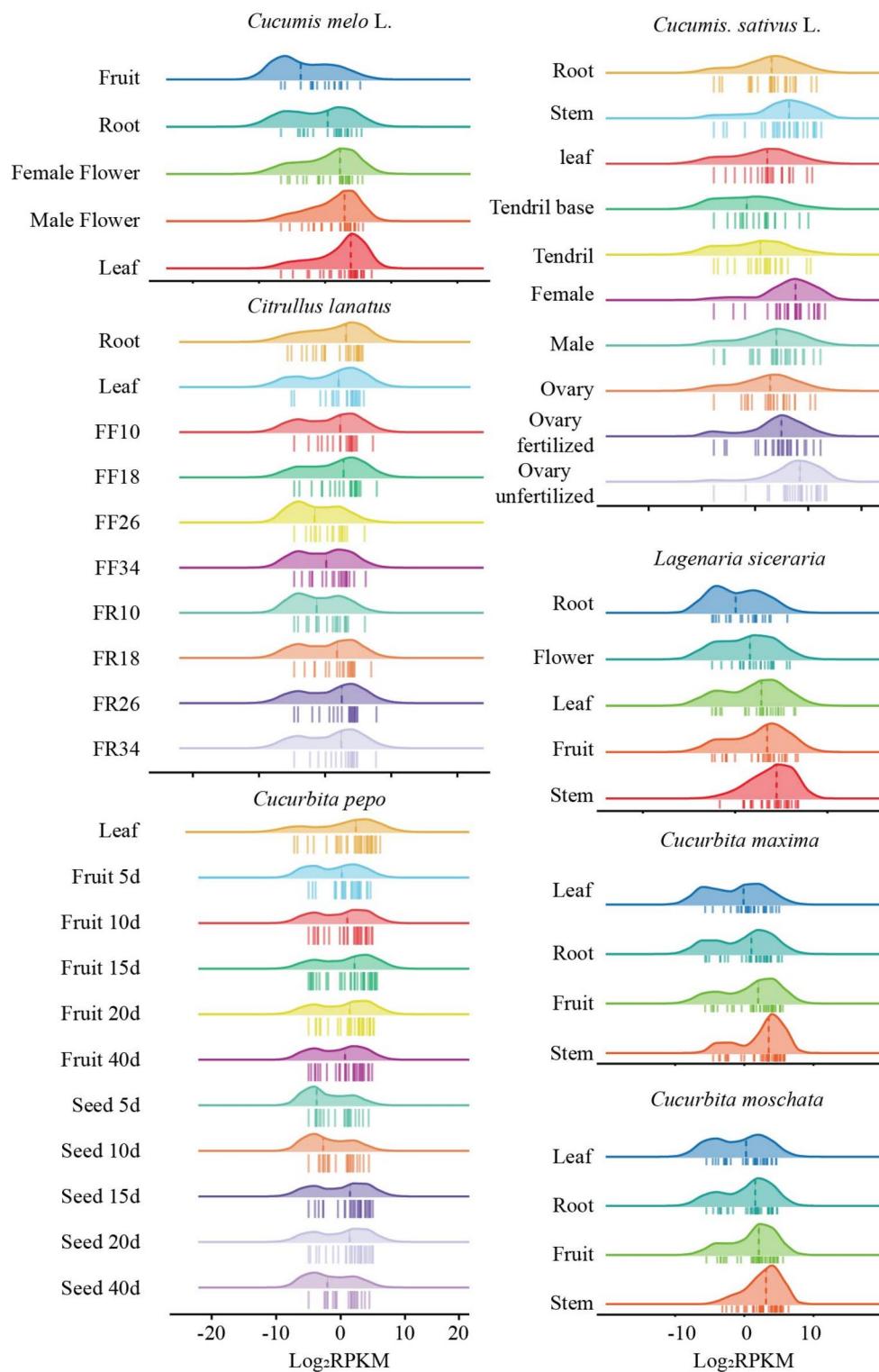


Fig. 6. Expression analysis of TCP genes in different tissues under normal growth conditions in the seven Cucurbitaceae species^{5,38,48,56}. Transcript levels of TCP genes were normalized to Reads Per Kilobase per Million mapped (RPKMs) and is displayed as the mean of three biological repeats. In the volcano plot, dashed lines above the peaks represent the median line, and the dashed lines below the peaks represent TCP genes in each species. FF, fruit flesh. FR, fruit rind.

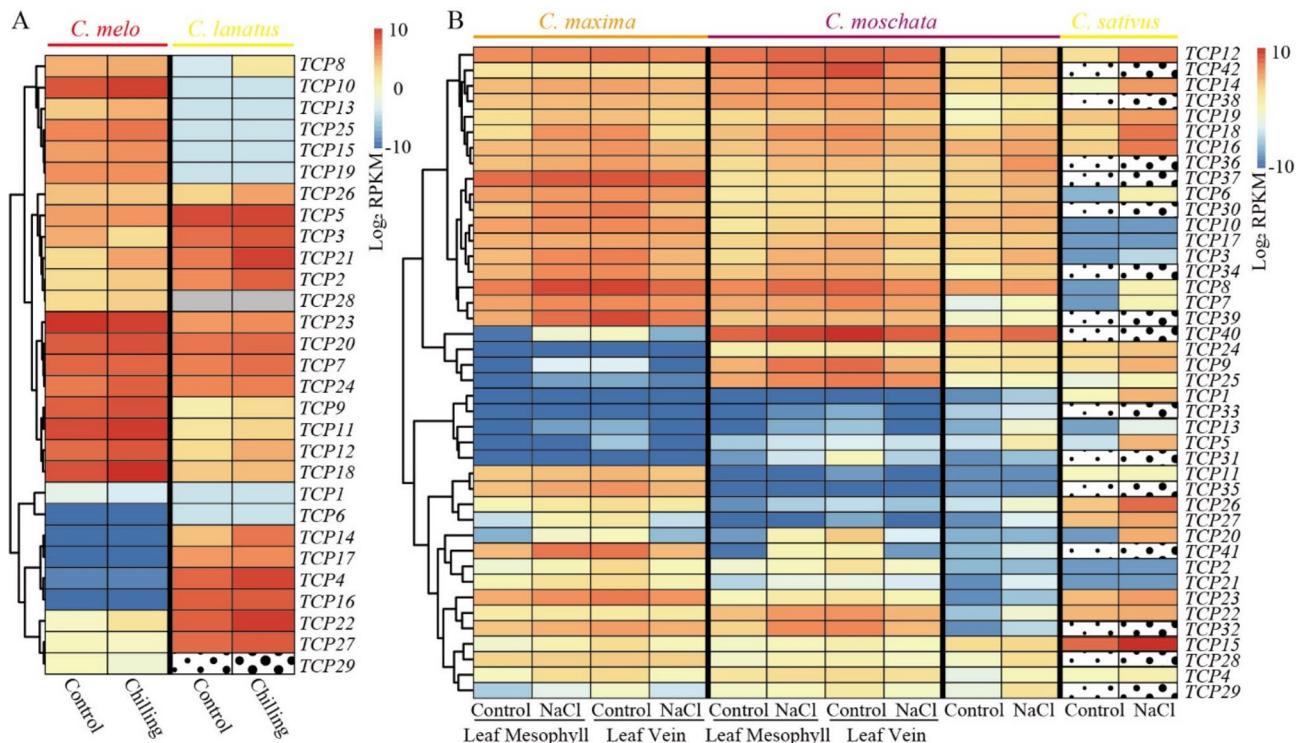


Fig. 7. TCP gene expression patterns under different abiotic stresses. Heatmap of TCP genes in response to cold stress in *C. melo* and *C. lanatus* (A) and salt stress in *C. maxima*, *C. moschata*, and *C. sativus* (B). RNA-seq data obtained from CuGenDB (<http://cucurbitgenomics.org/>) and public data were used^{14,34,52}. Color coding was based on the scale given. Boxes filled with dots indicate no detection in the species. Transcript levels were calculated as log2 transformed Reads Per Kilobase of exon model per Million mapped reads (RPKM).

Class II. Most plants have relatively similar numbers of TCP genes, for example there are 22 in rice, 29 in maize, 24 in *Arabidopsis*, and 30 in tomatoes, which is similar to the number of TCP genes in the seven cucurbit species. Notably, there is some variation in the size of these plant genomes; for example, the melon genome is 375 Mb, the cucumber genome is 243.5 Mb, and the *C. maxima*, *C. moschata* and *C. pepo* genomes are 269.9, 271.4, and 263 Mb, respectively²⁸. Therefore, it can be inferred that cucurbits contain a similar number of TCP genes, which are not necessarily related to the genome size of the species. In addition, according to the PI range of TCP genes in Cucurbita species, most of TCP genes are basic and hydrophilic proteins that are mainly distributed in the nucleus and may act in alkaline environments, suggesting that TCP proteins might play a role in regulating nuclear genes, which is in agreement with the results of our GO enrichment analysis. The results of the above analyses indicate that TCP genes in Cucurbitaceae are largely evolutionarily and functionally conserved.

Analyzing the evolutionary relationship of TCP genes aids the prediction of their functions. By analyzing CrTCP3 as a homologous protein of AtTCP11, previous authors deduced that this gene may regulate leaf and stem development⁶¹. Phylogenetic and structural domain analyses of TCP genes in different species revealed that TCP genes in Cucurbitaceae species are divided into Classes I and II, and Class II is further divided into the CIN and CYC/TB1 subclasses⁶². All cucurbit TCP proteins contain a typical TCP structural domain. Phylogenetic analyses showed that TCP genes from the same species tended to cluster together in both Class I and Class II analyses, which is consistent with the findings of previous studies on other species and demonstrates that genomic differences emerged during the evolution of cucurbit species^{9,62}. TCP genes play key regulatory roles in flower development. *PeCIN8/10* play an important role in orchid ovule development by regulating cell division⁶³. Moreover, CIN-type genes may play a key role in petal formation and are highly expressed in the ovule development and pistil initiation stages⁶⁴. This is consistent with the findings of the present study that CIN-type genes in cucurbit species play an important role in the development of female floral organs. Among the Class I-type genes, *CmTCP14* suppresses organ size and prolongs flowering time in chrysanthemums³⁰, and *AtTCP11* and *AtTCP16* are involved in early pollen development^{20,61}. Additionally, CIN genes play important regulatory roles in plant growth and development, particularly in the development of the lateral organs¹⁰.

The main causes of gene amplification are segmental duplication, tandem duplication, and WGD. Tandem duplication genes have multiple functions in plants⁶⁵. Tandem duplication of biosynthetic genes drives the diversity of plant secondary metabolites, which might be related to the evolution of certain unique traits⁶⁶. The rye genome contains 7,077 tandemly duplicated genes, which correlate with the abundance of secondary metabolites in rye⁶⁷, including *ScTCP* genes identified with three tandem duplicate gene pairs⁷. After gene duplication, most redundant copies of genes are exposed of during the long evolutionary process, but some duplicates are retained depending on the level of adaptation to the environment, which explains the differences in copy numbers

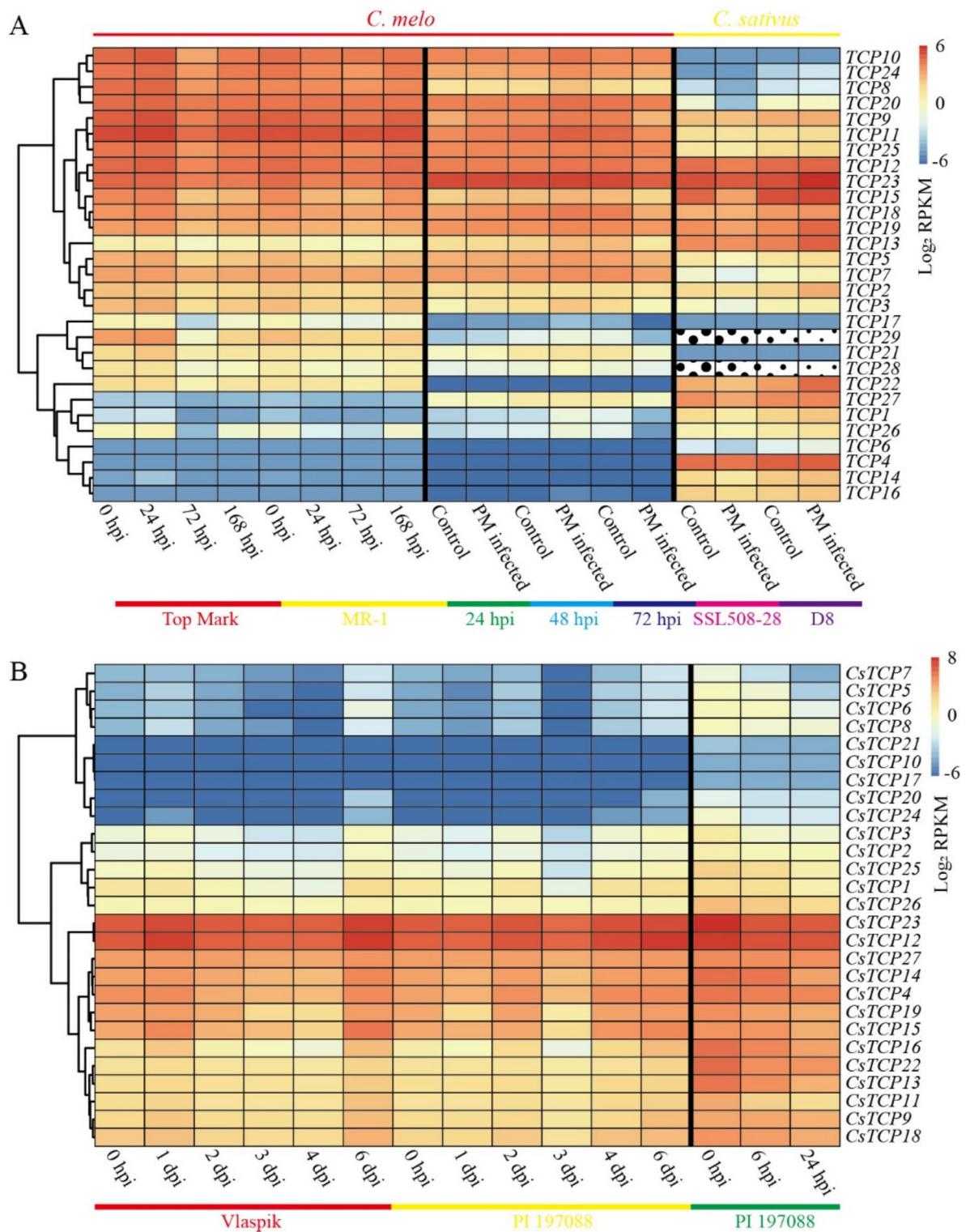


Fig. 8. Expression profile of TCP genes in response to biotic stresses. **(A)** Expression profiles of TCP genes in response to PM infection in *C. melo* (cultivars: Top Mark and MR-1, SBA number: PRJNA358655; cultivars: Rochet, SBA number: PRJNA434538) and *C. sativus* (cultivars: SSL508-28 and D8, SBA number: PRJNA321023). PM, powdery mildew. **(B)** Expression profiles of CsTCP genes in response to DM infection in cucumbers (cultivars: Vlaspik and PI 197088, SBA number: PNJNA285071; cultivars: PI 197088, SBA number: PNJNA388548). DM, Downy mildew. The axis shows the logarithmic normalized RPKM. hpi, hours after post-pathogen infection. dpi, days after post-pathogen infection.

between species⁶². Both Class I and Class II *TCP* genes have undergone continuous expansion, developing from a few members in algae and bryophytes to dozens of members in angiosperms, with some species having more than 50 members, such as soybeans, which have 55 *TCP* genes^{9,62}. The *TCP* gene family has gradually expanded and diversified as green plants have diversified, and similar changes have occurred in Cucurbitaceae species. Gene duplication and collinearity analyses showed that most *TCP* genes (155/230) arose from gene duplication events; however, the expansion of *TCP* genes may be caused by different mechanisms in different Cucurbitaceae species. Based on the evolutionary relationship among Cucurbitaceae, both the Benincaseae and Cucurbitae tribes experienced an initial WGD event, with species belonging to the Benincaseae and Cucurbitae tribes forming the sister clades²⁸. In our study, the number of *TCP* genes in the Cucurbitae tribe was higher than that in the Benincaseae tribe, which may be because the Cucurbitae tribe underwent another WGD event during its evolution. Both ancient and recent WGD events occurred in most angiosperms, which led to the expansion of the *TCP* gene family; this observation also supported our findings. Therefore, we conclude that the expansion of the *TCP* gene family in Cucurbitaceae was mainly driven by dispersed duplication and segmental duplications/WGD. In addition, collinearity analysis revealed the same high number of fragment repeats, and the purification selection was positive. Functional annotation analysis showed that *TCP* genes were not only involved in ‘regulation of cellular metabolic processes’, ‘regulation of metabolic processes’, ‘regulation of cellular processes’, ‘regulation of biological processes’, ‘biological regulation’, but also in response to light and hormones and tissue development. TFs regulate gene expression; therefore, *TCPs* have diverse functions, which is consistent with the findings of previous studies on the function of *TCP* genes^{7,68}.

TFs regulate gene expression at the transcriptional level by binding to *cis*-regulator elements^{69,70}. In this study, a series of *cis*-regulator elements were identified in the promoter regions of *TCP* genes, including growth-associated, hormone-associated, and stress response-associated *cis*-regulator elements⁷¹. The presence of different types of *cis*-regulator elements in the promoter regions, indicates that Cucurbitaceae *TCP* genes might be associated with a wide range of stress responses. The active components of secondary metabolites play an important role in plant adaptation to unfavorable environments and some environmental stressors can be used as inducers⁷². Previously, positive effects of UV-B radiation and MeJA application on TIA induction were confirmed¹⁵. In Ginkgo, *GbTCP6/11/13* were significantly up-regulated following exogenous MeJA administration²⁷. In the present study, light-responsive elements were the most prevalent in the promoters of *TCP* genes across species, suggesting that light is a key regulator of *TCP* gene function during plant growth and development. The second and third predominant *cis*-regulatory elements were ABA- and MeJA-responsive elements. Furthermore, these elements are widely distributed in the promoter region of *TCP* in cucurbit species. These results suggest that *TCP* genes may be involved in hormone signaling in cucurbit species. In addition, we identified other components involved in phloem organization and anaerobic induction, indicating the critical function of *TCP* gene in phloem homeostasis⁹.

TCP genes play an important role in the adaptation of plants to a wide range of biotic and abiotic stresses^{10,71}. Furthermore, *TCP* genes may have an evolutionarily conserved function in gene regulation, and comparative analyses analyzing upstream and downstream *TCP* signaling pathways indicate that *TCP* regulatory pathways are conserved among species. Our analysis of the transcriptional profiling libraries of *TCP* genes under abiotic (cold and salt stress) and biotic (powdery mildew and downy mildew) conditions showed that *TCP* genes were generally similar among different species of Cucurbitaceae. Preliminary studies have demonstrated the broad functional conservation of *TCP* genes in Rosaceae species, which is consistent with the results of this study¹⁵. However, further investigation is required to confirm our preliminary findings. Overall, analyzing the biological functions of these *TCP* genes will provide an important theoretical basis for improving the response of melons to environmental stresses.

Data availability

Publicly available datasets were analyzed in this study. The *C. melo*, *C. sativus*, *C. lanatus*, *L. siceraria*, *C. maxima*, *C. moschata* and *C. pepo* genome data presented in this study are openly available in CuGenDB at <http://cucurb.itgenomics.org/download>. The RNAseq data presented in this study are openly available in <https://www.ncbi.nlm.nih.gov/bioproject/>, PRJNA80169, PRJNA285071, PRJNA321023, PRJNA328189, PRJNA358655, PRJNA383830, PRJNA388584, PRJNA434538, PRJNA437579, PRJNA464060 and SRP012849.

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Author contributions

HJ, and SSW designed the experiments, supervised the study, and managed the projects. SSW and WLL performed most of the research and drafted manuscripts. SSW performed bioinformatics analysis and charting. HJ analyzed and discussed the results. All authors contributed to the article and approved the submitted version.

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Declarations

Competing interests

The authors declare no competing interests.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Additional information

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