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Comparative analysis of the *TCP* gene family in celery, coriander and carrot (family Apiaceae)

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Abstract

Apiaceae is one of the most important families in Apiales and includes many economically important vegetables and medicinal plants. The *TEOSINTE BRANCHED 1/CYCLOIDEA/PROLIFERATING CELL FACTOR 1/2* (*TCP*) gene family plays an important role in regulating plant growth and development, but it has not been widely studied in Apiaceae. In the present study, we identified 215 TCP family genes in six species of plant, of which 122 genes were present in three Apiaceae including 29 in celery (*Apium graveolens*), 43 in coriander (*Coriandrum sativum*), and 50 in carrot (*Daucus carota*). Whole-genome duplication likely contributed to *TCP* gene family expansion in Apiaceae. There were more paralogs in carrot than in coriander and celery, which was attributable to the greater number of tandem and proximal duplicated genes on chromosome 1. Nine microRNAs were found to regulate 20 *TCP* genes in the three Apiaceae species, with miR-319 having the most target genes. Several *TCP* genes showed high expression in the root, petiole and leaf of celery and coriander. These results provide a basis for comparative and functional genomic analyses of *TCP* genes in Apiaceae and other plants.

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INTRODUCTION

The Apiaceae family of plants includes more than 400 genera and 3,000 species[1,2]. Several Apiaceae species such as carrot (*Daucus carota*), celery (*Apium graveolens*), and coriander (*Coriandrum sativum*) are cultivated as a vegetable or for medicinal purposes worldwide[3,4].

Celery is an annual or biennial herbage species originating from the Mediterranean and Middle East[5,6]. Besides being a vegetable, celery is also used as a medicinal plant[7]. Coriander, which is also known as cilantro, is a popular herb and a major ingredient of curry powder[8]. Carrot is an economically important vegetable with a high nutritional value[9]. All three of these plants are diploid species although their chromosome number and genome size differ: celery and coriander each have 22 chromosomes (2n = 2x = 22) whereas carrot has 18 (2n = 2x = 18)[3,10,11], and the assembled genome size of celery is 3.33 Gb, which is larger than coriander (2.11 Gb) and carrot (421.5 Mb)[3,11,12].

The *TCP* gene family in plants is named after the first identified members—ie, *TEOSINTE BRANCHED 1* (*TB1*) in maize (*Zea mays*)[13], *CYCLOIDEA* (*CYC*) in snapdragon (*Antirrhinum majus*)[14], and *PROLIFERATING CELL FACTOR 1* (*PCF1*) and *PCF2* in rice (*Oryza sativa*)[15]. *TCP* genes regulate multiple processes in plant growth and development such as shoot branching[16], seed germination[17,18], gametophyte development[19,20], leaf development[21–25], leaf senescence[26–28], mitochondrial biogenesis[29], flower development[30–32] and cell cycle[29,33]. There are 24 *TCP* genes in *Arabidopsis thaliana*[34], 22 in rice[34], 24 in tomato[35], 19 in plum[26], 42 in switchgrass[36], 36 in carrot[37], and 32 in celery[38]. However, to date, no *TCP* genes have been identified in coriander. High-quality genome sequences of celery, carrot and coriander were recently released[3,11,12], which can facilitate comparative analyses of specific genes in Apiaceae.

In this study, we identified and characterized *TCP* genes in celery, coriander and carrot and performed comparisons with genes in *Lactuca sativa* (lettuce), *Vitis vinifera* (grape), and *Arabidopsis*. We mapped the *TCP* genes to chromosomes, identified orthologs and paralogs, detected collinearity and gene expansion or loss, and analyzed their expression patterns in plant tissues. Our results provide a basis for comparative studies on the function and evolution of *TCP* genes in plants.

MATERIALS AND METHODS

Identification of *TCP* family genes in celery, coriander and carrot

The genome sequences of coriander and celery were downloaded from the coriander genome database

(http://cgdb.bio2db.com) and celery genome database (http://celerydb.bio2db.com), respectively[3,10]. The *Arabidopsis* genome sequence was retrieved from The *Arabidopsis* Information Resource (TAIR10; http://www.arabidopsis.org). The sequences of carrot (version 2), lettuce (version 5), and grape (Genoscope.12X) were downloaded from Phytozome (https://phytozome.jgi.doe.gov/pz/portal.html)[11,39,40]. The Pfam database (https://pfam.sanger.ac.uk) was used to identify *TCP* family genes with the identifier PF03634 (E value < 1e−4)[41]. The Simple Modular Architecture Research Tool (SMART v9.0) database and Conserved Domains Database (CDD) were used for domain validation[42,43].

Phylogenetic analysis of *TCP* gene family

To analyze the evolutionary relationships of *TCP* genes in Apiaceae, multiple sequence alignment of the TCP amino acid sequences of *Arabidopsis*, grape, lettuce, carrot, coriander and celery was performed with ClustalW software (v2.0)[44] and a phylogenetic tree was constructed with the neighborhood-joining method (bootstrap = 1,000) using MEGA X[45]. The reconstructed *TCP* gene tree was compared to the actual species tree using Notung v2.9 software with default parameters[46,47].

Chromosome location, gene structure and conserved motif analysis

The location of coriander, celery and carrot *TCP* genes on chromosomes was drawn using Tbtools software and the files were saved in general feature format (gff)[48]. Gene structure was determined using Gene Structure Display Server 2.0 (https://gsds.cbi.pku.edu.cn)[49]. Conserved motifs were analyzed using Multiple Expectation maximizations for Motif Elicitation suite 5.2.0 (http://meme-suite.org)[50].

Analysis of orthologs and paralogs

Orthologous and paralogous *TCP* gene pairs in celery, coriander and carrot were analyzed using OrthoMCL software v2.0 (https://orthomcl.org/orthomcl)[51]. The relationships of the genes among the three species was depicted using Circos software (v0.69)[52].

Identification of collinear blocks and duplication types

MCScanX was used to identify collinear blocks and duplication types of the *TCP* genes[53]. Whole-genome sequences were searched against themselves using BLASTp (E value < 1e−5). We extracted *TCP* genes located in the collinear blocks using Perl scripts. The duplication type of *TCP* genes was estimated using the subprogram duplicate_gene_classifier.

Calculation of Ka/Ks and estimation of divergence time

The nonsynonymous rate (Ka), synonymous rate (Ks), their ratio (Ka/Ks) and divergence time among orthologous gene pairs of the three species were calculated using KaKs_Calculator 2.0[54]. The coding sequence of orthologous gene pairs were aligned using ClustalW (v2.0)[44]; AXTconvertor software (v1.0) was then used to convert the alignment file to axt format. Lastly, the Ka value, Ks value and their ratio were calculated based on the Nei–Gojobori method[54]. Ks was used to estimate the divergence time based on the formula T = Ks/2r, where r indicates neutral substitutions (5.2×10^{-9} for Apiaceae)[12].

Analysis of selective pressures

We used the maximum likelihood method and codon substitution models to determine the likelihood ratio of positive selection. We analyzed each branch of the phylogenetic tree to infer ω (the ratio of nonsynonymous to synonymous distances) using CodeML implemented in PAML4.9[47,55]. We adopted a complete deletion method for analyzing alignments with gaps and eliminated sequences with gaps in over 40% of their length. The likelihood ratio test between M0 and M1 and between M7 and M8 models were used to determine variation sites.

Identification of micro (mi)RNA target genes in the *TCP* gene family

Mature miRNA sequences of *A. thaliana* were downloaded from miRBase (release 22.1; http://www.mirbase.org)[56]. *TCP* genes that are miRNA targets were predicted using psRNATarget Schema v2 (2017 release)[57] with maximum expectation ≤ 3 and other default parameters. A miRNA–*TCP* gene network was constructed using Cytoscape v3.7.2 software[58].

Expression of *TCP* genes

TCP gene expression data in celery and coriander were extracted from RNA sequencing (RNA-seq) datasets previously published by our group[3,10] using Perl script; the values were normalized as reads per kilobase per million reads (RKPM). An expression heatmap was created using TBtools software (v1.0)[48].

RESULTS

Identification of *TCP* genes in three Apiaceae species

We identified 29 *TCP* genes in the genome of celery, 43 in coriander, and 50 in carrot (Supplemental Table S1 and S2). Additionally, 24 *TCP* genes were identified in *Arabidopsis* along with 20 in grape and 49 in lettuce (Supplemental Table S2). Thus, a total of 215 *TCP* genes were identified in the six species for further analysis.

Phylogenetic and functional analyses of *TCP* gene family

To classify the *TCP* gene family in plants, we constructed a phylogenetic tree of all 215 amino acid sequences from the six abovementioned species using MEGA X (Fig. 1). Consistent with the phylogenetic relationships described in *Arabidopsis* and grape, the phylogenetic tree had three groups according to the type of TCP protein domain including the *PCF*, *CINCINNATA* (*CIN*), and *CYC/TB1* classes.

In class *PCF*, there were ten *AgTCP*, 26 *CsTCP*, and 36 *DcTCP* genes; in class *CYC/TB1*, there were eight *AgTCP*, seven *CsTCP*, and seven *DcTCP* genes; and in class CIN, there were 11 *AgTCP*, ten *CsTCP* and seven *DcTCP* genes (Fig. 1 and Supplemental Table S2). Notably, in coriander and carrot there were more *TCP* genes in class *PCF* than in the other two classes.

The functions of most *TCP* family genes have been well studied in the model plant *Arabidopsis*. We inferred the function of homologous genes within the same taxonomic group in the phylogenetic tree in order to clarify the function of *TCP* genes in Apiaceae. For example, AT1G53230.1 (*AtTCP3*)

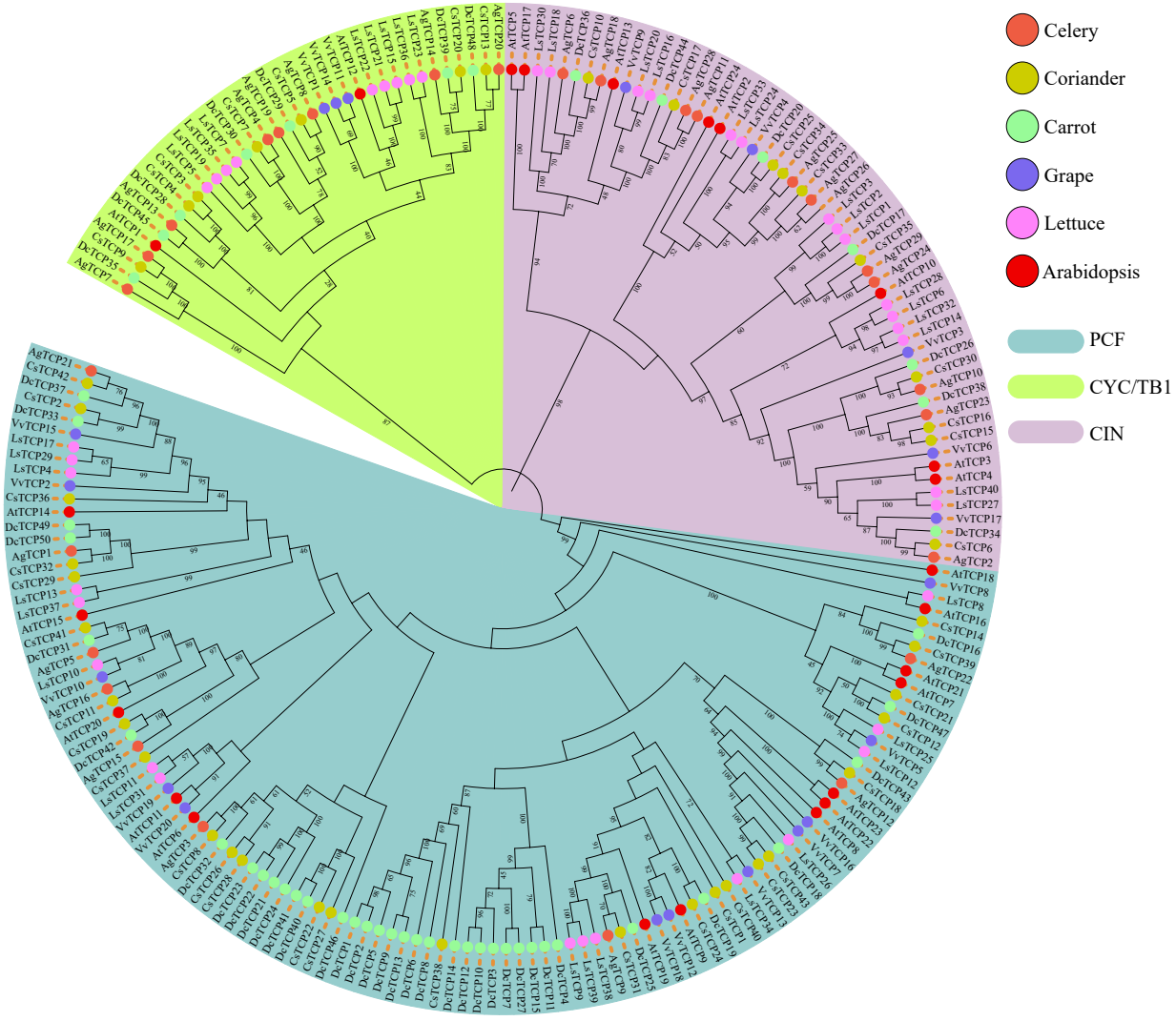

Fig. 1 Phylogenetic tree of *TCP* family genes in three *Apiaceae* species (carrot, celery and coriander) and lettuce, grape and *Arabidopsis*. The topology of the phylogenetic tree was determined using IQ-TREE with maximum likelihood (ML) based on the JTT+F+R8 model. The bootstrap was set to 1,000 replicates, and values > 40% are shown. The three classes were identified based on bootstrap values and phylogenetic topology.

is known to suppress the expression of *CUP-SHAPED COTYLEDON* (*CUC*), resulting in cotyledon fusion[24]. We identified three *TCP* genes—namely, *DcTCP34*, *AgTCP2*, and *CsTCP6*—that clustered together with *AtTCP3* (Fig. 1), suggesting that they are also related to cotyledon fusion. It may also be possible to deduce the function of other Apiaceae *TCP* genes based on the function of the homologous genes in *Arabidopsis*.

TCP gene family structure and conserved motifs

We carried out a gene structure analysis of *TCP* family genes to identify exons, introns and untranslated regions (Supplemental Fig. S1). Of the 122 *TCP* genes in Apiaceae, 94—including all 50 *DcTCP* genes—lacked introns. Most *TCP* genes had a single exon, although there were some exceptions. For example, *CsTCP15* (class *CIN*) had four exons and *DcTCP11* (class *PCF*) had three. In general, genes in the same class or subclass had similar gene structure and size.

As gene structure varied among genes, we performed a motif analysis to examine the structure in greater detail. We compared five motifs in Apiaceae species and found that motif 3 was found at the beginning of most genes, followed by motif 1 and motif 2 (Fig. 2). However, motif 2 was located at the start of the *CsTCP7*, *DcTCP15*, *CsTCP23*, *CsTCP36*, and *CsTCP2* and motif 5 was present at the beginning of *DcTCP11*. Almost all *TCP* genes had motif 1 except for *DcTCP* and *CsTCP* in class *PCF*, indicating that this motif is highly conserved and plays an important role in Apiaceae. Motif 3 was also present in most *TCP* genes and is likely conserved in Apiaceae.

Most genes in classes *CYC/TB1* and *CIN* lacked motif 2 except for *DcTCP17* and *CsTCP79*. Only five genes in class *CIN* had motif 4, which was present in all class *CYC/TB1* genes. Motif 5 was only found in class *PCF* and not in other classes. Interestingly, *DcTCP14* (class PCF) did not have any of the 5 motifs, suggesting that they were lost during the course of the evolution of carrot. Thus, genes in the same class had similar motif composition, indicating that they are functionally similar.

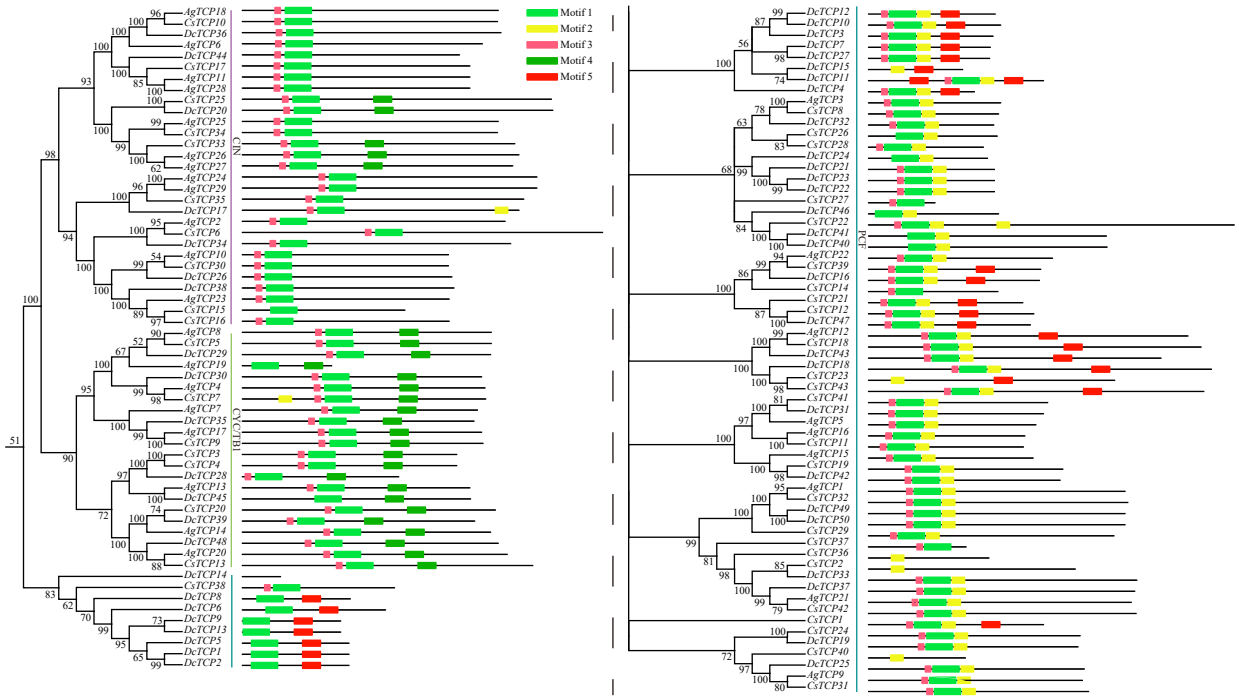

Fig. 2 Conversed motifs in *TCP* family genes of three *Apiaceae* species.

Chromosomal distribution of *TCP* genes in Apiaceae species

In celery, 27/29 *TCP* genes mapped to nine chromosomes (Fig. 3a and Supplemental Table S1). Two celery *TCP* genes did not map to any chromosomes, and no *TCP* family gene was found on chromosomes Agr6 and Agr10. There were five genes that mapped to chromosomes Agr3, Agr5, and Agr11 while only one was located on chromosomes Agr1 and Agr2.

In coriander, 35/43 *TCP* genes mapped to nine chromosomes (Fig. 3b and Supplemental Table S1); none were found on chromosome Csa8. Chromosome Csa9 had the most *TCP* genes (10), followed by chromosomes Csa7 (6) and Csa1 (5). Chromosomes Csa2 and Csa3 each had just one *TCP* gene.

The 50 carrot *TCP* genes were unevenly distributed across nine chromosomes (Dca1–9) (Fig. 3c and Supplemental Table S1). Interestingly, *TCP* gene expansion was observed on chromosome 1, which had 16 genes. Additionally, 15 genes (DcTCP1–15) were clustered together, mainly through tandem and proximal duplication.

Identification of orthologous and paralogous *TCP* genes in Apiaceae

We examined orthologous and paralogous gene pairs in Apiaceae and found that there were 22 orthologous gene pairs between any two of celery, coriander and carrot (Fig. 4 and Supplemental Table S3), indicating a close phylogenetic relationship between these species. There were only three paralogous gene pairs in celery and coriander (Supplemental Fig. S2 and Supplemental Table S4) but 29 were found in carrot.

The Ks value was calculated to estimate the divergence time of orthologous *TCP* gene pairs among celery, coriander and carrot (Fig. 5 and Supplemental Table S5). The divergence time ranged from 14.03 to 116.15 million years between celery and coriander *TCP* genes, 24.94 to 81.44 million years between celery and carrot, and 22.59 to 94.72 million years between coriander and carrot orthologous *TCP* gene pairs. Therefore, the divergence time of most *TCP* genes was earlier than that of any two species (celery vs coriander, 11–13 Mya; carrot vs celery or coriander, 20–23 Mya)[4].

Detection of duplication type for *TCP* family genes in Apiaceae

Various types of gene duplication can lead to the expansion of a gene family. We examined five types of gene duplication in celery, coriander and carrot (Fig. 6a, Table 1 and Supplemental Table S6)—namely, singleton, dispersed, proximal, tandem and whole-genome duplication (WGD). There were no singleton *TCP* gene in the three species of Apiaceae (Table 1). Dispersed and tandem duplications were the predominant types in celery and coriander. In celery, the percentage of genes showing dispersed duplication and WGD was 48.1% and 40.7%, respectively; in coriander, the percentages were 40.0% and 45.7%, respectively. WGD was also the predominant type in carrot (32.0%), which had a lower percentage of dispersed duplication (24.0%) than celery and coriander. Moreover, the percentage of the tandem type was higher in carrot (30.0%) than in celery (11.1%) and coriander (11.4%). These results demonstrate that WGD played an important role in *TCP* gene expansion in celery, coriander and carrot, which is supported by the previous suggestion that they underwent two WGD events since their divergence from lettuce[3].

TCP gene loss and duplication during the evolution of Apiaceae

We compared species and gene trees in celery, coriander and carrot to identify gene losses and duplications during the

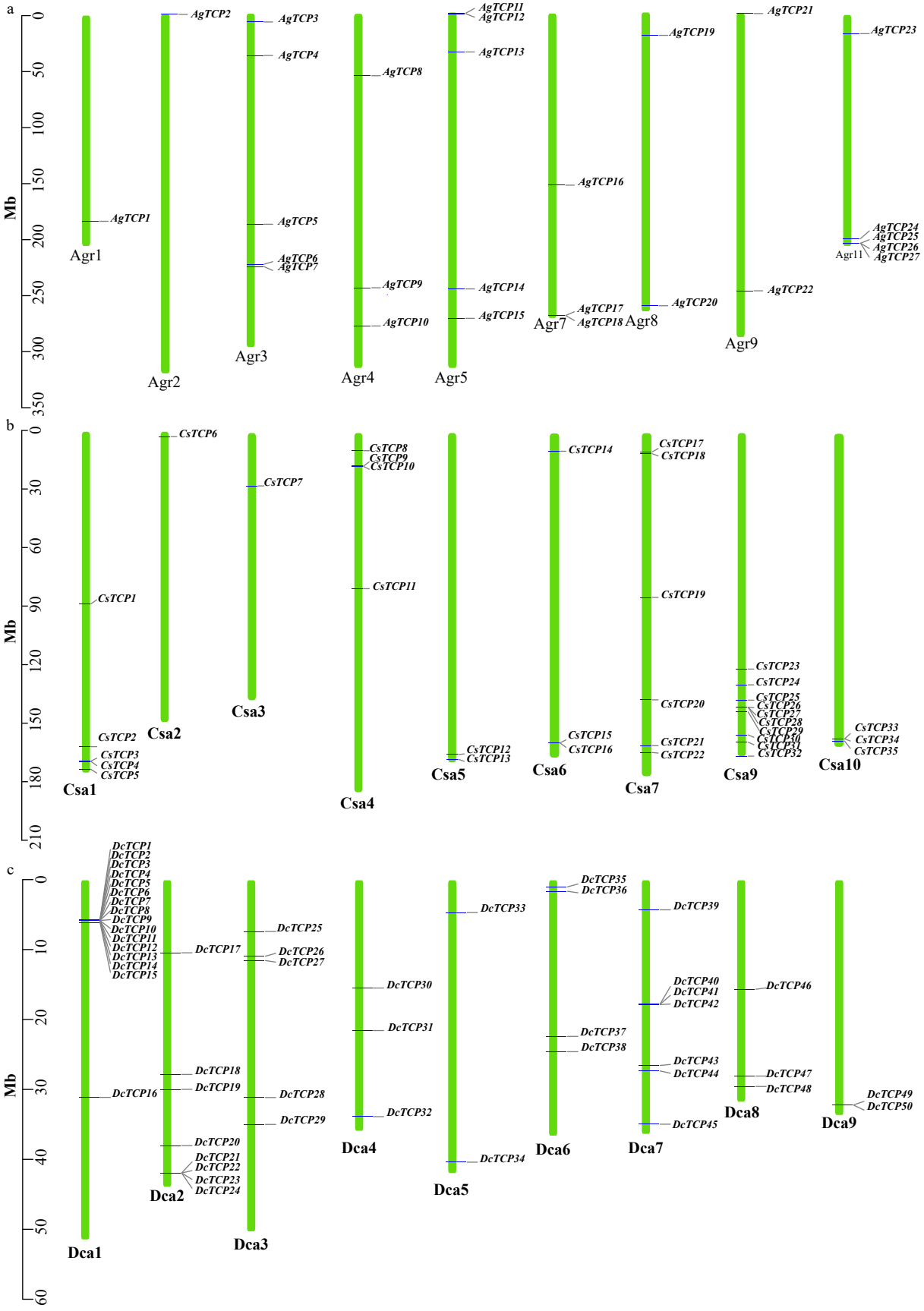

Fig. 3 Distribution of TCP transcription factors on each chromosome in three *Apiaceae* species. (a) Celery, (b) Coriander, (c) Carrot.

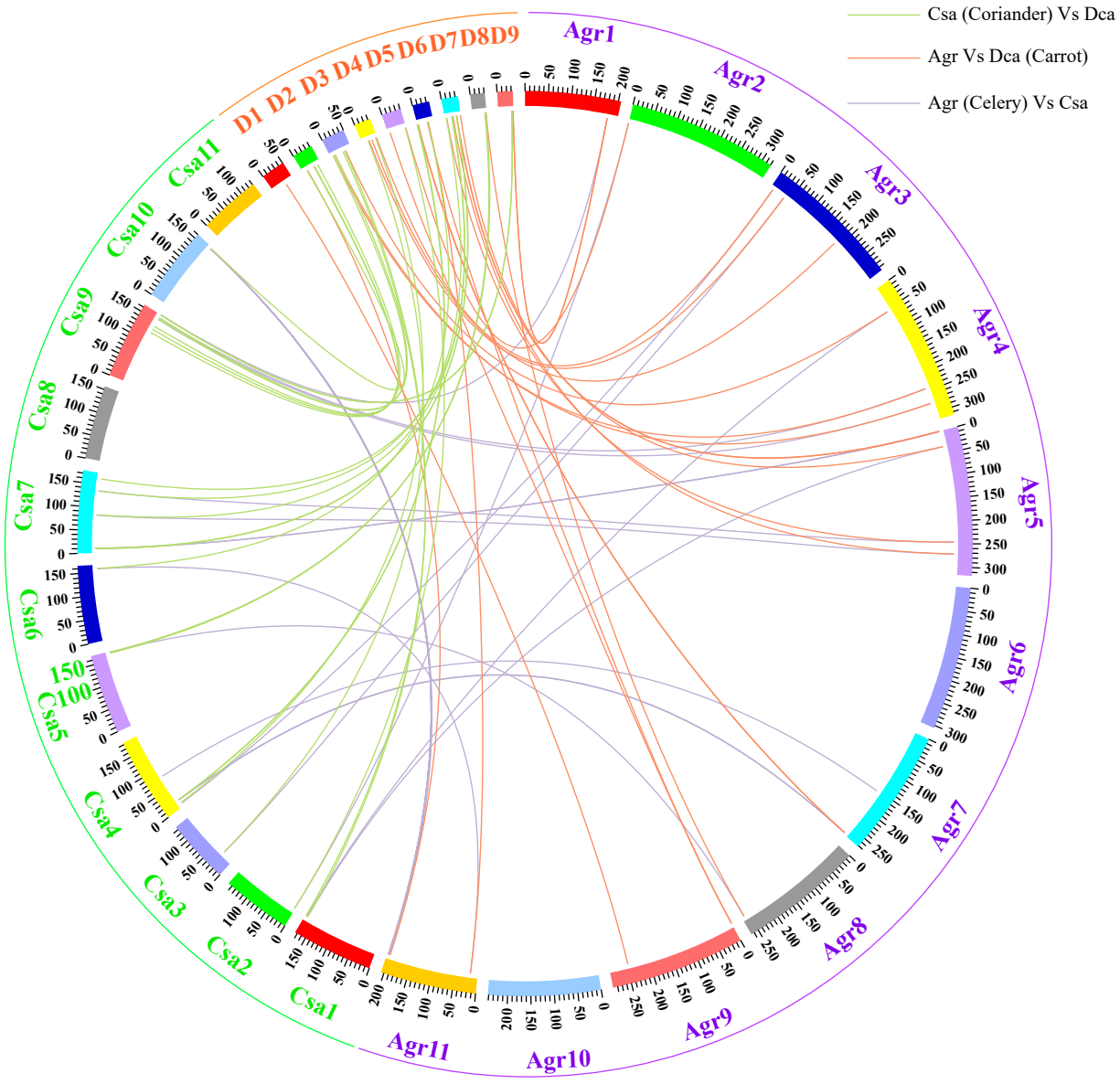

Fig. 4 Circle plot of orthologous *TCP* gene pairs among three *Apiaceae* species.

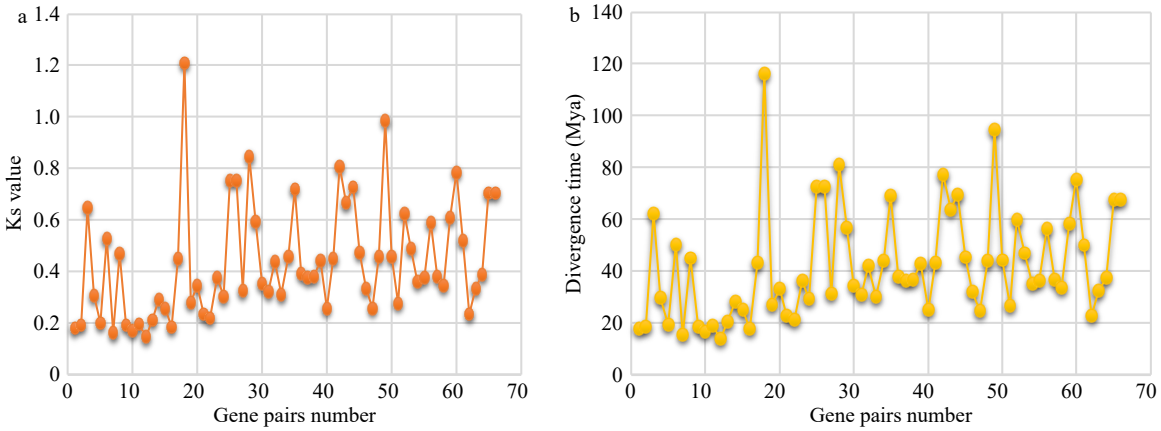

Fig. 5 Ks and divergence time of *TCP* orthologs. Ks values (a) and divergence time estimation (b) of orthologous gene pairs between any two of three *Apiaceae* species.

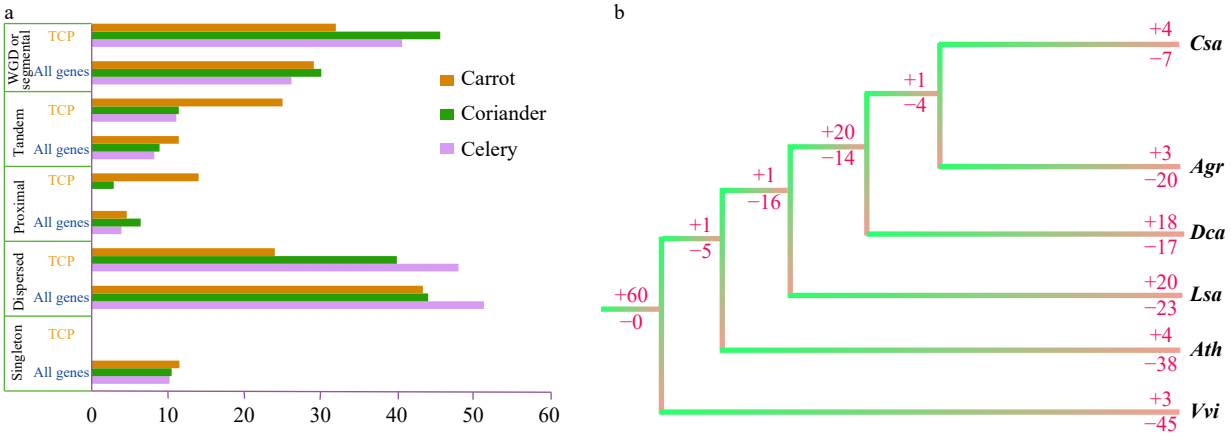

Fig. 6 Duplication and loss of *TCP* family genes. (a) Percentage of duplication types for *TCP* family genes and genes in the whole genome of three *Apiaceae* species. (b) Duplication (+) or loss (–) of *TCP* family genes in three *Apiaceae* species and three other representative species. Numbers after '+' and '–' are the number of genes. Agr, *Apium graveolens* (celery); Ath, *Arabidopsis thaliana* (*Arabidopsis*); Csa, *Coriandrum sativum* (coriander); Dca, *Daucus carota* (carrot); Lsa, *Lactuca sativa* (lettuce); Vvi, *Vitis vinifera* (grape).

Table 1. The identification of duplicated type for *TCP* family genes and all genes in *A. graveolens*, *C. sativum* and *D. carota*.

Duplication type[a]	Category[b]	*A. graveolens*	*C. sativum*	*D. carota*
Singleton	All genes	3,028	3,577	3,543
	TCP	0	0	0
	Percentage (%)	0	0	0
Dispersed	All genes	15,258	14,963	13,378
	TCP	13	14	12
	Percentage (%)	48.15	40	24
Proximal	All genes	1,167	2,161	1,428
	TCP	0	1	7
	Percentage (%)	0	2.86	14
Tandem	All genes	2,426	3,032	3,501
	TCP	3	4	15
	Percentage (%)	11.11	11.43	30
WGD/segmental	All genes	7,787	10,200	8,974
	TCP	11	16	16
	Percentage (%)	40.74	45.71	32
Total	All genes	29,666	33,933	30,824
	TCP	27	35	50

Note: [a] the classification of duplicate genes was conducted using the MCScanX program. WGD/segmental duplicates were inferred by the anchor genes in collinear blocks. Tandem duplicates were defined as paralogs that were adjacent to each other on chromosomes. Proximal duplicates were paralogs near each other, while interrupted by several other genes. Dispersed duplicates were paralogs that were neither near each other on chromosomes, nor do they showed conserved synteny. [b] TCP indicated the TCP family genes. Percentage (%) indicated the percentage of TCP family gene number among all genes.

evolution of the *TCP* gene family. There were more gene losses in celery (20) than in coriander (7) and carrot (17) but more gene duplications in carrot (18) than in coriander (4) and celery (3). In the common ancestor of coriander, celery and carrot, there were 20 gene duplications and 14 gene losses (Fig. 6b).

Positive selection of *TCP* family genes in Apiaceae

We analyzed natural selection in the evolution of *TCP* genes in Apiaceae (Fig. 7). Strong positive selection was observed at the major nodes of the phylogenetic tree, which may have contributed to the functional divergence of Apiaceae species. We detected 35 positive selection sites overall; most were in class *PCF* (29), followed by class *CYC/TB1* (5) and class *CIN* (1), indicating that *TCP* genes in class *PCF* underwent greater positive selection in the evolution of Apiaceae.

miRNA target *TCP* genes in Apiaceae

We next sought to identify *TCP* family genes in Apiaceae that are regulated by miRNAs. We found nine miRNAs that regulated 20 *TCP* family genes including five genes in celery, nine in coriander and six in carrot (Supplemental Table S7 and Fig. 8). Of the nine miRNAs, miR-319 had the most target genes (11), followed by miR-172 (3) and miR-181 (3) (Fig. 8). Specifically, miR-319 regulated four *TCP* family genes in celery, four in coriander, and three in carrot. Our results are supported by other studies demonstrating that miR-319 regulates *TCP* family genes[31,59–61]. We also found that four genes were regulated by more than one miRNA: *DcTCP20* was regulated by miR-319 and miR-837, *CsTCP33* was regulated by miR-319 and miR-8181, and *AgTCP26* and *AgTCP27* were regulated by miR-319 and miR-8181 (Supplemental Table S7 and Fig. 8).

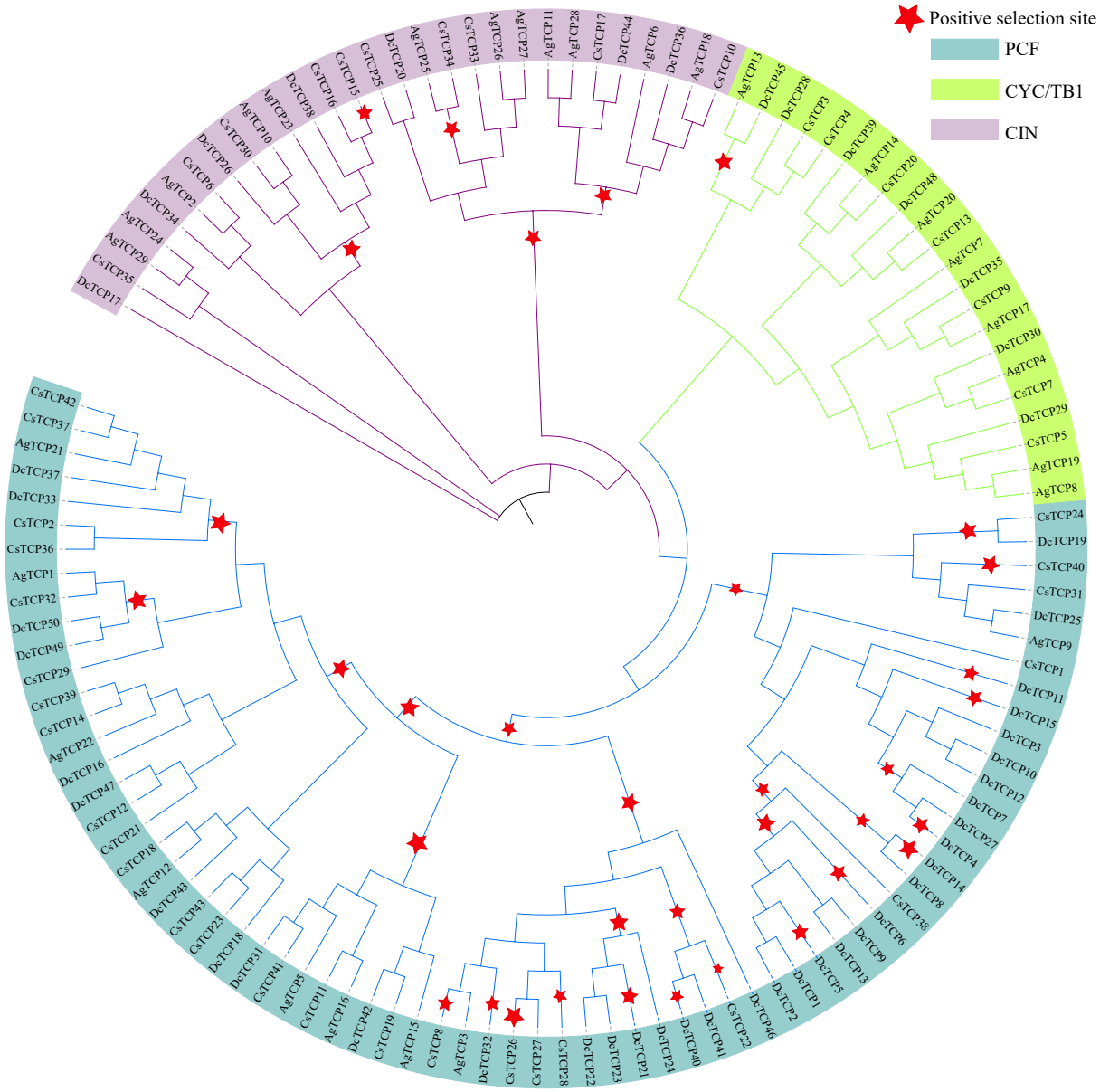

Fig. 7 Positive selection of *TCP* family genes in celery, coriander and carrot. Red stars represent branches in which positive selection occurred. The maximum likelihood (ML) phylogenetic tree was constructed using PhyML software.

Expression of *TCP* family genes in celery and coriander

We analyzed the expression patterns of *TCP* genes in root, petiole and leaf tissues of celery and coriander. In celery, all *TCP* family genes in class *CYC/TB1* had relatively low expression in the three tissues (Fig. 9a and Supplemental Table S8) whereas *AgTCP22* had the highest expression level (RPKM > 60), suggesting a key role in celery growth and development. In coriander and celery, all *TCP* family genes in class *CYC/TB1* were expressed at a relatively low level in the three tissues (Fig. 9b and Supplemental Table S8); meanwhile, several genes including *CsTCP39*, *CsTCP14*, *CsTCP12*, *CsTCP31*, and *CsTCP40* had high expression. *CsTCP6* and *CsTCP30* were more highly expressed in leaf than in the other two tissues.

DISCUSSION

Celery, coriander and carrot are typical members of the Apiaceae family. The draft genomes of these three species were recently released[3,11,12] and there have been several studies on *TCP* family genes in carrot and celery based on the sequences[37,38,62,63]. The latest versions of the celery, coriander and carrot genomes are of high quality with chromosomal-level assembly, allowing us to accurately and comprehensively analyze the *TCP* gene family in Apiaceae. To date there have been no reports on *TCP* genes in coriander. In this study, we identified 43 *TCP* genes in coriander as well as 29 in celery and 50 in carrot. Our results provide a resource for future studies on the *TCP* gene family in Apiaceae or related species.

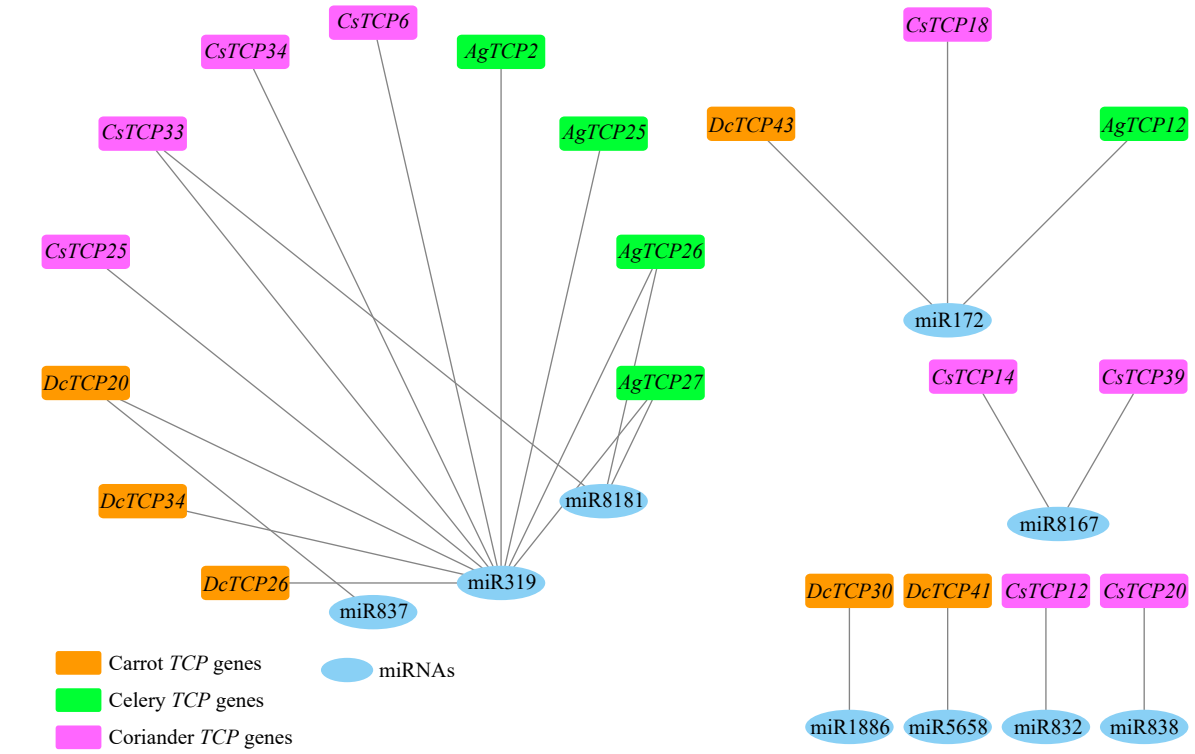

Fig. 8 Interaction network of miRNAs and target *TCP* family genes in carrot, celery and coriander.

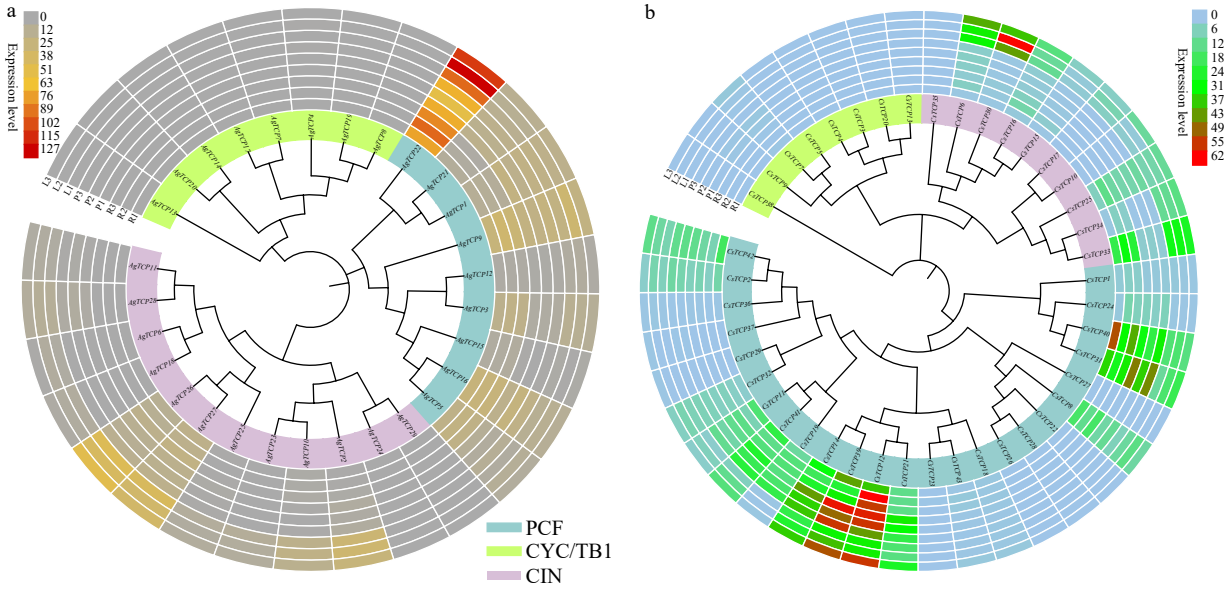

Fig. 9 Expression of *TCP* family genes in three replicates of plant tissues including root (R1, R2 and R3), petiole (P1, P2 and P3), and leaf (L1, L2 and L3). Hierarchical gene expression clustering of *TCP* genes in celery (a) and coriander (b). Expression levels were calculated based on RPKM.

TCP transcription factors have a 59-amino acid basic helix-loop-helix (bHLH) motif that is involved in DNA binding and protein–protein interaction[64]. The bHLH-like domain of TCP differs from the canonical bHLH in its basic region[34,65]. PCF1 and PCF2 interact with DNA-binding proteins that specifically bind to the *PROLIFERATING CELL NUCLEAR ANTIGEN* (*PCNA*) promoter[15]. Our analyses of gene structure and motifs of *TCP* genes in Apiaceae revealed similarities within the same class or subclass.

Gene duplication is the main mechanism underlying the evolution of complex phenotypes[66]. Many duplicated genes in plants were produced by WGD or whole-genome triplication[67–70]. Most duplicated genes were functionally redundant and had one of four fates during the course of evolution namely: conservation, neofunctionalization, subfunctionalization and specialization[67,71]. In conservation,

the ancestral function was maintained in both copies, thus preserving gene dosage[71]. In neofunctionalization, one copy retained the original function while the other acquired a novel function[71,72]. In subfunctionalization, both copies were required to preserve the ancestral gene function[71,73,74]. In specialization, subfunctionalization and neofunctionalization acted cooperatively, producing two gene copies that were functionally distinct from each other and from the ancestral gene[71,75]. Functional redundancies of *TCP* genes have been reported in *Arabidopsis*[76]. In carrot, 15 *TCP* genes were clustered on chromosome 1, and the number of paralogous gene pairs was greater in carrot (29) than in celery (3) and coriander (3). Although there were more gene losses than duplications in the evolution of celery, coriander and carrot, we found that WGD made a major contribution to *TCP* gene family expansion in Apiaceae, which is similar to what has been reported in most other gene families in higher plants[47,77–80].

The broad range of functions of *TCP* family genes in plants can be attributed to the diverse structures of different members. Most *TCP* genes are highly expressed in meristematic tissues, suggesting that their main function is to promote plant proliferation and growth[81]. However, some *TCP* genes, such as *CIN* and *CYC/TB1*, are known to negatively regulate plant proliferation and development[82] (lateral organ development for *CIN* genes and flower and lateral shoot development for *CYC/TB1* genes)[26]. Our gene expression analysis showed that *TCP* gene expression in celery (*AgTCP22*) and coriander (*CsTCP12*) was nearly 2x higher in root and petiole than in leaf, suggesting roles in plant growth and development.

In conclusion, we identified and characterized *TCP* genes in three Apiaceae species. We described their chromosomal location, exon–intron structure, motifs, collinearity, positive selection and expression patterns in plant tissues. These results provide a basis for investigations on the molecular networks regulating growth and development in Apiaceae and other plants.

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Conflict of interest

The authors declare that they have no conflict of interest.

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