

Genome-wide identification and expression pattern analysis of the CCT genes of *Akebia trifoliata*

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Abstract

Flowering is one of the important developmental events for many plants, and the CONSTANS/CONSTANS-LIKE/TIMING OF CAB1 (CCT) genes play key roles in this process. We identified a total of 46 *AktCCTs*, which are widely distributed across all chromosomes of the *Akebia trifoliata* genome. Their promoter regions contain hormone- and environment-responsive cis-acting elements. The average length and number of exons were 1,787.2 bp and 5.2, respectively, and the average number of amino acids, molecular weight and isoelectric point of putative protein were 388.4, 43.0 kDa and 6.3, respectively. Evolutionarily, *AktCCTs* experienced whole or segmental genome duplication and purifying selection and were divided into four classes (*COL*, *PRR*, *CMF* and *GTCC*) on the basis of sequence similarity and conserved domains, among which the *CMF* had the lowest gene length, number of exons, number of amino acids and molecular weights. Functionally, some *AktCCTs* expressed a detectable level. Otherwise, the expression of the *COL AktCCT17* significantly positively related with that of *AktFT2*, *AktFT4*, *AktFT5* and *AktGI1*, so that *AktCCT17* may be the crucial gene regulating the flowering behavior of *A. trifoliata* by "GI-CO-FT" model. Overall, these results provide valuable insights into the potential roles of these genes in regulating the biological process of flowering.

Introduction

Flowering is one of the most important biological behaviors in the plant kingdom, especially in angiosperms, which comprise approximately 300,000 extant species and dominate the vegetation of most terrestrial ecosystems (Crane et al. 1995). It not only has important ecological significance but also has substantial agricultural, horticultural and even ornamental value in practice (Wu et al. 2024). Differences in flowering time have an impact on flower morphology in addition to ensuring that plants are evolutionarily adapted to their specific geographical distribution and accomplish their life cycle to reproduce (Shan et al. 2019). However, flowering initiation is usually affected by environmental signals such as temperature and sunlight duration and endogenous changes such as developmental status and hormone levels (Liu et al. 2020), which are closely related to physiological processes such as photoperiod and vernalization (Liu et al. 2015). Therefore, detailing the characteristics of the genes involved in this process is important for understanding both the ecological distribution and flower development of a given plant species.

Plant photoperiod that has been discovered by more one hundred years (Garner and Allard 1920), refers the behavior that flowering time is influenced by the comparative length of light and dark periods in the diurnal cycle (Sawa et al. 2007), and hence flowering plants are classified into three types according to their response to photoperiod: day-neutral, short-day and long-day (Lumsden et al. 2002). Various studies have suggested that *CONSTANS/CONSTANS-LIKE/TIMING OF CAB1 (CCT)* gene families are the core elements affecting the plant photoperiod (Abe et al. 2003; Takahashi. 2017; Liu et al. 2020; Awal et al. 2022). The proteins encoded by *CCTs* commonly contain a conserved domain consisting of 43–45 amino acids at the C-terminus (Putterill et al. 1995) and are commonly localized in the nucleus (Robson et al. 2001). In addition, variations widely exist among intraspecific and interspecific members (Chen et al. 2010), which provides important evidence for classifying into subfamilies.

According to studies in *Arabidopsis thaliana*, *Oryza sativa*, *Sorghum bicolor*, and *Hordeum vulgare* (Cockram et al. 2012), the *CCT* family can be structurally divided into three subfamilies: *CONSTANS*-like (*COL*), containing one or two B-box domains; Pseudo response regulators (*PRR*), with a response-regulator (*REC*) domain beside a *CCT* domain; and the *CCT* motif (*CMF*), with only a *CCT* domain (Khanna et al. 2009; Cockram et al. 2012). In fact, a relatively special subclass called the *GTCC* subfamily containing the GATA domain also exists in some species such as *Cajanus cajan* and *Brassica rapa*, which sometimes accompanies a zinc finger protein expressed in the inflorescence meristem (Tribhuvan et al. 2022; Li et al. 2024). These findings indicate the high genetic diversity in the structure of the *CCT* family members among various species.

Since *CONSTANS (CO)*, a circadian clock regulatory gene belonging to the *COL* subfamily, was first cloned (Wenkel et al. 2006), various studies have functionally demonstrated that the *COL* subfamily could acts as the central regulatory factor in the photoperiodic pathway, and in the known "GI-CO-FT" model *CO* is first regulated by the upstream transcription factor named *Gigantea (GI)* and then it consequently regulates the downstream flowering locus *T (FT)* (Wu et al. 2014; Yang et al. 2014; Andrés et al. 2012). At the same time, some studies have also shown that *COL* genes have important functions in nuclear localization, protein–protein interactions and transcriptional regulation (Putterill et al. 1995; Kaewphalug et al. 2017), suggesting that they may be DNA-binding transcription factors, but the *CCTs* are not listed as a family in the Plant Transcription Factor Database (v5.0) (Jin et al. 2017), indicating that at least some *CCTs* lack the typical DNA-binding motif of classical transcription factors. In addition, some studies in the green algae *Chlamydomonas* and *A. thaliana* have shown that the *CCT* domain, which is homologous to the CCAAT-binding domain, can interact with transcription factors such as NF-YA, NF-YB and NF-YC with the typical DNA-binding motif to form multimers (Gnesutta et al. 2017; Lv et al. 2021), which further supports the view that *COL* indirectly regulates the expression of downstream genes, mainly through a complex pathway rather than directly binding the promoter region of the target genes. Ben-Naim et al (Ben-Naim et al. 2006) suggested that *COL* proteins usually lack a known DNA-binding motif and that their success in transcriptional regulation occurs mainly through the mediation of CCAAT binding factors, but some studies have shown that some *COL* proteins can directly bind DNA *in vitro* studies (Tiwari et al. 2010). Obviously, the details of the regulatory function and corresponding molecular mechanism of *COL* are not completely understood.

In addition, the *CMF* subfamily evolved from the *COL* subfamily, and thus, the functions of *CMF* families are very similar to those of *COL* subfamilies (Cockram et al. 2012). Compared with the *COL* and *CMF* subfamilies, the *PRR* subfamily is functionally more conserved and comprises important components of the circadian clock, usually interacting with circadian clock associated 1 (*CCA1*) and late elongated hypocotyl (*LHY*) (Makino et al. 2000; David et al. 2002; Nakamichi et al. 2020). Moreover, *PRRs* such as *TOC1* can influence the expression of other transcription factors, such as *CO*, *PIFs* and *GI*, by directly binding to their promoter region and even exerting self-regulatory effects (Hsu et al. 2012; Gendron et al. 2012). Therefore, more datasets from various species of different evolutionary clades would helpful to completely elucidate the complex regulatory network of the *CCT* family.

Akebia trifoliata (also called augmelon), which originates from the eastern edge of the Qinghai–Tibet Plateau and is representative of the Lardizabalaceae family, is a climbing perennial woody vine that is narrowly distributed in several regions of East Asia, including subtropical China south of the Yellow River (Chen et al. 2022; Dong et al. 2024), so that it has largely been ignored by both scientists and entrepreneurs in the past. However, it is becoming a very

popular topic in the field of edible wild plant domestication (Yu et al. 2021; Yang et al. 2024a) because of its multiple purposes, such as in traditional medicine (Yang et al. 2024b), for fresh fruit (Yang et al. 2021, Yi et al. 2025), as a woody oil crop (Guan et al. 2024; Han et al. 2025) and even as an ornamental plant (Yang et al. 2023). In addition, its value for theoretical study is also becoming increasingly appreciated because of its small genome size (Zhong et al. 2022a), short juvenile stage (Yang et al. 2021) and important evolutionary position (Zhong et al. 2022b), and it has been widely employed in various studies, such as those involving flower development (Zhong et al. 2022c; Zhong et al. 2024), phenylalanine metabolism (Guan et al. 2024; Li et al. 2024) and flavonoid biosynthesis (Zhong et al. 2022d; Yang et al. 2024b). However, to date, there are no reports about its photoperiod characteristics.

In fact, the most important reasons for using *A. trifoliata* to investigate the close involvement of the *CCT* family with the photoperiod are that we observed that it flowers in both spring and autumn each year in the *A. trifoliata* Germplasm Nursery of Sichuan Agricultural University at Chongzhou Research Station (Chen et al. 2022), and it can even fruit in both March and September each year in the commercially cultivated garden (28° 19' N, 101° 07' E) of Jiulong County, China, which indicates that *A. trifoliata* might be a day-neutral plant. In addition, abundant available omics data, such as genomic (Zhong et al. 2022a), transcriptomic (Chen et al. 2022) and metabolic (Zhong et al. 2022d) data, provide a good opportunity to comprehensively characterize the *CCT* family profile. Therefore, our objectives are to systemically identify the number and components of the *CCT* family in the *A. trifoliata* reference genome, to completely detail its structural and evolutionary characteristics and to finally determine their expression levels. These results would be helpful for further understanding the molecular mechanism of flowering in *A. trifoliata*.

Materials and Methods

Identification *CCT* family in *A. trifoliata*

Genome sequence annotation files of *A. trifoliata* (accession ID: GWHBISH000000000) were downloaded from the National Genomics Data Centre (<https://ngdc.cncb.ac.cn/>; accessed on 9 September 2024). The hidden Markov model of conserved *CCT* sequences (PF06203) (<http://pfam-legacy.xfam.org/>; accessed on 9 November 2024) was employed to identify *AktCCT*s. In addition, the sequences of all the proteins of *A. trifoliata* were then scanned by HMMER 3.0 using the HMM with an E value of 1×10^{-5} , in which the amino acid sequences of *Arabidopsis* *CCT*s were downloaded from the TAIR website (<https://www.Arabidopsis.org/>; accessed 9 September 2024) and used to identify *AktCCT*s. Only those *AktCCT*s that could be identified by the two methods were treated as the final identified *AktCCT*s. Moreover, the physicochemical properties of the predicted *AktCCT*s were determined using the ExPASy ProtParam website (<https://web.expasy.org/protparam/>; accessed on 9 November 2024).

Conserved protein structure and chromosomal localization of *AktCCT*

The MEME website (<https://meme-suite.org/meme/>; accessed 9 September 2024) was used with the default parameters to analyze the conserved motifs in the putative *AktCCT*s. Subsequently, TBtools (version 1.0876, CJ Chen, Guangzhou, China) was utilized to map the *AktCCT*s on the chromosomes, adopting the gff file of *A. trifoliata*. Additionally, TBtools was used to extract parameters such as gene length and gene location.

Calculation of Ka/Ks of homologous *AktCCT* pairs

The nonsynonymous substitution (Ka) to synonymous substitution (Ks) values (Ka/Ks) were calculated using TBtools-II software (v2.012, Chengjie Chen, China). Similarly, we performed intraspecific collinearity analysis using TBtools software to determine the duplication type.

Cis-acting elements of the *AktCCT* gene family

To predict putative cis-acting elements in the promoter regions of the *AktCCT*s, the 2,000 bp upstream sequences of the *AktCCT* coding regions were analyzed using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>, accessed on 10 September 2024). After deleting unnecessary cis-acting elements, the distribution of the remaining cis-acting elements of the *AktCCT*s was plotted using Basic Biosequence View in TBtools. In addition, the cis-acting elements were divided into hormone- and environment-responsive types according to their annotation.

Phylogenetic tree construction

The phylogenetic tree of both all *AktCCT*s and 37 reference *AtCCT*s was constructed via the N–J method with 1,000 bootstrap replicates using MEGA 11 software (v11.0.10, Auckland, New Zealand), in which 37 previously reported *AtCCT*s were downloaded from the Plant Transcription Factor Database website (<http://planttfdb.cbi.pku.edu.cn/>, accessed on 24 September 2024) (Tamura et al. 2021). In addition, the evolutionary relationships were constructed using Gene Structure View, and the online tool iTOL (<https://itol.embl.de/>, accessed on 9 October 2024) was used for subsequent visualization.

Subcellular localization of the proteins putatively encoded by *AktCCT*s

To predict the putative subcellular localization of the candidate proteins, in silico analyses were performed using multiple computational tools. Amino acid sequences were submitted to the following online prediction servers: WoLF PSORT (<https://wolfpsort.hgc.jp/>) for comprehensive prediction based on sorting signals and amino acid composition; Plant-mPLOC (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>) for plant-specific multi-label localization; and TargetP 2.0 (<http://www.cbs.dtu.dk/services/TargetP/>) for the identification of N-terminal sequences and prediction of mitochondrial, chloroplast, secretory pathway, or other localizations.

Differential expression of *CCT* in *A. trifoliata*

Transcriptome data for *A. trifoliata* leaves, sepals and stamens during flowering period were downloaded from the NGDC database (login number: PRJCA019245). Additionally, HISAT2 software (v2.1.0, Mihaela Pertea, USA) and DESeq2 (v1.36.0, Michael I. Love, Germany) were used to extract the FPKM

values from the transcriptomic data. We determined the expression of *AktCCTs* in these 3 tissues using HeatMap. Further mapping of the correlations between *AktCCTs* and both *AktGls* and *AktFTs* was performed using OriginPro (2024 (64-bit) SR1 10.1.0.178).

Statistical analysis

The significance of the differences in the characteristic parameters among the various groups was determined using SPSS (version 19.0; Armonk, NY: IBM Corp.), and correlation analysis of the expression levels was also performed using the same software.

Results

Chromosomal distribution and physiochemical characteristics of the *CCT* family in *A. trifoliata*

A total of 46 *CCT* genes were identified in the *A. trifoliata* reference genome, and they were orderly named *AktCCT1* to *AktCCT46* (Table 1, **Table S1**). Physically, all 46 *AktCCTs* were unevenly distributed all 16 chromosomes and most of them were mapped to regions near chromosome ends (Fig. 1), in which chromosome 3 had the most 7 *AktCCTs* while chromosomes 9, 13, 15 and 16 each had only one. According to the definition of gene clusters, the genes were assigned to 41 loci on the chromosomes, including 36 singletons and 5 gene clusters within 250-kb chromosomal regions, in which each cluster consisted of two *AktCCTs* (*AktCCT3/AktCCT4*, *AktCCT13/AktCCT14*, *AktCCT15/AktCCT16*, *AktCCT19/AktCCT20*, and *AktCCT26/AktCCT27*).

Note

The genes in red are distributed in the cluster, and the purple lines represent collinearity between the *AktCCTs*.

Main evolutionary experience of the *AktCCTs*

We found that 33 (71.7%) *AktCCTs* were produced by whole-genome duplication (WGD) or segmental duplication while and the remaining 13 (28.3%) *AktCCTs* including all *AktCCTs* on chromosomes 9, 15 and 16 were produced by dispersal duplication (Table 1). Interestingly, all 10 *AktCCTs* in cluster were produced by WGD or segmental duplication.

Table 1
CCTs identified in *A. trifoliata*.

Name	Genes family			Characteristics of putative proteins						DT
	CP	Begin	End	GL(bp)	NE	NAA	MW (kDa)	PI	SL	
AktCCT1	1	28523241	28526070	2829	5	436	49.1	8.1	N	D
AktCCT2	1	31772454	31779638	7184	6	408	44.9	5.8	N	W/S
AktCCT3	2	5902189	5912295	10106	11	542	40.1	4.9	N	W/S
AktCCT4	2	5976398	5978442	2044	7	296	31.9	9.1	N	W/S
AktCCT5	3	1015390	1019400	4010	4	340	38.6	6.0	N	W/S
AktCCT6	3	2575299	2579508	4209	3	292	33.7	7.1	N	W/S
AktCCT7	3	11202320	11204761	2441	2	382	43.2	5.8	N	D
AktCCT8	3	25181097	25189683	8586	4	481	54.8	5.8	N	D
AktCCT9	3	44086815	44102632	15817	13	422	45.5	5.0	N	W/S
AktCCT10	3	51615841	51624543	8702	8	712	78.9	6.5	N	W/S
AktCCT11	3	53009727	53014571	4844	7	282	32.0	4.8	N	W/S
AktCCT12	4	6504460	6511344	6884	5	400	44.5	4.4	N	D
AktCCT13	4	10441408	10453360	11952	12	347	38.3	4.9	N	W/S
AktCCT14	4	10456733	10466278	9545	7	254	27.5	8.2	N	W/S
AktCCT15	4	40953416	40973777	20361	11	341	37.6	5.1	N	W/S
AktCCT16	4	41035410	41059349	23939	7	310	33.9	5.0	N	W/S
AktCCT17	5	955460	960517	5057	4	480	54.1	5.1	N	W/S
AktCCT18	5	9772961	9774777	1816	2	329	37.3	5.4	N	W/S
AktCCT19	6	5801167	5811188	10021	9	767	83.6	6.3	N	W/S
AktCCT20	6	5976398	5978442	2044	3	268	30.6	5.6	N	W/S
AktCCT21	7	32202888	32210121	7233	8	558	63.2	6.2	N	D
AktCCT22	7	33382781	33385175	2394	2	362	40.0	5.1	C	W/S
AktCCT23	8	2406849	2408961	2112	3	226	26.0	7.7	N	W/S
AktCCT24	8	24582546	24584147	1601	3	217	25.9	5.1	N	D
AktCCT25	8	49018032	49020644	2612	2	381	42.2	5.5	C	W/S
AktCCT26	8	49876689	49879917	3228	2	403	45.7	8.6	N	W/S
AktCCT27	8	49976675	49987459	10784	7	317	35.2	7.2	N	W/S
AktCCT28	9	18517830	18520944	3114	2	414	45.6	5.4	C	D
AktCCT29	10	4329166	4330869	1703	2	359	39.6	5.6	C	W/S
AktCCT30	10	6725436	6742598	17162	8	270	29.3	6.8	N	W/S
AktCCT31	10	7217500	7220765	3265	3	389	44.5	8.7	N	W/S
AktCCT32	10	29942599	29944927	2328	4	320	36.5	5.1	N	W/S
AktCCT33	10	30988975	30995773	6798	4	289	32.6	6.1	N	W/S
AktCCT34	11	8023901	8027094	3193	4	259	29.6	5.0	N	D
AktCCT35	11	11833091	11837383	4292	4	282	32.0	6.3	N	W/S
AktCCT36	11	23047610	23048741	1131	3	224	26.6	4.9	N	D
AktCCT37	11	30582722	30584224	1502	2	156	17.9	9.6	N	D
AktCCT38	12	34672988	34674880	1892	4	322	36.9	5.8	N	W/S
AktCCT39	12	35707409	35711202	3793	4	274	30.9	5.0	N	W/S
AktCCT40	12	36585577	36592109	6532	7	727	81.0	5.6	N	W/S

Name	Genes family			Characteristics of putative proteins						DT
	CP	Begin	End	GL(bp)	NE	NAA	MW (kDa)	PI	SL	
<i>AktCCT41</i>	12	36873128	36878636	5508	8	703	78.6	5.8	N	W/S
<i>AktCCT42</i>	13	351777	358812	7035	5	414	45.4	5.4	N	W/S
<i>AktCCT43</i>	14	2520450	2525582	5132	7	722	80.5	8.2	N	D
<i>AktCCT44</i>	14	4925216	4932511	7295	9	725	80.0	8.7	N	W/S
<i>AktCCT45</i>	15	7240017	7240459	442	2	61	7.4	11.4	N	D
<i>AktCCT46</i>	16	29040983	29045522	4539	4	404	45.3	5.3	N	D

Note: CP: chromosomal position; GL: gene length; NE: number of exons; DT: duplication type; NAA: number of amino acids; MW: molecular weight; PI: isoelectric point; SL: subcellular localization; N: nuclear; C: chloroplast; D: dispersed; W/S: WGD/segmental.

In addition, 27 orthologous pairs related with 33 *AktCCTs* were detected, among which 8 *AktCCTs* (*AktCCT3*, *AktCCT7*, *AktCCT11*, *AktCCT14*, *AktCCT16*, *AktCCT29*, *AktCCT34* and *AktCCT41*) had 3 orthologous gene pairs while 20 *AktCCTs* (*AktCCT2*, *AktCCT8*, *AktCCT10*, *AktCCT12*, *AktCCT15*, *AktCCT17*, *AktCCT18*, *AktCCT19*, *AktCCT20*, *AktCCT23*, *AktCCT25*, *AktCCT26*, *AktCCT27*, *AktCCT30*, *AktCCT31*, *AktCCT32*, *AktCCT33*, *AktCCT39*, *AktCCT42* and *AktCCT44*) only had one orthologous gene pair. The Ka/Ks values varied from 0.15 (*AktCCT30* and *AktCCT25*) to 0.57 (*AktCCT40* and *AktCCT38*), and the average mean Ka/Ks was only 0.33 (**Table S2**).

Conserved structure, class and phylogenetic tree of the *AktCCTs*

We identified a total of 10 conserved motifs (from 1 to 10) with a varying width from 11 amino acids in Motif 6 to 50 amino acids in five motifs (Motif 2, 4, 5, 7 and 9) among 46 putative *AktCCTs* (Table 2), and we also identified five conserved domains (CCT, B-box, GATA, TIFY and REC) (Fig. 2; Table 2). By sequence comparing, we found that CCT, B-box and REC domain distributed across Motif 1 and Motif 6, Motif 2 and Motif 8, and Motif 5 and Motif 7, respectively, while GATA and TIFY only distributed within Motif 3 and Motif 4, respectively. On one hand, two *AktCCTs* (*AktCCT6* and *AktCCT26*) had only Motif 1 while 31 *AktCCTs* had the most 4 motifs (**Table S3**). On the other hand, Motif 1 distributed all 46 *AktCCTs* while both Motif 9 and Motif 10 only existed in five *AktCCTs*. Based on the existence of sequence encoding conserved domain, we classified 46 *AktCCTs* into four classes: 18 *CMFs*, 12 *COLs*, 9 *GTCCs*, and 7 *PRRs*. Many characters such as gene whole length and the exon number, and amino acids number and molecular weight of putative protein existed significant difference among various classes at $p = 0.05$ level. For example, the *COL* class had the lowest average gene length, number of exons while *GTCC* class had the lowest number of amino acids and molecular weight of putative protein. On the contrary, *PRR* had the greatest average gene length, exon number, amino acid number and molecular weights (Table 3).

In addition, the phylogenetic tree of the 46 *AktCCTs* was constructed according to the sequence component, in which 37 reference *AtCCTs* were also employed as the control. According to the phylogenetic tree, 46 *AktCCTs* were assigned into four distinct branches: α , β , γ and δ with 8, 9, 18 and 11 *AktCCTs*, respectively (Fig. 3), and the clustering results of 37 *AtCCTs* were well agreed by the previously reported classification (Huang et al. 2024). Interestingly, all 7 *PRRs* and 9 *GTCCs* were completely assigned into α and β branch, respectively, and similarly, all 18 *CMFs* except of *AktCCT45* and 12 *COLs* except of *AktCCT28* were assigned into γ and δ , respectively, which indicated that some DNA sequence beside that encoded conserved domain would be also experienced a fast evolution.

Table 2
Sequence annotation of motifs of 46 putative *AktCCTs*

Domain	Motif	Sequences	Width
CCT	Motif 1	REARLLRYREKRKERNFEKKIRYASRKALADRRP	34
B-box	Motif 2	CEFC EEAPAVIY CKADAAALCLTCDRNIHSANPLSRRHERTPJC PICGSQ	50
GATA	Motif 3	CQHCGISEKSTPMMRRGPAGPRT LCNACGLMWANKGTLRDL	41
TIFY	Motif 4	GTDL TL S FZGEVYVFD AV SPEKVQAVLLLLGGYEIPSAVPTIDIPSHNN	50
REC	Motif 5	HEICKNIP VIMMSS HDSVGVVFK MLKGAADFLVKPVRK NELRNLWQHVV	50
CCT	Motif 6	RVKGRFVKAKE	11
REC	Motif 7	LVESDDSTRQIVSALLRK CGYEVTA VADGLKAW EILKGRSH NIDLVL TEV	50
B-box	Motif 8	PASVYCRADSAYLC QBCD WNIHGANTLA	28
	Motif 9	IQKSISSGCLSSMDWIHG GAMRPNFLDFQGVDFE AAYGMRRAFSE GDIQT	50
	Motif 10	GDLFKAPEPIIEPTIGLD PMSAAISMISCGEDVIS PQTMKV	42
Note: the part in bold for conserved domain.			

Table 3
Multiple comparative analyses of the protein physicochemical properties of the *AktCCTs* in different subgroups

Group	GL			NE			NAA			MW (kDa)			PI		
	max	min	mean	max	min	mean	max	min	mean	max	min	mean	max	min	mean
COL	8586	1703	6065.5 ^c	13	2	5.28 ^{bc}	480	320	388.4 ^{bc}	54.1	36.5	43.0 ^{ab}	8.6	5	6.28 ^a
GTCC	23939	2044	6498.4 ^{ab}	12	2	5.54 ^c	542	296	368.5 ^c	44.5	31.9	40.4 ^b	9.05	4.37	6.31 ^a
CMF	6884	442	6227.2 ^{bc}	8	2	5.39 ^{ab}	727	61	393.9 ^{ab}	81	7.4	43.6 ^{ab}	11.4	4.9	6.22 ^a
PRR	10021	5132	6626.3 ^a	9	8	5.60 ^a	767	274	405.1 ^a	83.6	30.9	45.3 ^a	8.71	4.76	6.24 ^a
Note: GL, NE, NAA, MW and PI represent the gene length, number of exons, number of amino acids, molecular weight and isoelectric point, respectively. a, b and c indicate significance at the p = 0.05 level.															

Cis-acting components of the *AktCCTs*

By examining the 2,000 bp upstream regions of the *AktCCTs*, we identified a total of 1,220 functional cis-acting elements among all the *AktCCTs*, among which 530 and 690 cis-acting elements belong to 13 hormone- and 15 environment-responsive types, respectively (**Table S4**). Further analysis revealed that among the 13 kinds of hormone-responsive cis-acting elements, the number of cis-acting regulatory elements involved in MeJA responsiveness (MeJAR) and the number of cis-acting regulatory elements involved in root specificity (CRERS) were the greatest (193) and the lowest (1), respectively, and the sole CRERS was in the upstream region of *AktCCT17*. Similarly, among the 15 kinds of environment-responsive cis-acting elements, the number of light-responsive elements (LRMs) and the number of MYB binding sites involved in drought inducibility (MYBBS(D)) and the number of wound-responsive elements (W-Rs) were the greatest (257) and least (2), respectively. In addition, the number of cis-acting elements in each *AktCCT* exhibited large variation. For example, there were up to 59 (34 hormone-responsive types and 25 environment-responsive types) cis-acting elements in *AktCCT8*, whereas there were only 9 (4 hormone-responsive types and 5 environment-responsive types) cis-acting elements in *AktCCT1*.

Note

CEIAAR: cis-acting element involved in abscisic acid responsiveness; CEICCR: cis-acting element involved in cell cycle regulation; CEIDSR: cis-acting element involved in defense and stress responsiveness; CEIGR: cis-acting element involved in gibberellin responsiveness; CEILR: cis-acting element involved in light responsiveness; CEILTR: cis-acting element involved in low-temperature responsiveness; CEISAR: cis-acting element involved in salicylic acid responsiveness; CREEAI: cis-acting regulatory element essential for anaerobic induction; CREIAR: cis-acting regulatory element involved in auxin responsiveness; CREICC: cis-acting regulatory element involved in circadian control; CREIEE: cis-regulatory element involved in endosperm expression; CREILR: cis-acting regulatory element involved in light responsiveness; CRERS: cis-acting regulatory element, root specific; DPMC: element involved in the differentiation of palisade mesophyll cells; EFMEMA: element for maximal elicitor-mediated activation (2 copies); ELASI: enhancer-like element involved in anoxic-specific inducibility; GRE: gibberellin-responsive element; LRM: light-responsive element; MeJAAI: MeJAR essential for anaerobic induction; MeJAR: cis-acting regulatory element involved in MeJA-responsiveness; MYBBS(D): MYB binding site involved in drought-inducibility; MYBBS(FBGR): MYB binding site involved in flavonoid biosynthetic genes regulation; MYBBS(LR): MYB binding site involved in light responsiveness; PCMLR: part of a conserved DNA module involved in light responsiveness; PLRE: part of a light response element; PLRM: part of a light-responsive module; W-R: wound-responsive element; ZMR: cis-acting regulatory element involved in zein metabolism regulation.

Expression pattern analysis of the *CCT* family genes of *A. trifoliata*

The transcript levels of all 46 *AkCCTs* were analyzed in various tissues (leaves, sepals and stamens) at different developmental stages during flowering. The results showed that although the majority of *AktCCTs* had relatively low expression, but 19 *AktCCTs* had a detectable expression level in leaf. *AkCCT27* exhibited high expression across all tissues during whole period (Fig. 5). In addition, *AkCCT26* was highly expressed at sepals and stamens. Previous studies showed that *FT* and *GI* were involved in the CO related pathway (Mizoguchi, 2005), and they should also be used to determine the expression profile of the *AktCCTs* in the known "GI-CO-FT" model, so that we further identified two *AktGIs* (*AktGI1* and *AktGI2*) and seven *AktFTs* (from *AktFT1* to *AktFT7*) (**Table S5**). In addition, we found that *AktFT2* and *AktFT4* was highly expressed in leaf and that the expression of both *AktGIs* was very low in all tissues at all time points, but the expression of *AktGI1* was detectable (Fig. 5). Interestingly, *AktFT2*, *AktFT4* and *AktFT5* as well as *AktGI1* had significantly co-expressed relationship with many *AktCCTs* such as *AktCCT17* (Fig. 6).

Note

Red represents a positive correlation, blue represents a negative correlation, and the darker the colour, the stronger the correlation. * for the significance at the level of p = 0.05.

Discussion

To adapt to seasonal and environmental changes, plants have specifically evolved a regulatory mechanism that optimizes developmental events via the light response pathway (Takagi et al. 2023), in which CCT family members play very important roles in various processes, such as flowering, circadian rhythms and even the abiotic stress response (Liu et al. 2020). This suggests that the structure and function of CCTs may have experienced rapid variation and divergence during evolution. In the present study, we found that among the 46 *AktCCTs*, many physicochemical parameters, such as gene length, number of

exons, number of amino acids, and the molecular weight and isoelectric point of the putative proteins, differed substantially (Table 1). In fact, structural diversity may be favored by various evolutionary experiences. Previous studies have demonstrated that WGD is an important evolutionary force in almost all organisms, especially plants, and moreover, WGD is usually related to different clades (Jiao et al. 2011). For example, a recent report suggested that θ WGD and ω WGD favored the survival of eudicots and the thriving of core eudicots around the Jurassic-Cretaceous boundary and the Middle Cretaceous, respectively (Zhong et al. 2022c). Here, 33 (71.7%), including all 10 *AktCCTs* found in gene clusters out of the 46 *AktCCTs*, were produced by WGD or segmental duplication (Table 1), which could have presented an important opportunity for numerous variations in *AktCCTs* to evolve. In addition, the small *Ka/Ks* value suggested that *AktCCTs* might have undergone purifying selection during evolution and maintained a conserved function (**Table S2**), which helps *A. trifoliata* quickly eliminate damaged mutants, especially individuals whose light-responsive pathways are disrupted (Wang et al. 2011; Hsu et al. 2014).

The chloroplast is the most important organ related to the light-responsive process (Schwenkert et al. 2022), and interestingly, all 4 *AktCCTs* (*AktCCT22*, *AktCCT25*, *AktCCT28* and *AktCCT29*), encoding proteins localized in the chloroplast, only had two exons and they belonged to the *COL* type. (Fig. 3; Table 1). Moreover, their promoters contained both MeJAR hormone-responsive and LRM environment-responsive cis-acting elements (Fig. 4; **Table S5**). On the other hand, their compartmental distribution and cis-acting element component indicated that they may retain the basic function of responding to changes in light, further suggesting that the *AktCCTs* might be older. In addition, on the basis of the knowledge of *CCTs*, the *COL* subfamily is the oldest, whereas other subfamilies, especially *CMF*, are usually the evolved offspring of *COLs*. Therefore, how *COLs* produced other subfamilies is one of our concerns.

In total, the most distinct difference between the *COLs* and other types was the existence of a B-box (Cockram et al. 2012; Tribhuvan et al. 2022; Li et al. 2024). In this study, we found that among the various subfamilies, the gene length, number of exons, number of amino acids and molecular weight of the *PRR* members were the largest. In contrast, the number of amino acids and molecular weight of *GTCC* were the smallest while the gene length and exon number of *COL* was the smallest (Table 3). Based on this information, we propose that the *CMFs* could have been produced from *COLs* mainly by losing the DNA regions putatively encoding the B-box conserved domain, whereas the *PRRs* and *GTCCs* might have been produced mainly via the insertion of new DNA sequences into the encoding and non-encoding regions of the corresponding B-box sequence, respectively.

Various studies have suggested that *CO* or *COL* can directly regulate the key downstream flowering gene *FT* by altering its status and that this process is regulated by the upstream gene *GI*; thus, a known "GI-CO-FT" regulatory model was proposed (Fowler et al. 1999; David et al. 2006; Sawa et al. 2007; Imaizumi et al. 2010). From the perspective of the system phylogenetic tree, most *AktCCTs* such as *AktCCT17* were distributed into the β branch that contained the key *Arabidopsis* flowering regulatory gene *CO* (*At5g15840*) (Figs. 3). Otherwise, two *AktCCTs* (*AktCCT17* and *AktCCT27*) obviously upregulate in leaves (Fig. 5, Fig. 6). Both *AktCCT17* and *AktCCT27* exhibited a significant co-expression with *AktFT2*, *AktFT4*, *AktFT5* and *AktGI1* involved in "GI-CO-FT" model, but the unique cis-acting elements binding with the sole root-related CRERS transcription factor existed in *AktCCT17* (**Table S4**). Moreover, previous studies showed that flowering is highly related with root behavior (Zou et al. 2020), so that *AktCCT17* could play a key role in flowering of *A. trifoliata*.

Overall, genome duplication events, especially WGD, afforded an important opportunity for the diversification of the *AktCCTs*, and at the same time, strong purifying selection facilitated their evolution toward favorable environmental adaptation. In addition, the sequences encoding conserved domains and the other sequences in the *AktCCTs* may have evolved in parallel to further enrich the structural and functional diversity. Finally, *AktCCT17* may play a very important role in regulating flowering.

Declarations

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 potential conflicts of interest.

Supplementary Material

The Supplementary Material for this article can be found online.

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Author Contribution

DL: formal analysis, Software, Writing - Original Draft - Visualization. XY, HY, YX, CC, FT, YH and JL: Obtained the data - Participated in the data analysis, Discussion. XL: Proofreading the language of the manuscript. PL: Conceptualization, Writing - Review and Editing, Project - Administration, Funding Acquisition. All the authors contributed to the discussion of the results, reviewed the manuscript and approved the final article. Details of all funding sources

should be provided, including grant numbers if applicable. Please ensure to add all necessary funding information, as after publication this is no longer possible.

Data Availability

The datasets presented in this study can be found in online repositories. The names of the repositories/repositories and accession number(s) can be found in the article/Supplementary Material.

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Figures

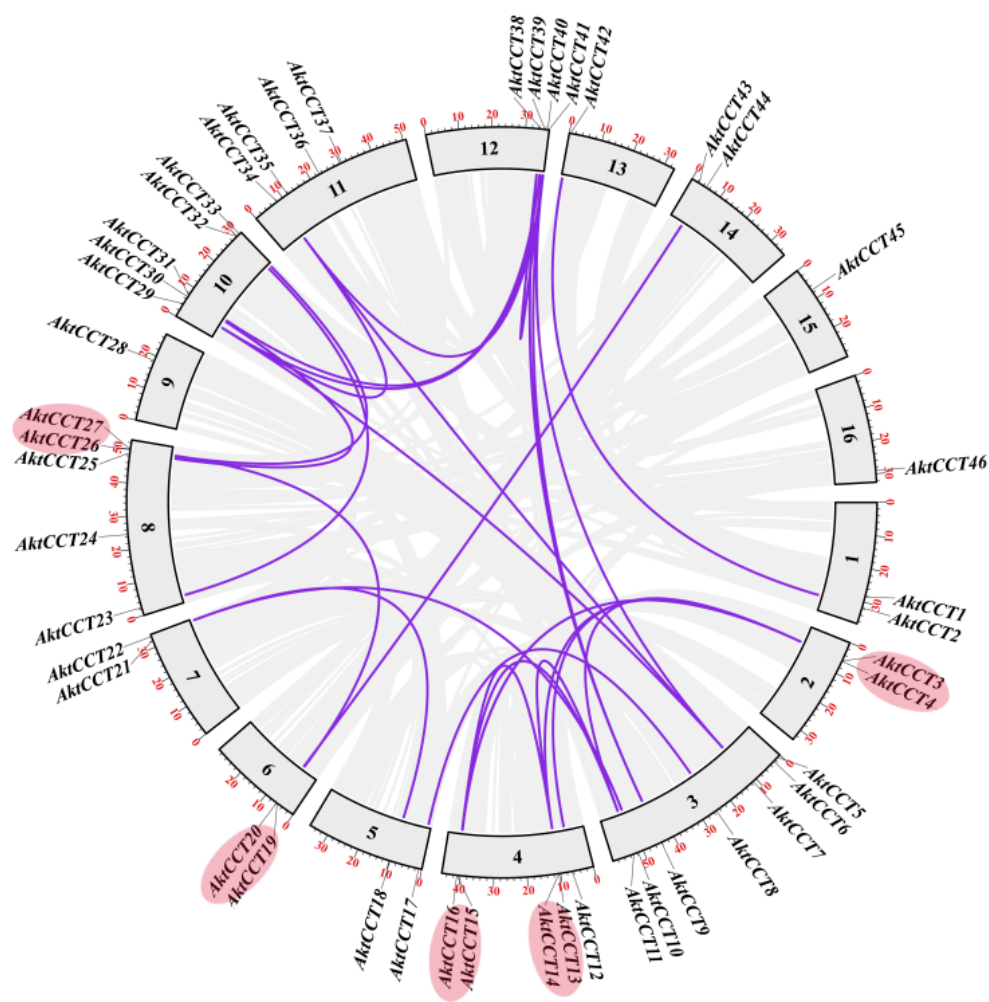


Figure 1

Distribution and collinearity of the AktCCTgenes on chromosomes in *A. trifoliata*.

Note: The genes in red are distributed in the cluster, and the purple lines represent collinearity between the AktCCTs.

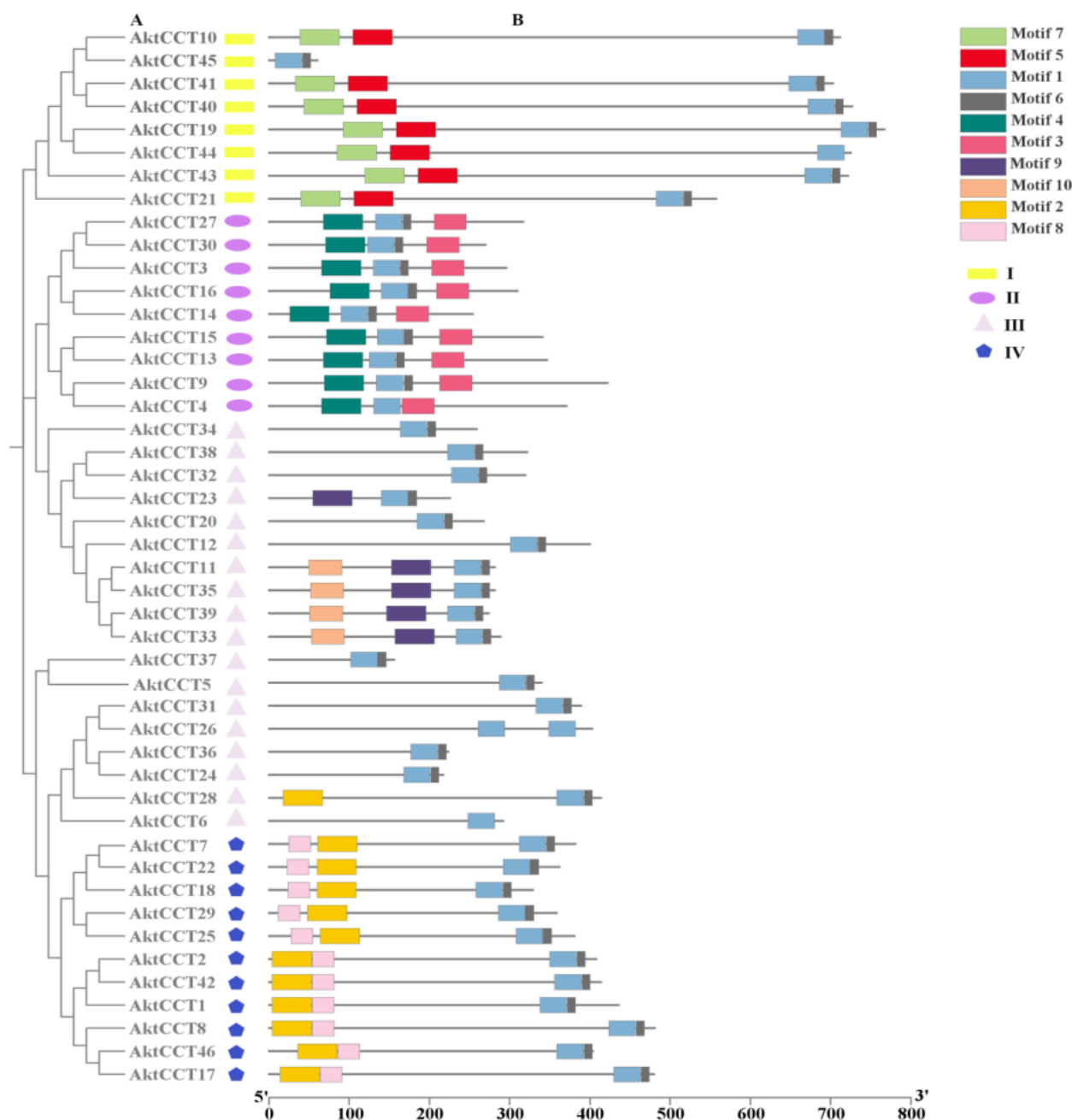


Figure 2

Gene and protein structure analyses of the AktCCT family. A for phylogenetic analysis of the AktCCTs; B for the distribution of conserved motif in the putative AktCCTs

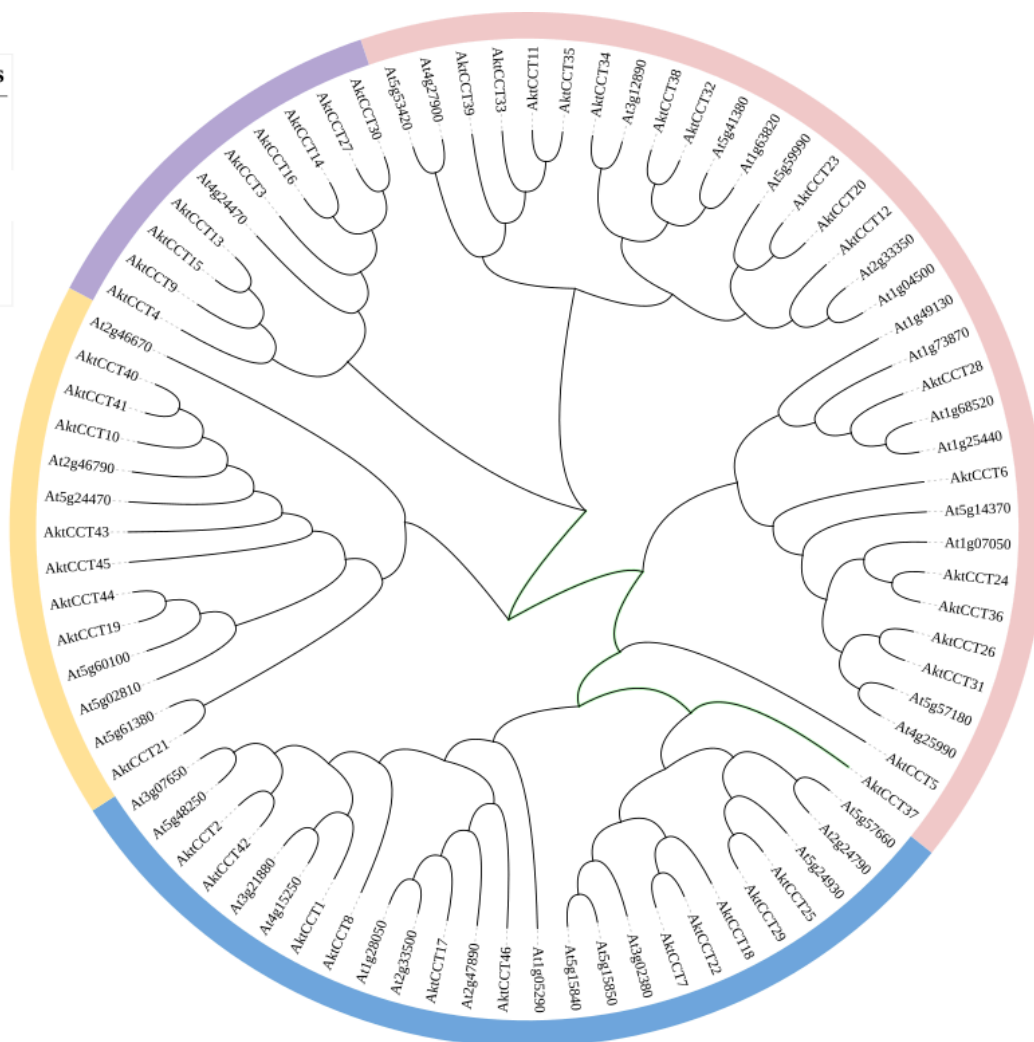


Figure 3

Phylogenetic tree consisting of the 46 AktCCTs and 37 AtCCTs

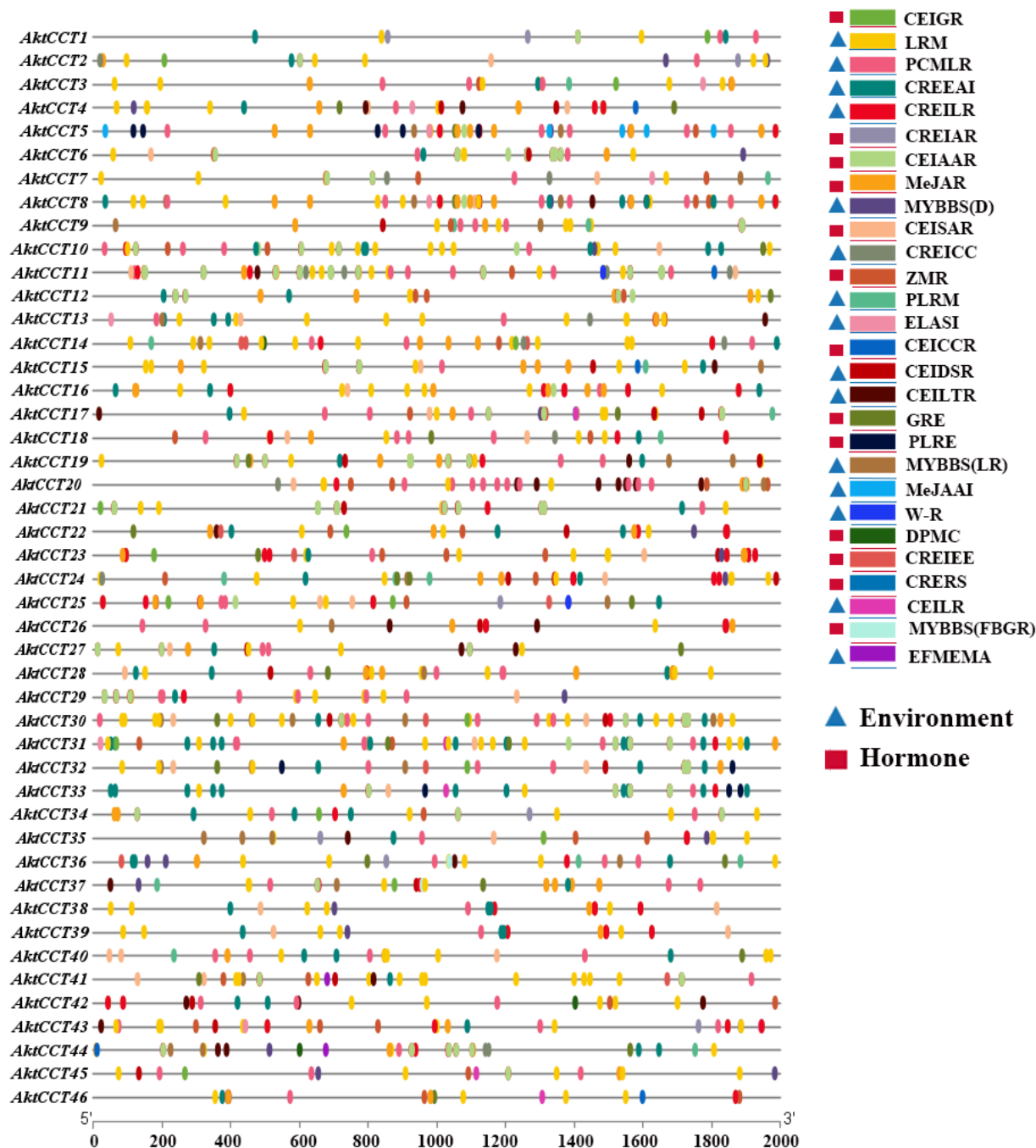


Figure 4

Distribution of cis-acting elements within the upstream 2000 bp region of the *AktCCTs* in *A. trifoliata*

Note: CEIAAR: cis-acting element involved in abscisic acid responsiveness; CEICCR: cis-acting element involved in cell cycle regulation; CEIDSR: cis-acting element involved in defense and stress responsiveness; CEIGR: cis-acting element involved in gibberellin responsiveness; CEILR: cis-acting element involved in light responsiveness; CEILTR: cis-acting element involved in low-temperature responsiveness; CEISAR: cis-acting element involved in salicylic acid responsiveness; CREEAI: cis-acting regulatory element essential for anaerobic induction; CREIAR: cis-acting regulatory element involved in auxin responsiveness; CREICC: cis-acting regulatory element involved in circadian control; CREIEE: cis-regulatory element involved in endosperm expression; CREILR: cis-acting regulatory element involved in light responsiveness; CRERS: cis-acting regulatory element, root specific; DPMC: element involved in the differentiation of palisade mesophyll cells; EFMEMA: element for maximal elicitor-mediated activation (2 copies); ELASI: enhancer-like element involved in anoxic-specific inducibility; GRE: gibberellin-responsive element; LRM: light-responsive element; MeJAAI: MeJAR essential for anaerobic induction; MeJAR: cis-acting regulatory element involved in MeJA-responsiveness; MYBBS(D): MYB binding site involved in drought-inducibility; MYBBS(FBGR): MYB binding site involved in flavonoid biosynthetic genes regulation; MYBBS(LR): MYB binding site involved in light responsiveness; PCMLR: part of a conserved DNA

module involved in light responsiveness; PLRE: part of a light response element; PLRM: part of a light-responsive module; W-R: wound-responsive element; ZMR: cis-acting regulatory element involved in zein metabolism regulation.

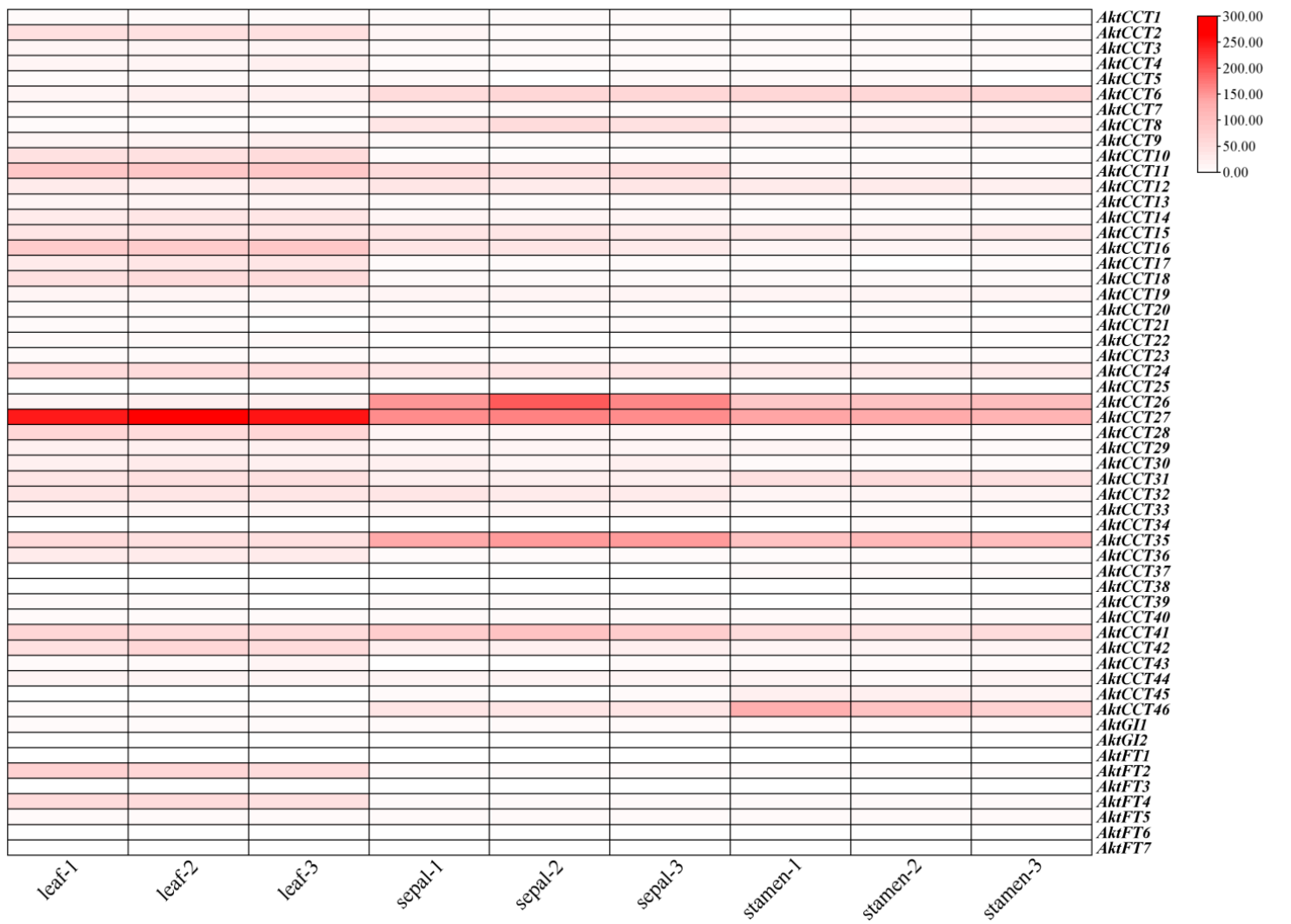


Figure 5

Expression level of 46 AktCCTs, 2 AktGIs and 7 AktFTs in various tissues of *A. trifoliata* at different developmental stages

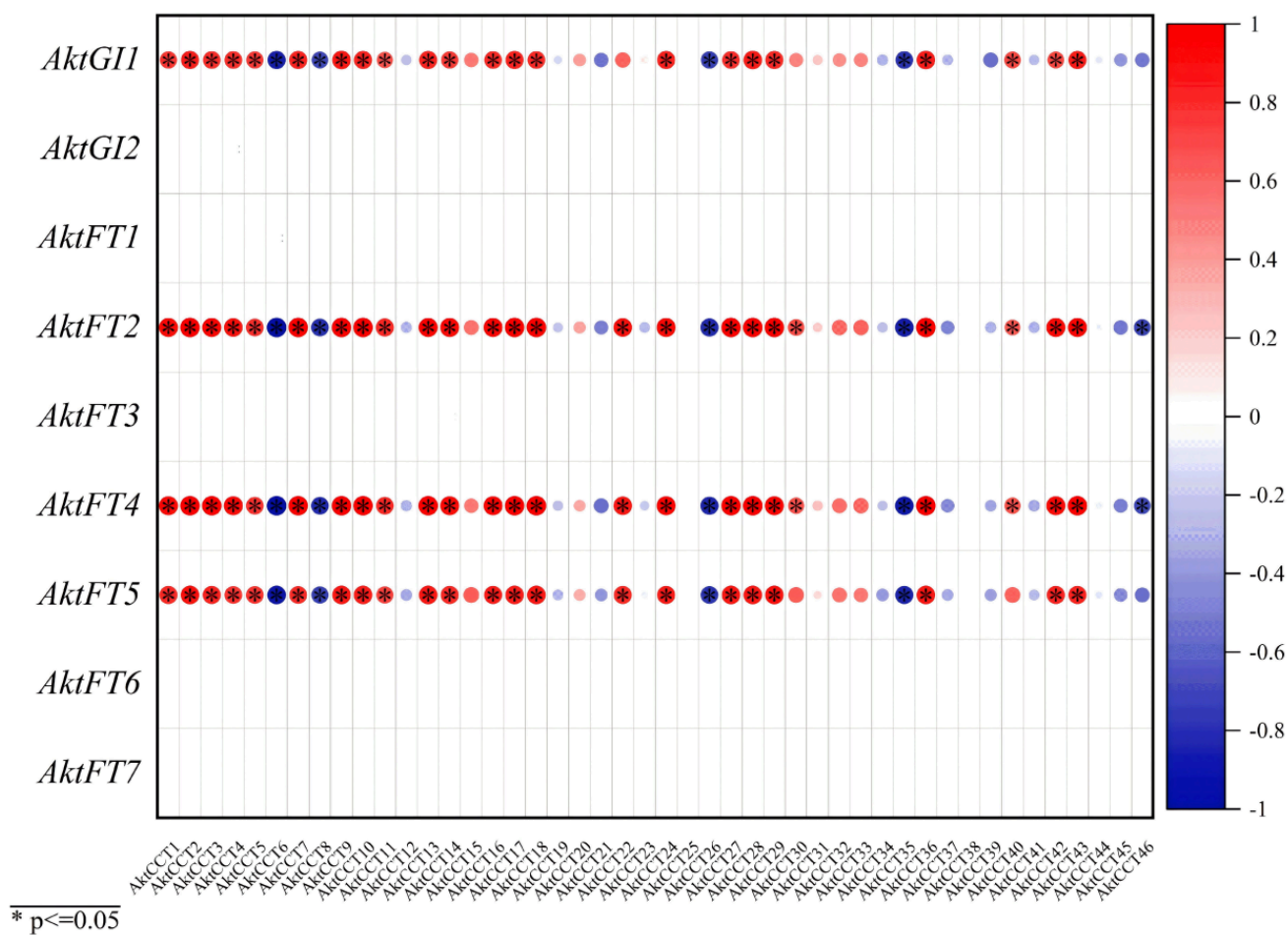


Figure 6

Co-expression between 46 AktCCTs and both 2 AktGIs and 7 AktFTs

Note: Red represents a positive correlation, blue represents a negative correlation, and the darker the colour, the stronger the correlation. * for the significance at the level of $p = 0.05$.