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Differential response of *AP2/ERF* genes under hormone and light in *Coptis chinensis*

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Abstract:

Background: The AP2/ERF transcription factor family is crucial for regulating plant growth, development, and responses to environmental stimuli. However, the genomic characterization and functional dynamics of this family in the medicinal plant *Coptis chinensis* remain largely unexplored.

Results: A total of 125 *CchAP2/ERF* genes were identified in the *C. chinensis* genome and classified into four subfamilies: AP2 (25), ERF (94), RAV (4), and Soloist (2). Chromosomal localization and synteny analyses indicated that the expansion of the CchAP2/ERF family was primarily driven by whole-genome and segmental duplications. Significant divergence in the cis-acting elements of duplicated gene pairs suggested functional neofunctionalization or subfunctionalization. Gene structure analysis revealed lower intron polymorphism in the ERF and RAV subfamilies compared to the AP2 and Soloist subfamilies, implying a potential capacity for rapid transcriptional activation. Expression profiling demonstrated distinct spatial and temporal patterns, with specific genes exhibiting tissue-specific (e.g., *CchERF02* in roots, *CchERF03* in leaves) and age-dependent (e.g., *CchERF50* and *CchERF53*) regulation. Furthermore, qRT-PCR analyses under hormone and light treatments identified *CchERF45* as a central regulatory hub, exhibiting remarkable induction by abscisic acid (peaking at 5 μmol), methyl jasmonate, red light,

a 12 h/12 h photoperiod, and moderate light intensity (2K Lux). Conversely, *CchERF14* was significantly suppressed by both ABA and MeJA, implicating it as a potential negative regulator in hormone signaling crosstalk.

Conclusion: This study comprehensively elucidates the evolutionary divergence and expression dynamics of the *CchAP2/ERF* family in *C. chinensis*. The identification of key transcription factors, particularly *CchERF45* and *CchERF14*, provides crucial genetic targets for understanding hormone and light signaling networks, offering valuable genetic resources for the molecular breeding and targeted cultivation of *C. chinensis*.

Keywords: *Coptis chinensis*, AP2/ERF, Hormone, Light, Expression analysis

Introduction

The AP2/ERF transcription factor family represents the largest group of transcription factors in plants, playing a pivotal role in plant growth, development, and stress responses [1-3]. Characterized by the presence of at least one AP2 domain, comprising 60-70 highly conserved amino acids that form a typical helix-turn-helix structure [4], the AP2/ERF family is subclassified into the AP2, ERF, RAV, and Soloist subfamilies based on the number and characteristics of AP2 domains [5]. AP2/ERF transcription factors bind to target genes through the conserved YRG and RAYD motifs within their AP2 domains, regulating gene transcription [6]. Members of the AP2/ERF family are crucial in various plant biological processes and stress responses. For instance, transcription factors from the DREB subfamily can bind to the DRE/CRT cis-acting elements [7], activating the expression of downstream

genes involved in stress resistance [8]. AP2/ERF transcription factors are extensively involved in plant responses to various abiotic stresses, such as drought [9], salinity [10], heat [11], and cold [12]. These transcription factors are essential for plant stress adaptation and serve as valuable genetic resources for breeding stress-resistant crops. Additionally, AP2/ERF transcription factors play a significant role in the biosynthesis of plant secondary metabolites, such as regulating the synthesis of flavonoids [13], carotenoids [14], and lignin [15]. Therefore, AP2/ERF transcription factors are vital in plant growth, development, and stress responses, making them important candidates for studying plant physiology and molecular mechanisms.

Coptis chinensis Franch., a perennial herb of the Ranunculaceae family and the Coptis genus, is predominantly found in high-altitude mountainous regions in the southwestern part of China [16]. Recognized as a traditional Chinese medicinal herb, *C. chinensis* is renowned for its therapeutic properties, including heat-clearing, dampness-drying, fire-purging, and detoxifying effects, earning it the title of “King of Chinese Medicine” [17]. The primary bioactive constituent in *C. chinensis* is berberine [18], which possesses a plethora of pharmacological activities, such as anti-inflammatory, antimicrobial, antiviral, and anticancer effects [19-22].

In recent years, with the advancement of molecular biology techniques, the in-depth study of plant growth, development, and stress response mechanisms has led to an increasing focus on transcription factors as key regulators of gene expression [23, 24]. The AP2/ERF transcription factor family, one of the largest in plants, plays a crucial role in governing various biological processes, including plant growth and stress responses [25]. Although research on the AP2/ERF family has made headway, investigations in medicinal plants like *C. chinensis* are still in their

infancy. This study aims to analyze the gene structure, conserved domains, evolutionary relationships, and expression patterns in different organs of the AP2/ERF transcription factor family members in *C. chinensis*, providing a theoretical basis for further elucidating the molecular mechanisms underlying the growth, development, and pharmacological properties of *C. chinensis*.

Materials and methods

Identification of *CchAP2/ERF* genes

Genome sequences and annotation files of *C. chinensis* were obtained from public databases (https://www.bic.ac.cn/IMP/) [26]. AP2/ERF protein sequences of Arabidopsis thaliana were retrieved from the TAIR website (https://www.arabidopsis.org/). Two methods were employed to identify members of the *CchAP2/ERF* gene family in *C. chinensis*: i) Bidirectional blastp: Firstly, local Blast programs (E-value ≤ 1e−5) were used to search for AP2/ERF protein sequences in the *C. chinensis* genome using Arabidopsis AP2/ERF protein sequences as queries. The results were then searched against the swissprot database (E-value ≤ 1e−5), and the output proteins were manually filtered. ii) Conservation domain-based: The AP2/ERF domain (PF00847) was downloaded from the Pfam database (http://pfam.xfam.org), and the Advanced HMMER Search program in TBtools was used to identify *C. chinensis* AP2/ERF proteins. The results of the two methods were combined, and the CchAP2/ERF proteins were confirmed using NCBI CD-Search (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Proteins without complete conserved domains or redundant sequences were manually removed. The CDS sequences and protein sequences of *CchAP2/ERF* genes were obtained using the Gtf/Gff3 Sequences Extract and Batch Translate CDS to Protein plugins in TBtools, respectively, for subsequent analysis. The Molecular

Weight, Theoretical pI, Instability Index, Aliphatic Index, and Grand Average of Hydropathicity of CchAP2/ERF proteins were analyzed using the Protein Parameter Calc program in TBtools. The Subcellular localization of CchAP2/ERF proteins was predicted using the online analysis program WoLF PSORT (http://wolfpsort.org/).

Chromosomal map, gene duplication, and collinearity analysis of *CchAP2/ERF* Genes

The gene density and the positions of the *AP2/ERFs* on the chromosomes, analysis of gene duplication events, tandem duplication, and segmental duplication were analyzed using the subprograms Gene Location Visualize from GTX/GFF and Dual Systeny Plot for MCscanX in the TBtools software. Additionally, the subprogram WGDI in TBtools was employed to validate gene duplication and collinearity analysis results, and generate Dot plots. All the analysis results were visualized using the TBtools software.

Analysis of conserved motifs, gene structures, phylogenetic tree and gene promoters

The conserved motif of CchAP2/ERF was analyzed through the online analysis program MEME 5.5.5 (https://meme-suite.org/meme/tools/meme) with parameters set to 10 motifs, model being Any Number of Repetitions (anr), and other parameters at default. NCBI CD-Search was employed to predict the conserved structural domains of CchAP2/ERF. The exons and introns information in the *CchAP2/ERF* family genes were retrieved from the annotated genome of *C. chinensis*. The phylogenetic relationship between *C. chinensis* itself, and between *C. chinensis* and Arabidopsis, was analyzed using the subprogram One Step Build a ML Tree in TBtools. This program utilized Muscle for sequence alignment, trimAL for trimming the aligned sequences, and

IQ-tree for constructing the Maximum Likelihood (ML) phylogenetic tree (UltraFast BootStrap Number: 5000). According to the IQ-tree output and BIC values, the best-fit model was determined to be VT+F+R8. The online software iTOL (https://itol.embl.de/) was used to beautify the tree. The upstream 2000 bp sequence of the start codon (ATG) of the *CchAP2/ERF* genes was extracted using the GXF Sequences Extract subprogram in TBtools and submitted to the PlantCARE online service (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for analysis of cis-acting elements [27]. All analysis results were visualized using TBtools software.

Expression profile analysis of *CchAP2/ERF* genes

To analyze the expression profiles of *AP2/ERF* genes in *C. chinensis* across different years, locations, and organs, 17 *C. chinensis* RNA-Seq datasets were obtained from the NCBI public database (https://www.ncbi.nlm.nih.gov/sra), including datasets from different ecotypes (Shizhu, Zunyi, Zhenping, accession number: PRJNA361017), 3-year-old Enshi plants (root, stem, leaf, accession number: PRJNA662860) , 4-year-old plants (petiole, leaf, root, rhizome, accession number: PRJNA662860) [19], and 5-year-old plants (rhizome, stem, leaf, flower, accession number: PRJNA649082). These files were converted into paired-end sequencing Fastq files using the subprogram SRA to Fastq in the TBtools software. With the *C. chinensis* genome as the reference, the expression levels of all genes in *C. chinensis* were obtained using the subprogram Kallisto Super Wrapper in TBtools, and the expression data of *C. chinensis AP2/ERF* genes were extracted. These data were visualized using the HeatMap from TBtools software.

qRT-PCR analysis of *CchAP2/ERF* genes

Callus tissue of *C. chinensis*, provided by our laboratory, was subcultured in a basic medium consisting of MS medium supplemented with 2,4-D (1.5 mg/L) and 6-BA (1 mg/L). The culture was maintained at (25±1)°C in the dark, with subculturing carried out every 20 days. Healthy callus tissues with dense structures were selected as experimental materials for further processing.

For the ABA treatment, three variable control groups were established: a control group (CK), an ABA treatment group with concentrations at 1, 5, and 10 μmol/L, and a MeJA treatment group with concentrations at 50, 100, and 200 μmol/L. Sampling occurred at 0 d (CK), 0.5 d, 1 d, 7 d, and 14 d in each group. In the light treatment study, a control group was maintained in a dark environment, while three additional groups were exposed to different light qualities, light periods, and light intensities. The light quality group was exposed to white, red, blue light, and UV radiation, the light period group to 12 h day/12 h night and 16 h day/8 h night cycles, and the light intensity group to 2K Lux and 10K Lux. Each treatment group consisted of 3 bottles, each containing 3 uniform callus tissues. Samples were collected at 0 h, 12 h, 24 h, 36 h, and 48 h, rapidly frozen in liquid nitrogen, and stored at -80°C for subsequent analysis.

Total RNA was extracted from the samples using the Trizol method (Total RNA Extract Reagent, Beijing Coolaber), and cDNA was synthesized with NotionScript qPCR First-strand cDNA Synthesis Mix (with dsDNase), Beijing Coolaber, Beijing Coolaber Science and Technology Co., Ltd.). For real-time fluorescent quantitative PCR (qPCR), the SYBR GREEN dye method was employed on the qPCR instrument. The reaction mixture comprised 20 μL with the following components and volumes: SYBR GREEN Premix 10 μL, RNase - Free ddH_2O 8 μL, upstream primer (F) 0.5 μL, downstream primer 0.5 μL, and cDNA template 1 μL. The qPCR amplification protocol consisted of pre-denaturation at 95°C for 2 min (1 cycle), denaturation at

95°C for 15 s (40 cycles), annealing at 60°C for 15-30 s (40 cycles), and extension data collection at 72°C for 30 s (40 cycles). Each sample was analyzed in triplicate, using *CchTCTP* as a housekeeping gene, and relative expression levels were determined using the $2^{-\Delta\Delta Ct}$ approach.

Statistical analysis

All results are represented as means ± SE (standard error). Excel 2019 was used for data processing. Statistical analysis of the physiological data was conducted using DPS (Version 12.01 for Windows, DPS, Shenzhen, China). Significant differences among treatments were determined using one-way ANOVA and Duncan's multiple comparison analysis ($p < 0.05$). A simple graph was drawn with Origin 2019.

Results

Identification and physical characteristics of CchAP2/ERF

A total of 125 *AP2/ERF* genes were identified in the *C. chinensis* genome through Hidden Markov Model (HMM) and BLAST searches. The physicochemical characteristics of the CchAP2/ERF proteins were further analyzed (Table S1). The results showed that the molecular weights of the 125 AP2/ERF proteins ranged from 8,000 to 74,000 Da. The theoretical isoelectric points, instability indices, aliphatic indices, and grand averages of hydropathicity were in the ranges of 4.13-10.96, 22.78-81.6, 42.76-96.79, and －1.143 to －0.05, respectively. Subcellular localization predictions indicated that most CchAP2/ERF proteins were localized in the nucleus, with only small amounts localized in the chloroplast, cytoplasm, mitochondria, and plastid. CchAP2_12, CchERF12, -38, -43, -51, -52, -76, and -82 were localized in the mitochondria, while

CchERF43 was localized in the plastid. These results suggest that CchAP2/ERF proteins may play diverse roles in various biological processes in *C. chinensis*.

Chromosomal localization, microsynteny, and gene duplication events of *CchAP2/ERF*

CchAP2/ERF genes were distributed across 9 chromosomes, with chromosome 2 having the most genes (19) and chromosome 9 having the fewest genes (6). Certain *CchAP2/ERF* genes were arranged in clusters on chromosomes, likely due to tandem duplication of the genome. Additionally, *CchAP2* and *ERF* genes were distributed across all chromosomes, while RAV-type genes were found on chromosomes 2, 5, and 7, and CchSoloist genes on chromosomes 3 and 7. Chromosome 7 contained the most diverse types of *CchAP2/ERF* genes (Figure 1).

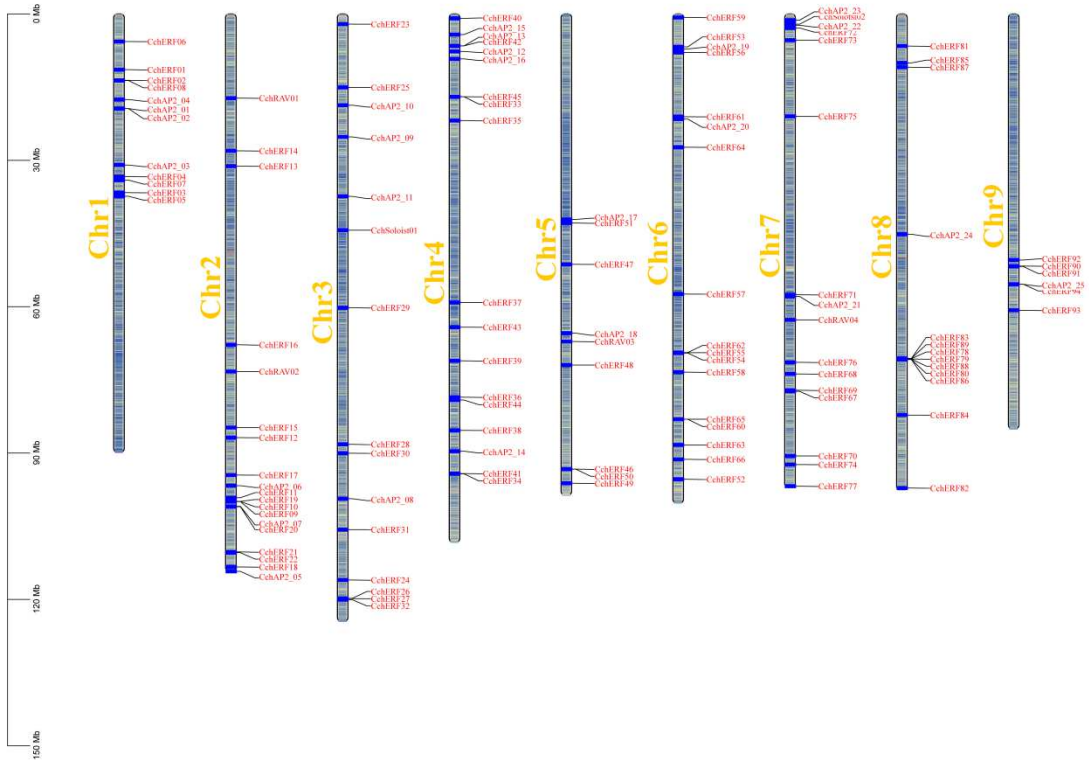


Figure 1: Chromosomal location and distribution of *AP2/ERF* genes in the *C. chinensis* genome. Chromosomes are depicted as rectangles, with the legend on the left indicating the length of each chromosome. The varying colors along these rectangles represent gene density.

The dark blue segments within the chromosomes denote the positions of *AP2/ERF* genes.

Intra-species collinearity analysis revealed that only 2 pairs of *CchAP2/ERF* genes were collinear (*CchAP2_05* & *CchAP2_19*, *CchERF48* & *CchERF71*), except for CchSoloist genes. This suggests that homology is more likely to be found between genes in the same subfamily, indicating a higher probability of similar biological functions. Furthermore, chromosomes 8 and 9 showed no collinearity (Figure 2, Table S2). These results may be attributed to previous whole-genome duplication (WGD) or chromosomal segmental duplication. Dot plots were obtained to clarify whether the occurrence of these gene pairs was related to extensive gene duplication events (Figure 3，Table S2). The Dotplot of *C. chinensis* vs. *C. chinensis*, particularly on chromosomes 5, 6, and 8, indicated possible genome doubling events, with red lines on the diagonal indicating self-tandem duplications . The coloring of blocks in *C. chinensis* around Ks=0~0.25 might represent the main time points of *CchAP2/ERF* gene duplication events.

To clarify the orthologous genes and evolutionary relationships among different species and the generation and functional differentiation of *AP2/ERF* homologous genes, comparative genomics analyses were performed on *C. chinensis*, *A. thaliana*, and *O. sativa*. Few synteny blocks were observed between *C. chinensis*, *A. thaliana*, and *O. sativa*, with 2 and 3 pairs, respectively (Figure 4).

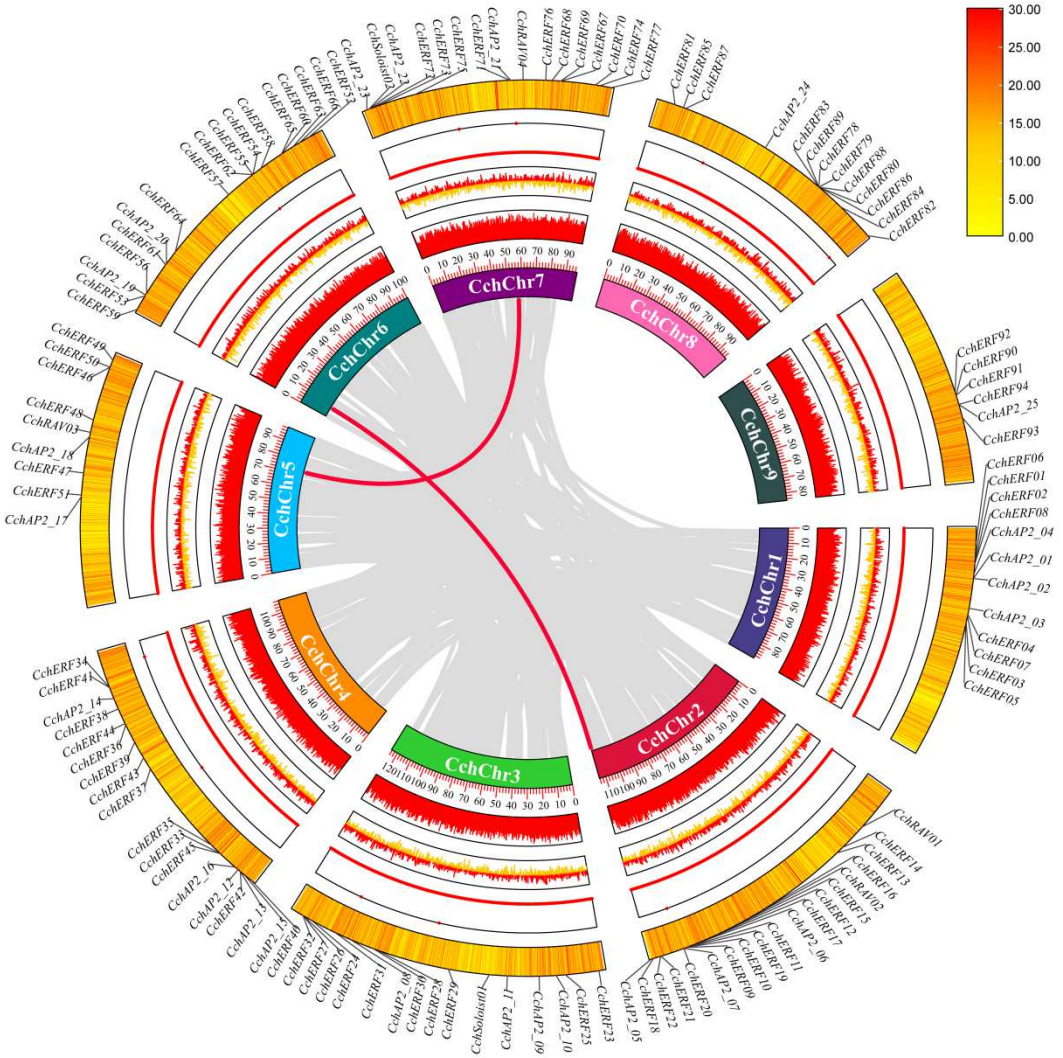


Figure 2: Microsynteny mapping of *AP2/ERF* genes in *C. chinensis* genome. The central sector bars indicate the length of the chromosomes. Progressing from the innermost to the outermost part of the outer ring, the following data are represented: GCratio (bar chart), GC skew (line graph), N ratio (dot plot), gene density (heatmap), and *AP2/ERF* genes. Gray lines connecting the chromosomes depict all collinear regions across the genome, while red lines highlight the collinear modules specific to *AP2/ERF* genes.

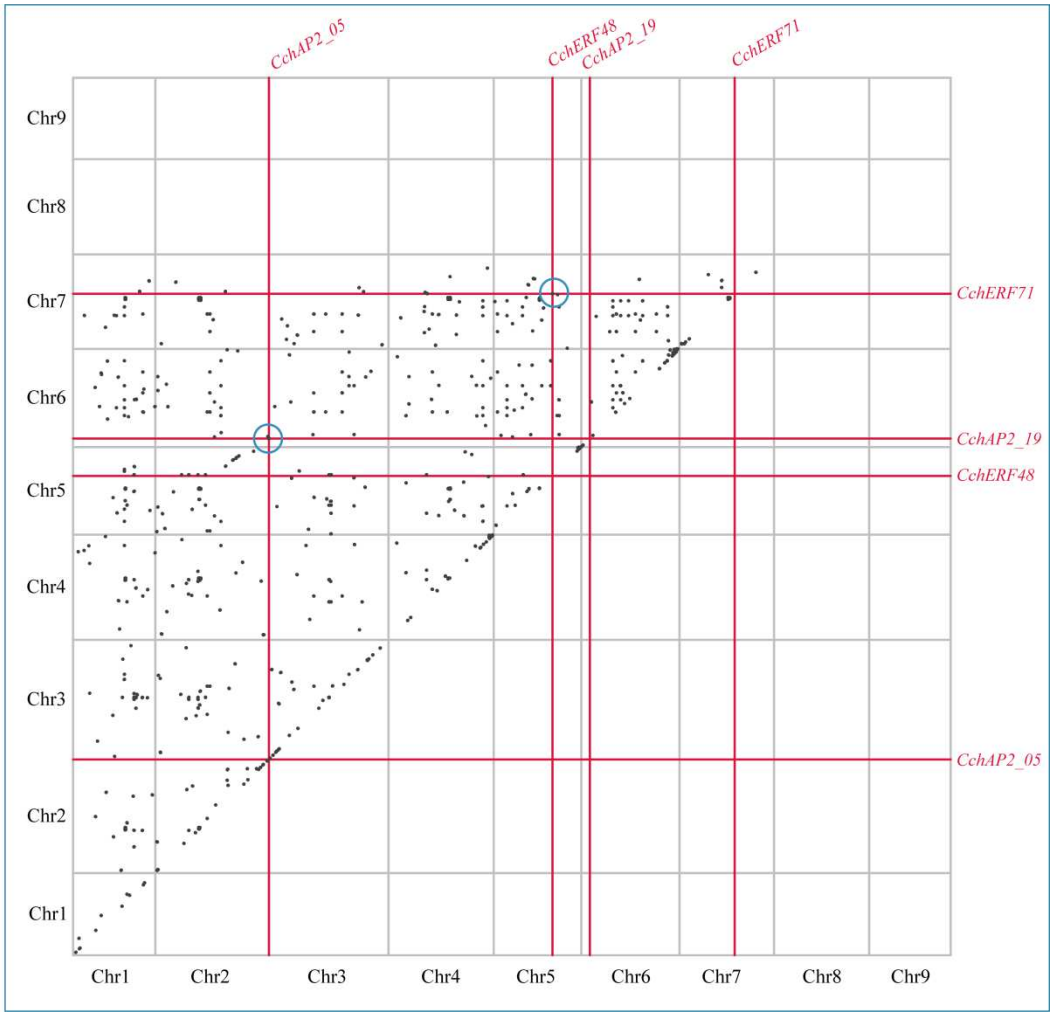


Figure 3: Dotplot of duplication events in *C. chinensis* genes. Each black dot signifies a collinear module between genes. The red cross lines delineate the collinear modules specifically between *AP2/ERF* genes of *C. chinensis*, and these are further emphasized with light blue circles. This indicates that these gene pairs may have undergone whole genome duplication or segmental duplication.

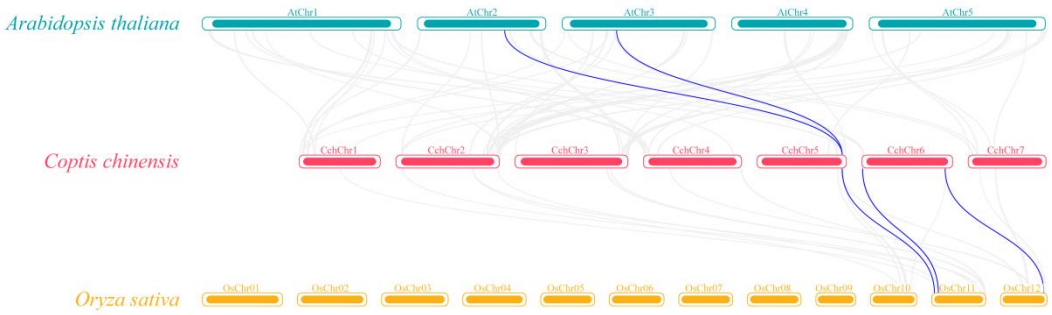


Figure 4: Colinearity results of AP2/ERF genes between *C. chinensis* and other species. *O. sativa* and *A. thaliana* were selected to establish a synteny block with *C. chinensis*. The gray lines indicate collinear regions of genes shared between the two species, with the violet lines specifically representing the collinear regions of *AP2/ERF* genes.

Gene structure and conserved motif analysis of CchAP2/ERF

Gene structure and conserved motif analysis are presented in Figure 5A. All CchAP2/ERF proteins possess Motif 1, except for CchAP2_3, CchAP2_12, CchAP2_14, CchERF04, CchERF06, CchERF37, CchERF39, and CchERF68. Additionally, Motif 3 is present in most CchAP2/ERF proteins, with 86 proteins containing this motif, followed by Motif 2 and Motif 5.

The CchAP2/ERF proteins were submitted to the NCBI online website for prediction of conserved domains. Based on previous research and the structural characteristics of AP2/ERF proteins [28, 29], the 125 CchAP2/ERF proteins were classified into the AP2, ERF, RAV, and Soloist subfamilies, designated as CchAP2_01 to CchAP2_25, CchERF01 to CchERF94, CchRAV01 to CchRAV04, and CchSoloist01 to CchSoloist02, respectively (Figure 5B). The results indicate that 112 CchAP2/ERF proteins contain at least one AP2 domain, while 13 proteins do not have an AP2 domain but contain one or two AP2 subfamily domains. Four CchAP2/ERF proteins (CchAP2_11, -13, -15, CchERF42) contain a B3_DNA domain, and four proteins (CchRAV01, -02, -03, -04) contain a B3 domain.

The exon-intron structure analysis of AP2/ERFs revealed that the number of introns in the 125 AP2/ERFs ranged from zero to nine. The number of introns varied significantly among different subfamilies. *CchAP2* and *CchSoloist* showed higher polymorphism, with intron numbers

ranging from 1-9, except for *CchAP2_25*. In contrast, *CchERF* and *CchRAV* showed lower polymorphism, with most genes having fewer than three introns, except for *CchERF43* and *CchERF39*. These results provide important and effective information for a deeper understanding of the evolutionary history and functions of CchAP2/ERF proteins (Figure 5C).



Figure 5: Gene structure and conserved motif analysis of CchAP2/ERF. Distributions of conserved (A), structural domain (B), and gene structure (C). The numbers enclosed within the boxes denote the various Motif types (A). The legends situated beneath Subfigures A and

B indicate the count of amino acids, whereas the legend below Subfigure C signifies the length of the genes.

Phylogenetic tree of CchAP2/ERFs and AtAP2/ERFs

To gain a deeper understanding of the evolutionary relationships and classification of CchAP2/ERF, a phylogenetic tree of AP2/ERF from Arabidopsis and *C. chinensis* was constructed (Figure 6). Based on reported literature and domain analyses [5, 28], an initial classification of AP2/ERF was conducted. The results showed that 25 genes belonged to the AP2 subfamily, 94 genes to the ERF subfamily, 4 genes to the RAV subfamily, and two genes to the Soloist subfamily. Additionally, the CchERF subfamily was divided into 11 groups (Figure 6): ERF-I (5 genes), ERF-II (6 genes), ERF-III (22 genes), ERF-IV (6 genes), ERF-V (2 genes), ERF-VI (7 genes), ERF-VI-L (2 genes), ERF-VII (10 genes), ERF-VIII (11 genes), ERF-IX (10 genes), and ERF-X (4 genes). The Soloist and AP2 subfamilies clustered together, but due to low sequence similarity, CchSoloist formed a separate cluster. CchAP2_05 & CchAP2_19 and CchERF48 & CchERF71 clustered together, supporting our collinearity results (Figure 2), indicating that these gene pairs were likely produced by Whole Genome Duplication or Segmental Duplication. These classification results are related to the motifs and sequence characteristics of CchAP2/ERF, suggesting that they may have different biological functions.

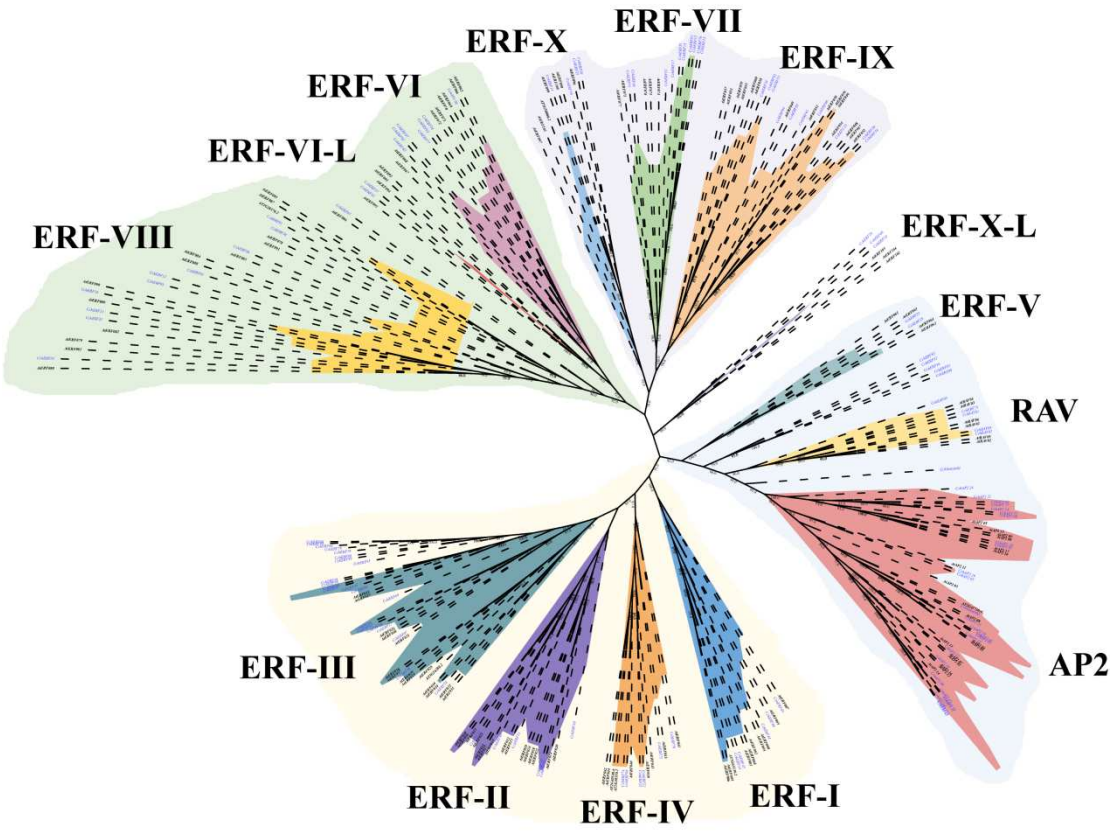


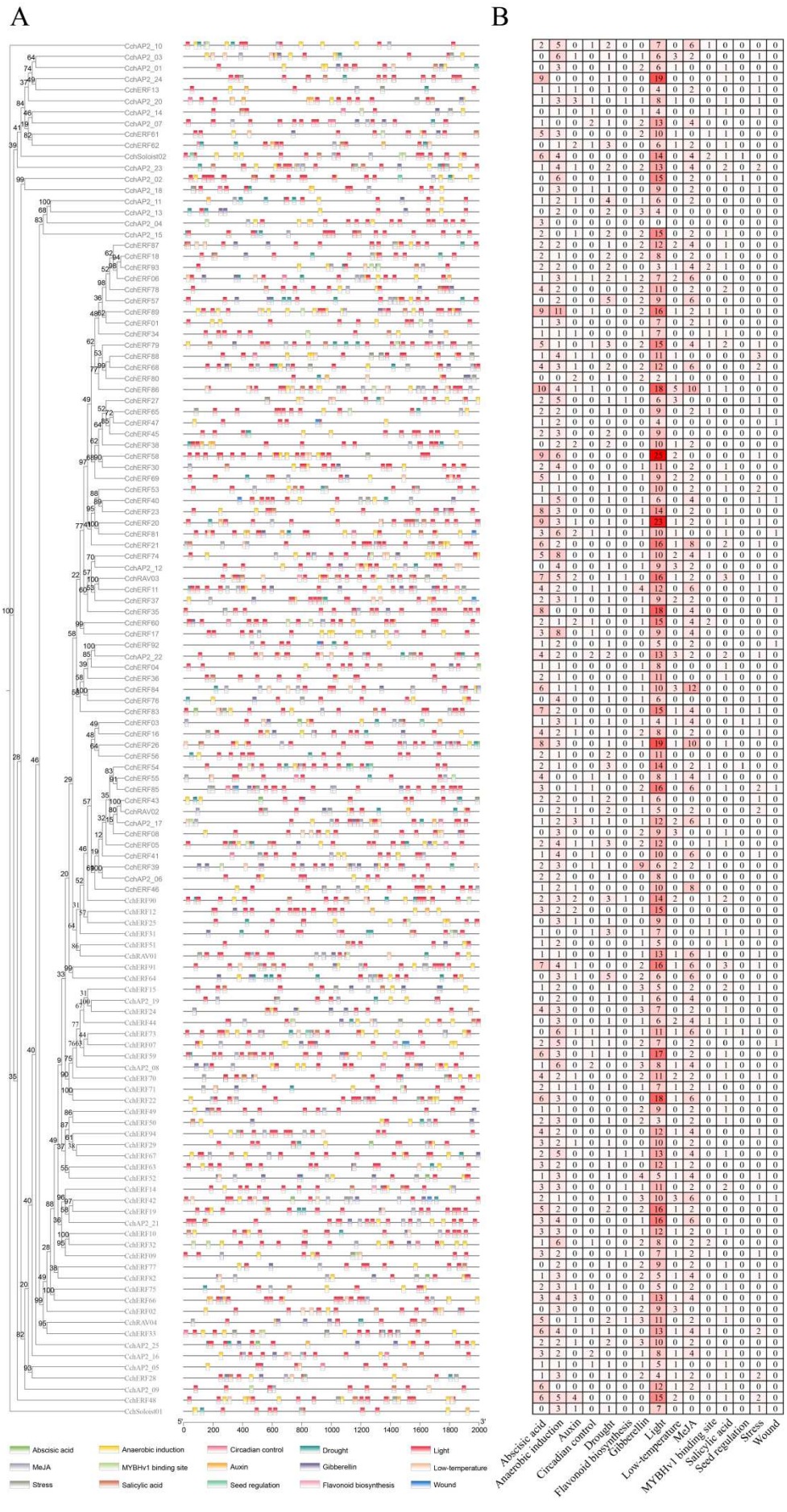
Figure 6: Phylogenetic relations of CchAP2/ERF and AtAP2/ERF. The tree was constructed using IQ-TREE. The branch values indicated UltraFast BootStrap values, and only the percentages of replicate trees greater than 70 are shown on the branches.

Analysis of the cis-acting elements of *CchAP2/ERF*

Promoter cis-acting elements can influence the expression activity of their own genes or bind with other regulatory proteins, controlling the initiation of transcription and the expression level of genes. The analysis of cis-acting elements in genes is crucial for understanding the mechanisms of gene expression regulation. In the promoter regions of these *CchAP2/ERF* genes, there are 15 common cis-acting elements involved in hormone regulation, environmental responses (including light), and other biological processes (Figure 7A). Among them, the cis-acting elements in the promoters of *CchAP2/ERF* genes are enriched in four biological processes: Abscisic acid response,

Anaerobic induction response, Light response, and MeJA response. While other biological processes show enrichment, the number of elements is lower. Additionally, it was found that all genes except *CchAP2_04* contain cis-acting elements for Light response, indicating the significant role of *CchAP2/ERF* genes in the Light response process. However, the number of cis-acting elements in the promoters of *CchAP2/ERF* genes is less common in the processes of Seed regulation and Wound response. The types of cis-elements in different subfamilies are not significantly different, with the main difference being the number of elements. Finally, the number of cis-acting elements in the *CchAP2_05* & *CchAP2_19* and *CchERF48* & *CchERF71* gene pairs was compared (Figure 2), showing that there are significant differences in both the types and numbers of cis-acting elements among the gene pairs (Figure 7), suggesting that after Whole Genome Duplication or Segmental Duplication in *C. chinensis*, the functions of AP2/ERF genes have diverged, which may have helped the plant adapt to its external environment.

In summary, each *CchAP2/ERF* gene contains abundant cis-acting elements, indicating the important molecular biological functions of AP2/ERF transcription factors in the life history of *C. chinensis*. This information helps us understand the roles and regulatory mechanisms of these genes in different biological processes.


A
B
CchAP2_10
CchAP2_03
CchAP2_01
CchAP2_24
CchERF13
CchAP2_20
CchAP2_14
CchAP2_07
CchERF61
CchERF62
CchSoloist02
CchAP2_23
CchAP2_02
CchAP2_18
CchAP2_11
CchAP2_13
CchAP2_04
CchAP2_15
CchERF87
CchERF18
CchERF93
CchERF06
CchERF78
CchERF57
CchERF89
CchERF01
CchERF34
CchERF79
CchERF88
CchERF68
CchERF80
CchERF86
CchERF27
CchERF65
CchERF47
CchERF45
CchERF38
CchERF58
CchERF30
CchERF69
CchERF53
CchERF40
CchERF23
CchERF20
CchERF81
CchERF21
CchERF74
CchAP2_12
CchRAV03
CchERF11
CchERF37
CchERF35
CchERF60
CchERF17
CchERF92
CchAP2_22
CchERF04
CchERF36
CchERF84
CchERF76
CchERF83
CchERF03
CchERF16
CchERF26
CchERF56
CchERF54
CchERF55
CchERF85
CchERF43
CchRAV02
CchAP2_17
CchERF08
CchERF05
CchERF41
CchERF39
CchAP2_06
CchERF46
CchERF90
CchERF12
CchERF25
CchERF31
CchERF51
CchRAV01
CchERF91
CchERF64
CchERF15
CchAP2_19
CchERF24
CchERF44
CchERF73
CchERF07
CchERF59
CchAP2_08
CchERF70
CchERF71
CchERF22
CchERF49
CchERF50
CchERF94
CchERF29
CchERF67
CchERF63
CchERF52
CchERF14
CchERF42
CchERF19
CchAP2_21
CchERF10
CchERF32
CchERF09
CchERF77
CchERF82
CchERF75
CchERF66
CchERF02
CchRAV04
CchERF33
CchAP2_25
CchAP2_16
CchAP2_05
CchERF28
CchAP2_09
CchERF48
CchSoloist01
5'
3'
0
200
400
600
800
1000
1200
1400
1600
1800
2000
Abscisic acid
Anaerobic induction
Auxin
Circadian control
Drought
Flavonoid biosynthesis
Gibberellin
Light
MeJA
Low-temperature
MYBHv1 binding site
Salicylic acid
Seed regulation
Stress
Wound

299

Figure 7: Cis-acting elements map of the promoter of CchAP2/ERF. Various colors denote different cis-acting elements, with illustrations of these elements located at the bottom of the figure (A). The heatmap illustrates the distribution and frequency of the cis-acting elements (B). The intensity of the color corresponds to the quantity, with darker shades indicating a higher abundance of elements.

Expression profile of *CchAP2/ERF*

An examination of the numerical ranges in the Different places dataset reveals a wide range of expression levels, varying from 0 to several hundred (Table S2), indicating significant differences in expression levels among genes across different samples. Some genes exhibit very high expression levels in specific samples (Figure 8), such as *CchERF01* in the CQ_3_Root sample and *CchERF05* in the CQ_3_Rhizome sample. These genes with high expression levels may play crucial roles in the respective samples. It is evident that different genes exhibit distinct expression patterns across various samples. Some genes show relatively low expression across all samples, such as *CchAP2_08* and *CchERF18*. Conversely, some genes exhibit high expression in certain samples but low expression in others, like *CchAP2_07*, which has a high expression in the Shizhu sample but low expression in others. Comparing gene expression levels across different samples reveals certain variations. Genes may have high expression in some samples and low expression in others, potentially due to differences in sample source, growth environment, or other factors.

Observing the expression levels of the same gene across different plant organs reveals specific expression patterns (root-specific expression, leaf-specific expression), such as

CchERF02, which is only expressed in roots and at a much higher level than other organs; *CchERF14*, with significantly higher expression in roots than other organs; *CchERF03*, which is only expressed in leaves among all organs; and *CchERF06*, with significantly higher expression in leaves than other organs. Comparing the expression changes of the same gene across different growth years, it is observed that the expression levels of *CchERF50* and *CchERF53* increase significantly with age. Comparing the expression patterns of different genes in the same plant organ, *CchERF08* and *CchERF09* show similar expression patterns, primarily expressed in roots; *CchERF13* and *CchERF33* show similar expression patterns, primarily expressed in leaves. Additionally, *CchERF38* and *CchERF45* exhibit high expression across multiple organs, potentially playing key roles in plant growth processes.

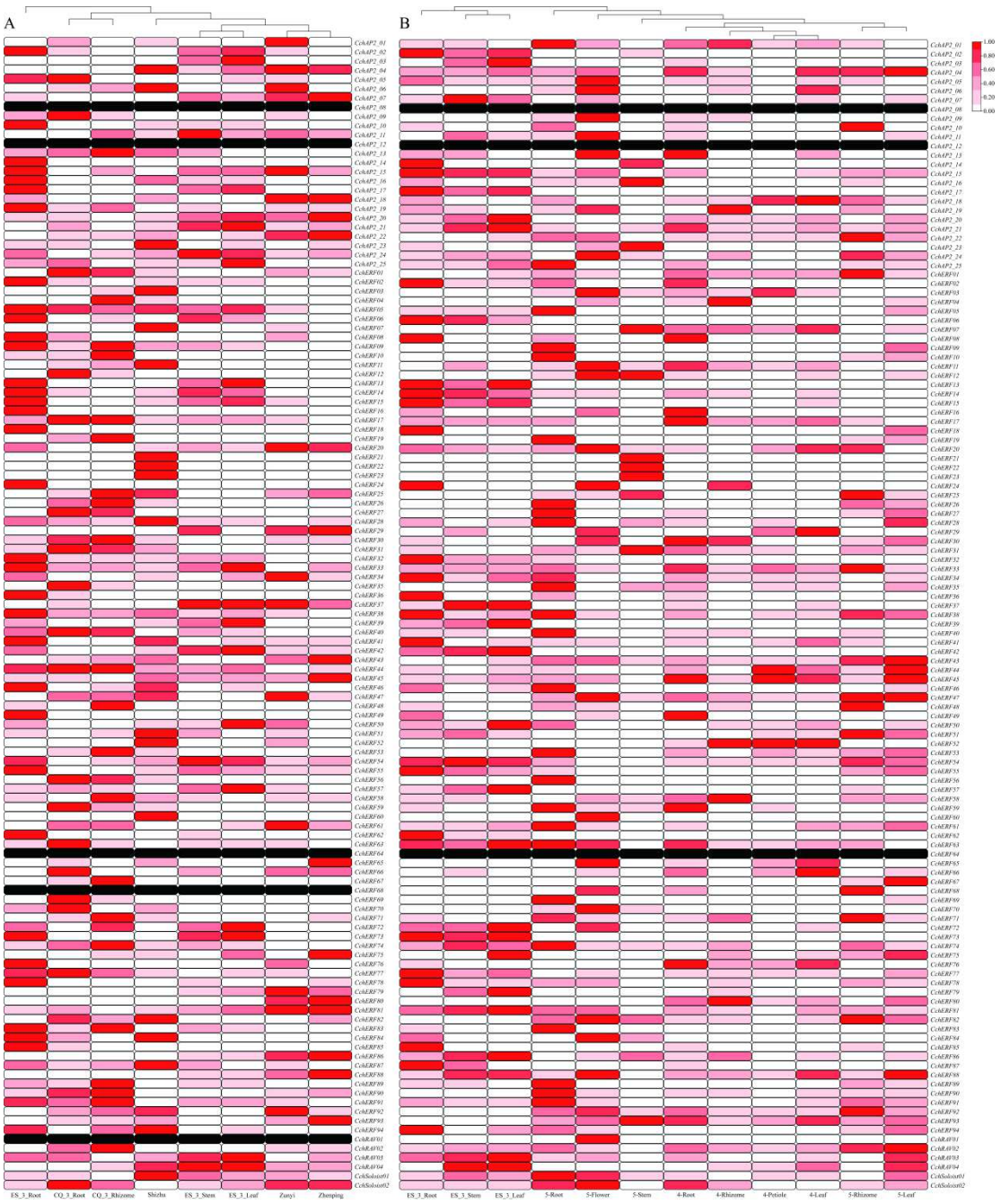


Figure 8: Expression pattern of *CchAP2/ERF* under different organs and different plants. Using parameters of Bias Correlation, a Kmer Size of 31, Bootstrap set to 0, and ThreadNum set to 2, all values were normalized using the $\log_2$ (TPM) transformation. The intensity of the color scale corresponds to the gene expression levels, with darker shades indicating higher expression. Modules depicted in black signify genes with an expression value of 0.

In summary, these results aid in understanding the expression patterns of different genes in various plant organs and growth stages, facilitating the identification of key genes and functional regulatory mechanisms. Genes such as *CchERF02*, *-08*, *-09*, *-14*, *-38*, *-45*, *-50*, and *-53* can be considered as candidate genes for subsequent studies on their molecular functions.

Expression profile of *CchAP2/ERF* in response to hormone

The cis-acting elements in the promoters of *CchAP2/ERF* genes were enriched in Abscisic acid response, Light response, and MeJA response. The role of *CchAP2/ERF* genes in the response mechanism of these biological processes was further investigated by qRT-PCR. In the ABA treatment group, *ERF45* exhibited the most significant expression response. Treated with 1 μmol ABA, *ERF45* expression peaked at 14 d, approximately 17-fold higher than at other time points (p<0.05). Under 5 μmol ABA treatment, *ERF45* expression at 14 d was remarkably high, reaching about 116 times, the strongest response among all genes. At 14 d with 10 μmol ABA treatment, *ERF45* expression was around 9.2 times higher than the control, although lower compared to the 5 μmol treatment. *ERF02* and *ERF09* showed relatively weak expression responses to ABA, with most time points exhibiting no significant changes (p>0.05). *ERF14* had a similar expression trend to *ERF45* but with lower levels, reaching approximately 35 times at 14 d under 5 μmol ABA treatment. *ERF08* maintained low expression levels across all ABA treatment concentrations, showing no significant difference from the control at most time points.

In the MeJA treatment group, *ERF45* also exhibited the most significant expression response. Treated with 50 μmol MeJA, *ERF45* expression peaked at approximately 15-fold at 14 d, significantly higher than at other time points (p<0.05). Under 100 μmol MeJA treatment, *ERF45*

361 expression at 14 d reached about 42-fold, the strongest response among all genes. In contrast, at
362 14 d with 200 μmol MeJA treatment, *ERF45* expression was around 4.7 times higher than the
363 control, although lower compared to the 100 μmol treatment. *ERF02* and *ERF09* showed
364 relatively weak expression responses to MeJA, with most time points exhibiting no significant
365 changes (p>0.05). *ERF14* had a similar expression trend to *ERF45* but with lower levels, reaching
366 approximately 20 times at 14 d under 100 μmol MeJA treatment. *ERF08* maintained low
367 expression levels across all MeJA treatment concentrations, showing no significant difference
368 from the control at most time points.

369 *ERF09* and *ERF45* were significantly induced by both ABA and MeJA treatments, with
370 *ERF45* showing the highest expression under 5 μmol ABA at 7 d (approximately 116-fold
371 increase), and *ERF09* showing a 41-fold increase under 100 μmol MeJA at 7 d, indicating that
372 these genes are key transcription factors in hormone response. *ERF02* and *ERF08* were more
373 sensitive to ABA, particularly at the 5 μmol concentration, exhibiting a sustained high level of
374 expression, and had a weaker response to MeJA. *ERF14* was strongly suppressed in all hormone
375 treatments, with expression levels decreasing by over 30-fold after 0.5 d of 10 μmol ABA and 200
376 μmol MeJA treatments, suggesting a potential role in negatively regulating the hormone response.

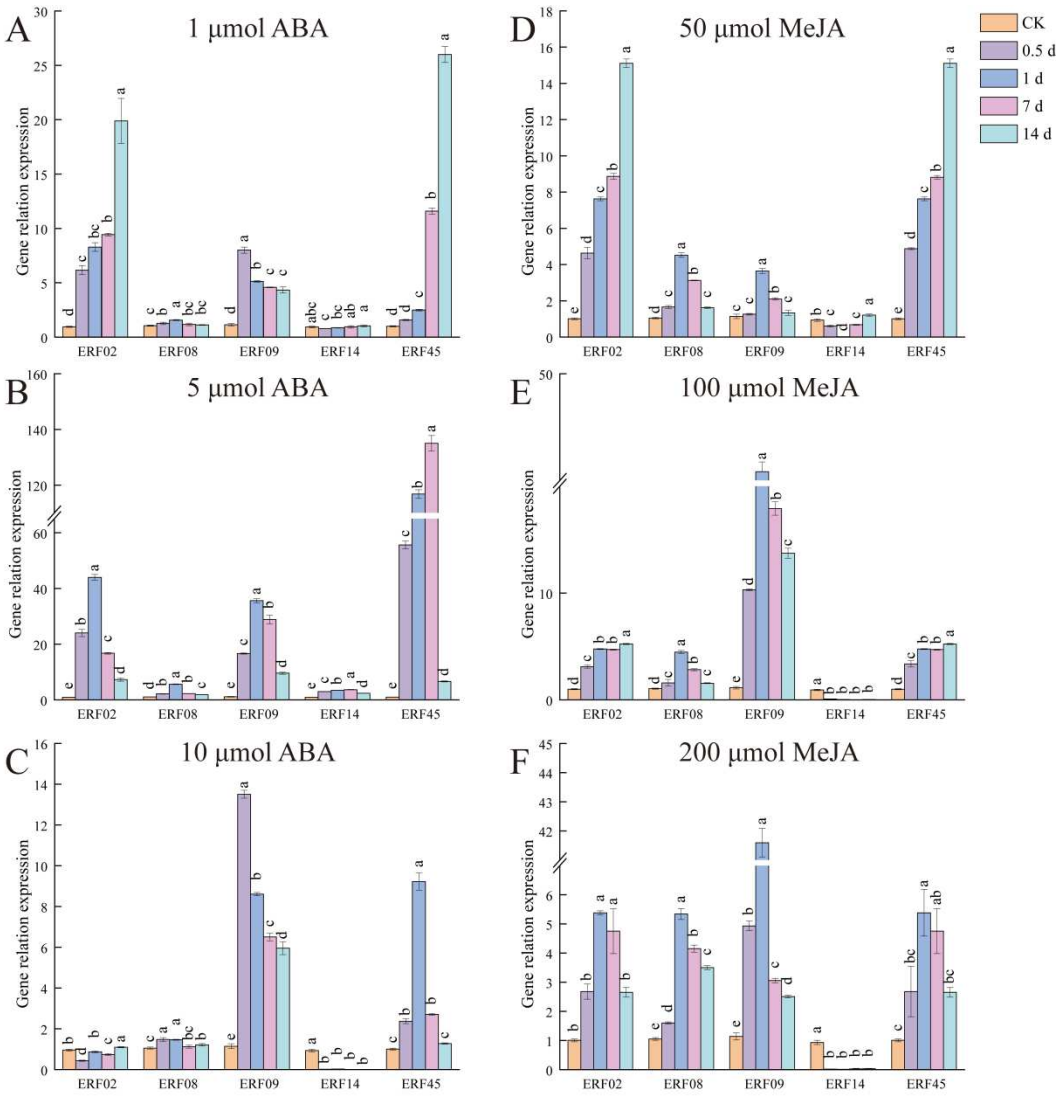


Figure 9: Expression pattern of *CchAP2/ERF* under various concentrations of ABA and MeJA hormones. The significance marks (a, b, c, d, e) denote the statistical differences among various time points within a single treatment group ($p < 0.05$). Identical letters signify no significant difference, whereas different letters denote significant differences.

Expression profile of *CchAP2/ERF* in response to light

In the light quality treatment group, *ERF45* exhibited the most significant expression response among the five genes. Under red light treatment, *ERF45* reached its peak response at 24

h, approximately 17.9-fold higher than under white light (2.7-fold), blue light (7.2-fold), and UV (8.7-fold) treatments (p<0.05). *ERF14* also showed significant expression responses to all light treatments, albeit at lower levels than *ERF45*. *ERF02* and *ERF09* had relatively weak responses to light quality treatments, with no significant differences from the control at most time points. *ERF08* maintained low expression levels across all light treatments, showing no significant differences from the control at most time points.

In the light period treatment group, *ERF45* exhibited the most significant expression response among the five genes. Under 12 h/12 h light period treatment, *ERF45* reached its peak response at 24 h, approximately 27.9-fold higher than under 16 h/8 h light period treatment (18.2-fold) (p<0.05). *ERF14* also showed significant expression responses to all light period treatments, albeit at lower levels than *ERF45*. *ERF02* and *ERF09* had relatively weak responses to light period treatments, with no significant differences from the control at most time points. *ERF08* maintained low expression levels across all light period treatments, showing no significant differences from the control at most time points.

In the light intensity treatment group, *ERF45* exhibited the most significant expression response among the five genes. Under 2K Lux light intensity treatment, *ERF45* reached its peak response at 24 h, approximately 26.6-fold higher than under 10K Lux light intensity treatment (7.6-fold) (p<0.05). ERF14 also showed significant expression responses to all light intensity treatments, albeit at lower levels than *ERF45*. *ERF02* and *ERF09* had relatively weak responses to light intensity treatments, with no significant differences from the control at most time points. *ERF08* maintained low expression levels across all light intensity treatments, showing no significant differences from the control at most time points.

In all light treatment groups, *ERF45* exhibited the most significant expression response among the five genes. Under red light and 12 h/12 h light period treatments, *ERF45* reached its peak response at 24 h. *ERF14* also showed significant expression responses to all light treatments, albeit at lower levels than *ERF45*. *ERF02* and *ERF09* had relatively weak responses to all light treatments, with no significant differences from the control at most time points. *ERF08* maintained low expression levels across all light treatments, showing no significant differences from the control at most time points.

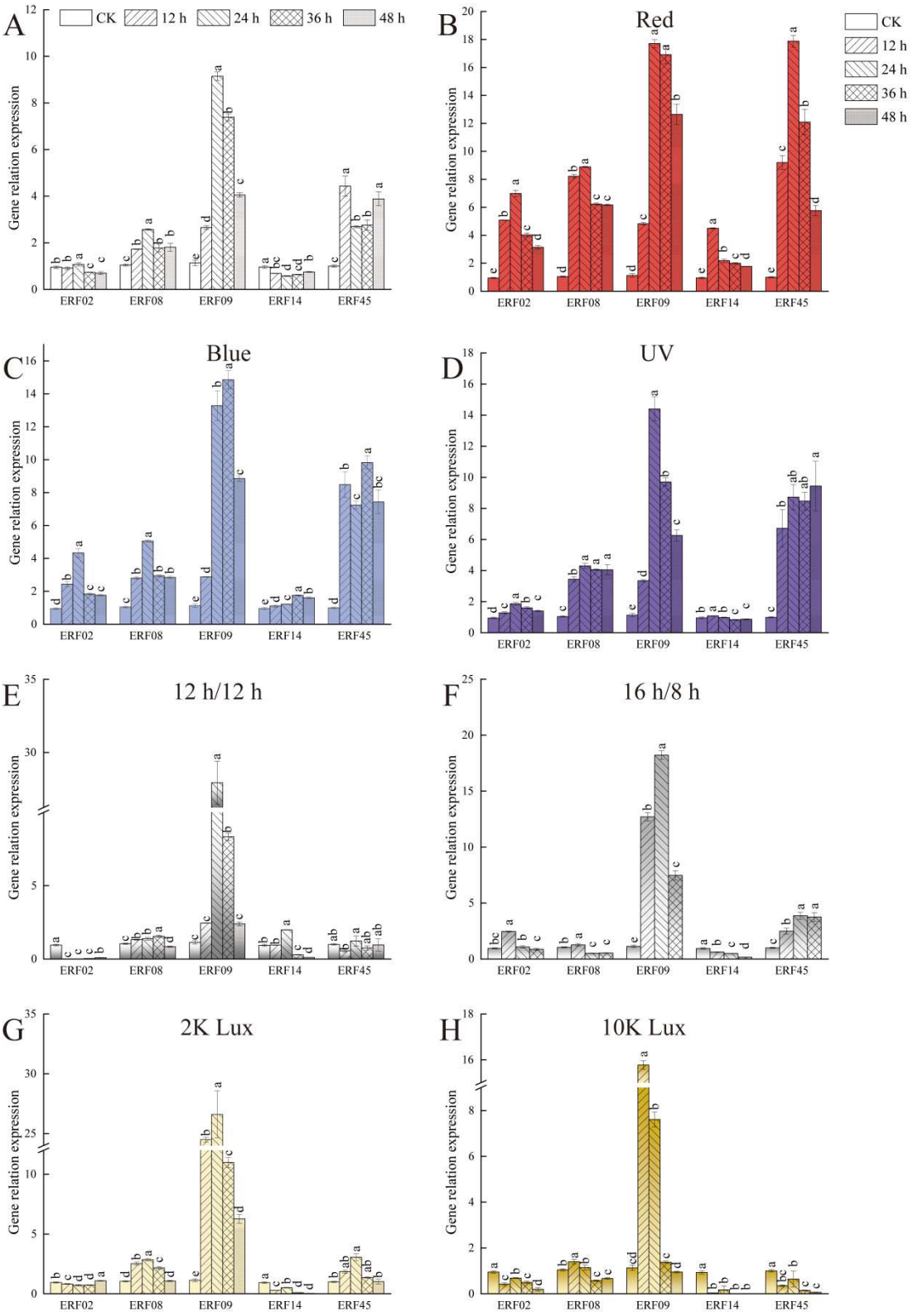


Figure 10: Expression pattern of *CchAP2/ERF* under different light condition. The significance marks (a, b, c, d, e) denote the statistical differences among various time points within a single treatment group ($p < 0.05$). Identical letters signify no significant difference,

whereas different letters denote significant differences.

Discussion

The AP2/ERF transcription factor family is pivotal in regulating plant development and mediating responses to environmental stimuli. In this study, 125 CchAP2/ERF genes were identified in the *Coptis chinensis* genome, a count comparable to those found in other vascular plants, suggesting a conserved evolutionary foundation for this family. The expansion of the *CchAP2/ERF* family is largely driven by whole-genome duplication (WGD) and segmental duplication events, as evidenced by the collinear gene pairs (*CchAP2_05* & *CchAP2_19*, *CchERF48* & *CchERF71*) and the synteny blocks on chromosomes 5, 6, and 8. The lack of collinearity between chromosomes 8 and 9 likely represents chromosomal rearrangements or evolutionary divergence occurring post-duplication, which has disrupted ancestral synteny. Furthermore, the relatively few collinear pairs among Soloist genes imply a lineage-specific evolutionary trajectory or rapid divergence, minimizing synteny conservation. Notably, significant divergence in the types and numbers of cis-acting elements between these duplicated gene pairs highlights neofunctionalization or subfunctionalization. This regulatory divergence following duplication is a crucial evolutionary mechanism that equips *C. chinensis* with enhanced transcriptional plasticity to adapt to complex environmental cues [30].

Structural and phylogenetic analyses provided deeper insights into the functional divergence of the CchAP2/ERF family. The conservation of Motif 1 and Motif 3 across the majority of proteins underscores their indispensable roles in maintaining the structural integrity of the AP2 DNA-binding domain. Conversely, the variation in intron-exon structures reflects evolutionary

diversification; the higher intron polymorphism in the AP2 and Soloist subfamilies contrasts sharply with the intron-poor nature of the ERF and RAV subfamilies. In plants, genes with fewer introns are typically associated with rapid transcriptional activation in response to stress [31, 32], suggesting that CchERF and CchRAV genes may be primed for swift responses to hormonal and environmental triggers. Phylogenetically, the clustering of *C. chinensis* AP2/ERF proteins with their Arabidopsis orthologs allows for functional inference. The limited synteny blocks observed between *C. chinensis*, *A. thaliana*, and *O. sativa* indicate that while the core AP2/ERF lineages were established before the divergence of monocots and eudicots, species-specific expansions and gene loss have shaped the current repertoire, likely reflecting adaptations unique to the ecological niche of *C. chinensis*.

The expression profiles of *CchAP2/ERF* genes demonstrate pronounced spatial, temporal, and conditional specificity, reinforcing their multifaceted roles in plant biology. Tissue-specific expression patterns, such as the root-dominant expression of *CchERF02* and *CchERF14* and the leaf-specific expression of *CchERF03* and *CchERF06*, suggest specialized regulatory functions in organogenesis and potentially in the tissue-specific biosynthesis of secondary metabolites characteristic of *C. chinensis*. Furthermore, the age-dependent upregulation of *CchERF50* and *CchERF53* implies their involvement in developmental transitions or the accumulation of bioactive compounds over the plant's life cycle. The integration of cis-element enrichment data with qRT-PCR validation confirmed that *CchAP2/ERF* genes are central to hormonal and light signaling pathways. Notably, *CchERF45* emerged as a master transcription factor, exhibiting massive induction under both ABA and MeJA treatments. Intriguingly, its response to ABA demonstrated a non-linear, bell-shaped dose-response curve, peaking at 5 μmol and declining at 10

μmol. This suggests a sophisticated feedback inhibition mechanism or receptor saturation at higher hormone concentrations, which prevents runaway stress responses. Similarly, the strong suppression of *CchERF14* by both ABA and MeJA indicates its potential role as a negative regulator, fine-tuning the hormone signaling network to maintain cellular homeostasis.

Light signaling is intimately linked with hormonal pathways to coordinate plant growth and stress adaptation [33]. The distinct responsiveness of *CchAP2/ERF* genes to varying light conditions highlights their regulatory versatility. *CchERF45* again stood out as the most responsive gene, particularly to red light, a 12 h/12 h photoperiod, and moderate light intensity (2K Lux). The preferential activation of *CchERF45* by red light strongly suggests crosstalk between the AP2/ERF family and phytochrome signaling pathways. The diminished expression of *CchERF45* under higher light intensity (10K Lux) further implies that its protective or regulatory functions may be optimized for shaded or understory environments, which aligns with the natural habitat of *C. chinensis*. The consistent, albeit lower, co-expression of *CchERF14* alongside *CchERF45* across light treatments suggests a potential cooperative or compensatory regulatory module operating between these two factors. In contrast, the minimal responsiveness of *CchERF02*, *CchERF08*, and *CchERF09* to light and hormone treatments suggests they may be governed by alternative signaling cascades or act under specific conditions not captured in this study.

Conclusion

In conclusion, this study systematically identified and characterized 125 AP2/ERF transcription factors in the *C. chinensis* genome. Evolutionary analyses revealed that WGD and

segmental duplication, followed by structural and promoter cis-element divergence, have driven the expansion and functional diversification of this family. Expression profiling demonstrated that *CchAP2/ERF* genes exhibit distinct spatial and developmental expression patterns, and play critical roles in mediating ABA, MeJA, and light signaling pathways. Notably, *CchERF45* was identified as a central hub integrating multiple hormonal and light signals, while *CchERF14* was implicated as a potential negative regulator in hormone crosstalk. These findings provide a robust genomic framework and identify key candidate genes for future functional validation, offering valuable genetic resources for the molecular breeding and targeted cultivation of *C. chinensis*.

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Authors' contributions

HC and PD conceived and designed the experiment. QA, XYM, SBL, XL, MTM and YXW performed the experiments. QA, MTM and YXW analyzed all the data. QA wrote the manuscript. PD and HC revised the manuscript. All of the authors read and approved the final manuscript.

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Availability of data and materials

All data generated or analyzed in this study are included in this published article and its supplementary material. Table S1: Physical characteristics and subcellular localization of CchAP2/ERFs. Table S2: Collinear module of CchAP2/ERFs.

Declarations

Ethics approval and consent to participate

Experimental research and studies on plants in this study, including the collection of plant material, are complied with the institutional, national, and international guidelines and legislation. We conducted the experimental research on cultivated *Coptis chinensis* in accordance with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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