

Article

Transcriptomic Profiling Across Developmental Stages of *Camellia petelotii* (Merr.) Sealy Flower

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Abstract

Background: The *Camellia* genus is widely recognized for its remarkable diversity in floral morphology and coloration, with *Camellia petelotii* (Merr.) Sealy being particularly notable for its rare golden-yellow flowers, which possess exceptional ornamental value. Despite its horticultural significance, the molecular mechanisms governing its flowering process remain poorly elucidated, presenting a substantial barrier to effective conservation and breeding initiatives. **Methods:** To address this knowledge gap, we conducted a comprehensive transcriptomic analysis, focusing on three distinct developmental stages of *C. petelotii* floral organs: the alabastrum stage (S1), the half-opened flower stage (S2), and the full bloom stage (S3). These samples were subjected to high-throughput sequencing using the Illumina platform. Following rigorous quality control and alignment with the reference genome, we performed transcript assembly and integrated comprehensive gene annotation data with quantitative gene expression profiles. **Results:** Our analysis identified 18,732 differentially expressed genes (DEGs) showing significant expression changes across developmental stages. Notably, we identified 134 DEGs as potential flowering-related genes, which were functionally associated with key pathways involved in floral regulation, including plant hormone signal transduction (e.g., *AUX/IAA*, *ARF*, *SAUR*, *GH3*, *JAR4*, *GID1* and *SOC1*), starch (SS, SUS, BAM) and sucrose metabolism (*HK*, *FrK*, and *GH32*), circadian rhythm regulation (e.g., *PIF3*, *ELF3*, *LHY*, and *PRR*), and the Autonomously pathway. Building upon these findings, we have proposed a comprehensive model illustrating the regulatory network underlying flowering transition in *C. petelotii*. The reliability of the transcriptomic data was demonstrated through the validation of 11 genes using quantitative real-time PCR (qRT-PCR). **Conclusions:** These insights not only enhance our understanding of the molecular basis of flowering in this species but also provide a valuable theoretical framework for future genetic improvement and breeding programs of *C. petelotii*.

Keywords: *Camellia petelotii* (Merr.) Sealy; differentially expressed genes; flower development; RNA-seq; transcriptomics

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1. Introduction

Camellia plants, renowned for their diverse and vibrant floral displays, are highly prized ornamental species in horticulture. Among the numerous garden cultivars, the most prevalent flower colors include red, pink, and white [1]. Notably, golden-yellow flowering varieties, though relatively rare within the *Camellia* genus, hold exceptional ornamental value [2]. A particularly significant species is *Camellia petelotii* (Merr.) Sealy, distinguished by its striking golden-yellow blossoms. As one of the few yellow-flowered *Camellia* species [3], it is predominantly found in southern China and northern Vietnam. This species represents a crucial germplasm resource for breeding yellow-flowered cultivars [4,5], making it particularly valuable for horticultural development and conservation efforts.

Plant flowering represents a complex morphological development process and a critical stage in the formation of floral organs. This process is regulated by the combined influence of internal and external factors, with the quality of differentiation directly determining the ornamental value of the plants [6,7]. As unique structures in angiosperms [8], flowers play a crucial role in plant reproduction, marking a vital phase in the plant life cycle and serving as an essential mechanism for species survival and propagation [9,10]. The flowering process can be systematically categorized into five distinct developmental phases: initial differentiation, sepal differentiation, petal differentiation, stamen differentiation, and pistil differentiation [8]. A substantial amount of research has demonstrated that the main regulatory pathways of the plant flowering process included plant hormones [11], starch and sugar metabolism [12], photosynthesis [13], circadian rhythm and biological clock [14], vernalization [15], and the autonomous flowering pathway [16]. Specifically, the plant hormone indole-3-acetic acid (IAA) could accelerate the flowering process of *Rosa chinensis* [17]. Sugars, acting as providers of energy and signal molecules, were found by Lebon et al. [18] to respond positively to the flowering of grapes. A series of vernalization-related genes, including *VRN1*, *VRN2*, *VIN1*, and *VIN2*, were identified in wheat, indicating that wheat flowering was influenced by the vernalization pathway [19]. In these pathways, the main typical genes involved in the regulation of the flowering mechanism included *FLOWER LOCUS T (FT)*, *CONSTANS (CO)*, *SUPPRESSOR OF CONSTANS 1 (SOC1)*, *LEAFY (LFY)*, *APETALA1 (AP1)*, *APETALA2 (AP2)*, and *APETALA3 (AP3)*, which induced the plant to flower [16,20].

During the development of *C. petelotii* petals, distinct phenotypic changes occur [21,22]. The process initiates with the formation of small, green petals, which undergo an early growth phase predominantly driven by cell division, alongside the onset of pigment biosynthesis. At this stage, petal cells exhibit slow expansion, while pigment accumulation proceeds rapidly, ultimately leading to visible pigmentation and the emergence of yellow-colored petals. Subsequently, further increases in petal size are achieved through cellular enlargement, culminating in the attainment of the final petal dimensions [23]. Several studies have conducted transcriptome analyses on *C. petelotii*, primarily focusing on flavonoid biosynthesis [5], light response mechanisms [24] and leaf metabolic compounds [25]. However, research on the molecular mechanisms underlying the flowering process of *C. petelotii* remains limited, with most studies focusing primarily on phenotypic observation and hormonal regulation, and the molecular regulatory networks governing its flowering are poorly understood. Consequently, elucidating the transcriptional regulation of flowering in *C. petelotii* and uncovering the genetic mechanisms controlling floral organ development are of significant importance for plant breeding, particularly for ornamental species with prominent floral traits in horticultural applications [10,26].

High-throughput sequencing technology was utilized to conduct transcriptome analysis, enabling a comprehensive understanding of gene functional expression, biological

processes, and molecular mechanisms in a species under specific environmental conditions. In this study, the floral organs of *C. petelotii* were examined at three developmental stages: alabastrum (S1), half-opened flower (S2), and full bloom (S3). Using the Illumina sequencing platform, transcriptome sequencing was performed on these floral organs, followed by rigorous data filtering. The filtered data were then aligned to a specific reference genome, and transcript assembly was carried out using StringTie (version 2.2.3). Through this transcriptomic analysis, gene expression patterns during different stages of floral organ development were elucidated, and key regulatory genes associated with floral organ development were identified. This research provides a theoretical foundation for the molecular breeding of *C. petelotii*.

2. Materials and Methods

2.1. Plant Materials

The research specimens of *C. petelotii* were collected from the Flower Germplasm Resource Nursery at the Baiyun Campus of Zhongkai University of Agriculture and Engineering, located in Guangzhou City, Guangdong Province, China. Given that February marks the peak blooming period for *C. petelotii*, flower samples were collected from flowering plant on a clear February morning in 2024. The flowering stages were categorized into three distinct phases: alabastrum (S1), half opened flower (S2), and full bloom (S3), corresponding to different developmental levels (Figure 1). The fresh flower samples were frozen with liquid nitrogen after collection and then stored in a -80°C refrigerator for subsequent transcriptome experimental analysis. In addition, three biological replicates were collected for each developmental stage (S1, S2, and S3).

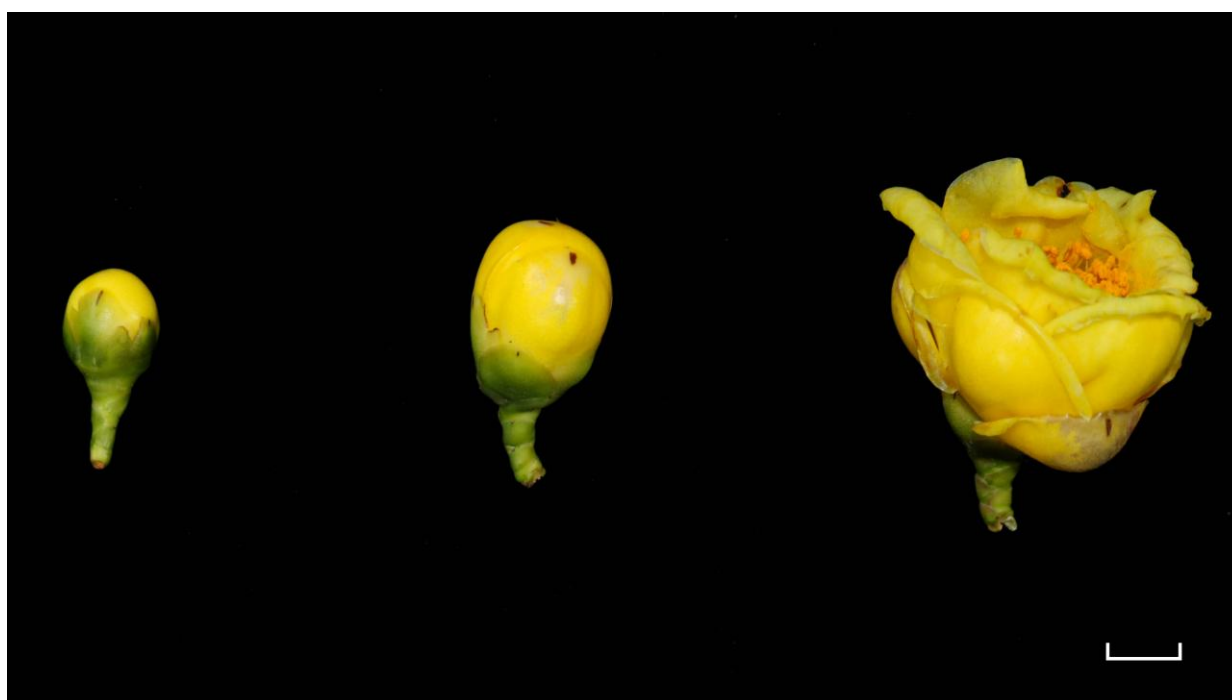


Figure 1. The phenotypes observed at various developmental stages of the flower are as follows (from left to right): floral bud (S1), half-opened flower (S2), and fully bloomed flower (S3). (S1) completely closed conical floral buds with smooth surfaces and no petal exposure, tightly enclosed by pale green sepals; (S2) apical dehiscence of floral buds with initial petal expansion and chromatic transition from pale green to light yellow; (S3) complete anthesis with fully expanded golden-yellow petals and well-developed floral morphology.

2.2. RNA Extraction, Library Construction and Transcriptome Sequencing

RNA isolation, purification, and quantification, as well as cDNA library construction and RNA sequencing, were carried out as described in previous studies [27]. All samples were ground on dry ice, and total RNA was extracted from the flowers of the samples using the CTAB-PBIOZOL reagent kit and ethanol precipitation method. To determine the purity, concentration, and integrity of the RNA extracted in this study, the NanoPhotometer® spectrophotometer (IMPLEN, Westlake Village, CA, USA), the Qubit® 4.0 fluorometer with the Qubit® RNA Assay Kit (Life Technologies, Carlsbad, CA, USA), and the Qsep400® high-throughput bio-analyzer (BiOptic, Changzhou, Jiangsu, China) were used for examination, measurement, and assessment. Sequencing libraries were generated using the Illumina® NEBNext®Ultra™ RNA Library Prep Kit (NEB, Ipswich, MA, USA), and the cDNA libraries were then sequenced on the Illumina sequencing platform at Metware Biotechnology Co., Ltd. (Wuhan, China).

After passing the library check, the different libraries were sequenced in Illumina after pooling them according to the effective concentration and the target sequencing output data volume, yielding 150 bp paired-end reads. The basic principle of sequencing is to synthesize and sequence at the same time. Four types of fluorescently labeled dNTPs, DNA polymerase, and junction primers were added to the sequencing flow cell for amplification. When extending the complementary strand of each sequencing cluster, each fluorescently labeled dNTP added emits corresponding fluorescence, and the sequencer captures the fluorescence signals and converts the light signals into sequencing peaks through computer software, so as to obtain the sequence information of the fragment to be tested.

2.3. Data Filtering and Gene Quantification

Fastp (version 0.23.2) was used to filter and select the raw data, primarily for removing reads with adapters. When the content of N's in either sequencing read exceeded 10% of the read's nucleotide length, or when the number of low-quality ($Q \leq 20$) nucleotides in either sequencing read was greater than 50% of the total nucleotide length of that read, the paired reads were discarded. The sequencing data were initially processed using fastp to eliminate low-quality reads, ensuring that only high-quality, clean reads were retained for subsequent assembly and analytical procedures [27,28]. Then, we utilized the HISAT2 software (version 2.2.1) to perform sequence alignment between the reference genome (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/013/676/235/GCA_013676235.1_HZAU_G240_1.0/, accessed on June 13, 2024) and the clean reads, from which we derived the alignment efficiency. Utilizing the information from the aligned clean reads, we employed StringTie [29] for transcript assembly. The assembled transcripts were then compared with the genome's annotation information using GffCompare v0.12.x, facilitating the discovery of new transcripts or genes. Equally important was the tally of reads for each gene based on the alignment results and the gene's position on the reference genome. The Fragments Per Kilobase of exon model per Million mapped fragments (FPKM) values for each gene were calculated using gene length, which were employed for transcript-level quantification [30].

2.4. Transcript Assembly and Gene Annotation

Subsequently, we extracted new gene sequences from the genome and used diamond [31] to align these new genes with sequences from the Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), NR, Swiss-Prot, TrEMBL, and KOG databases, obtaining single-gene annotation results with an E-value cutoff of 1×10^{-5} . The iTAK [32] software was employed for the identification of transcription factors in this genome, which also integrated two databases, PlnTFDB [33] and PlantTFDB [34].

2.5. Identification and Functional Annotation of DEGs

DESeq2 version 1.22.1 [35] was employed to assess fold changes and false discovery rates (FDRs) among samples. A threshold of $FDR < 0.05$ and $|\log_2 \text{Fold change}| \geq 1$ was set to identify differentially expressed genes (DEGs). The R (v4.0.3) software package clusterProfiler (v3.18.1) was utilized to conduct KEGG and GO enrichment analysis on the DEGs.

2.6. Statistical Analysis

Microsoft Excel 2020 was used for preliminary data organization, and the SPSS software (version 29.0, SPSS, Inc., Chicago, IL, USA) was utilized for the visualization of results, which were expressed as the mean $\pm$ standard deviation (SD) of at least three measurements. One-way analysis of variance (ANOVA) and Duncan's method were employed to analyze the significance of differences among samples, with the level of significance set at 0.05.

2.7. Validation of DEGs by Quantitative Real-Time PCR

The expression profiles of 11 flowering-related DEGs in *C. petelotii* were experimentally validated using qRT-PCR (Supplementary Table S1 displays gene-specific primers). The actin gene (internal control) was used as an internal control for normalization of gene expression. Total RNA from the flower buds of *C. petelotii* was extracted using the same methods described above for RNA-Seq. RT-qPCR was conducted using RealUniversal Color PreMix (SYBR Green) according to the manufacturer's instructions (Tiangen, Beijing, China). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted according to the approach described by Wang et al. [36]. The comparative CT method ($2^{-\Delta\Delta CT}$ method) was subsequently used to quantify gene expression.

3. Results

3.1. Transcriptome Sequencing

To uncover the molecular mechanisms underlying flower development in *C. petelotii*, we employed RNA-Seq technology to analyze the transcriptome sequencing of nine samples from flower organs at three different developmental stages (S1, S2, S3). This generated nine cDNA libraries, and on the Illumina platform, we obtained a total of 45,792,382 to 66,687,468 raw reads. After removing reads containing adapters or poly-N and low-quality reads, we collectively acquired 67.05 Gb of clean data, with each sample's clean data exceeding 6 Gb. The Q30 base percentage reached 95% or above, and the GC percentage was between 44.35% and 45.08% for the high-quality data used in the analysis (Supplementary Table S2). By mapping the clean reads from the nine cDNA libraries to the reference genome for alignment, the alignment efficiency for each library was as follows: 35,364,192 (71.39%), 33,436,256 (71.69%), 31,751,680 (71.80%), 37,742,043 (73.07%), 34,639,135 (72.36%), 31,599,879 (72.54%), 43,406,486 (70.82%), 35,374,409 (70.26%), and 36,992,872 (71.23%) (Supplementary Table S2). The proportion of reads mapped to exons was between 78.23% and 80.62%. Then, utilizing the FPKM values of the genes, we calculated the overall trend of gene expression changes across the samples, which was visually presented using a boxplot (Figure 2A). At the same time, the correlation coefficient (B.Pearson) (Figure 2B) indicated that samples from the same stage had high reproducibility, while the principal component analysis (Figure 2C) showed that samples from different developmental stages exhibited differences. These results indicated that the sequencing quality was sufficient for further analysis.

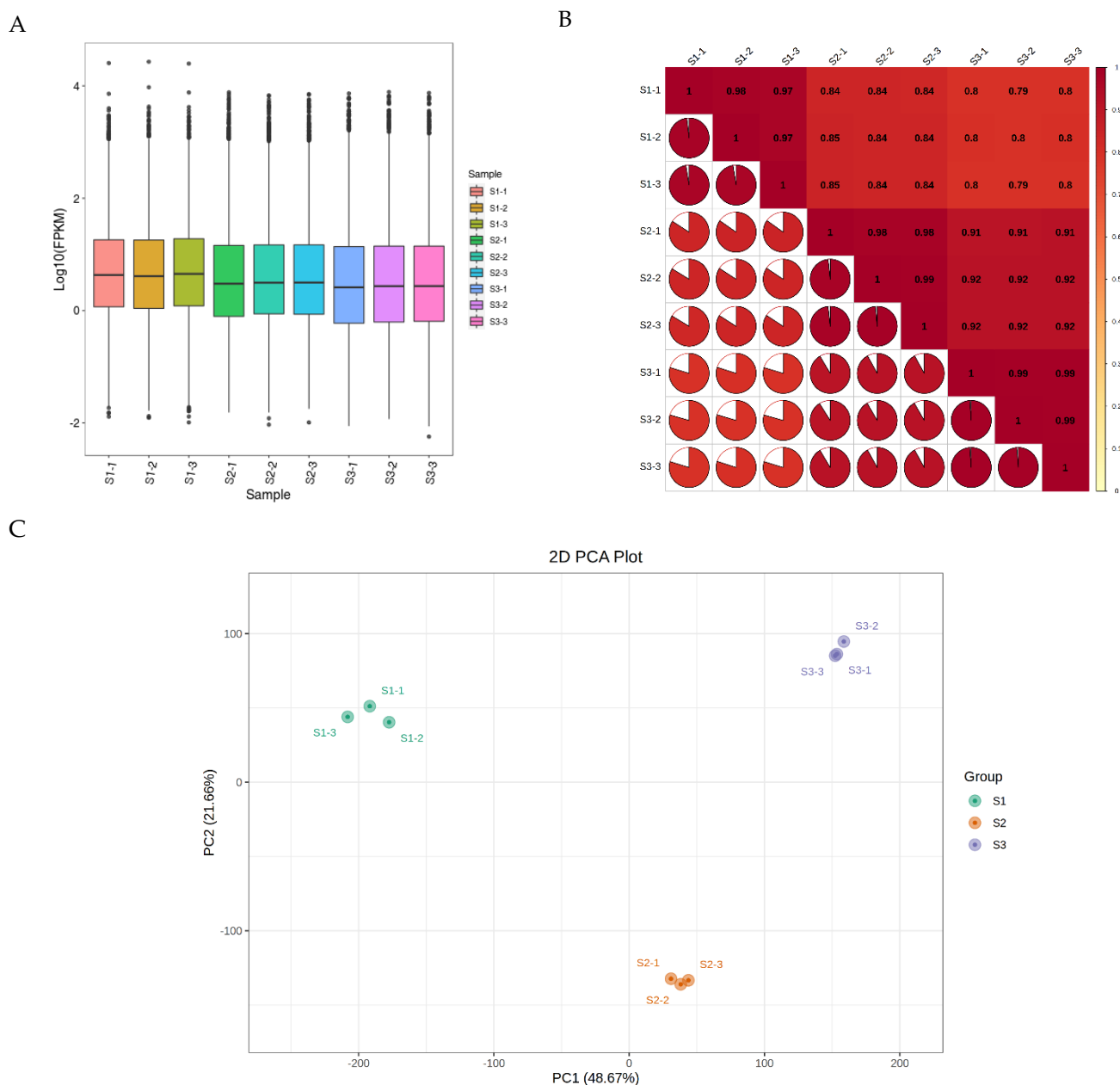


Figure 2. Presents the reliability analysis of transcriptome data. (A). The horizontal axis represents different samples, while the vertical axis indicates the logarithm of the expression values; (B). The horizontal and vertical axes represent different samples, with the color intensity indicating the strength of positive correlation—the redder the color, the stronger the positive correlation; (C). The horizontal and vertical axes represent the contribution of PC1 and PC2, respectively. The degree of aggregation within groups and the degree of dispersion between groups demonstrate the reliability of the samples.

3.2. Functional Annotation and Classification

To elucidate the molecular mechanisms underlying flower development in *C. petelotii*, it was necessary to clarify the functions and types of genes in the genome. Therefore, we analyzed the annotation results of the genome with six databases (KEGG, GO, NR, Swiss-Prot, TrEMBL, and KOG). Swiss-Prot successfully annotated 33,268 genes. The data from NR showed that 48,958 genes were successfully annotated. The distribution of the best matches with the gene sequences indicated that *C. sinensis* (84.79%) had the highest number of matches with the samples, followed by *C. lanceoleosa* (8.45%), *C. sinensis* var. *sinensis*

(3.59%), *Actinidia rufa* (0.19%), and Others (2.98%) (Figure 3A). In the comparison with the TrEMBL database, 48,334 genes had completed functional annotation. Through analysis with the KOG database, we found that 28,475 genes were successfully annotated (Figure 3B), and these genes were divided into 25 categories. The top three categories with the highest proportions were the R category (General function prediction only) with 5305 genes, the T category (Signal transduction mechanisms) with 2925 genes, and the O category (Posttranslational modification, protein turnover, chaperones) with 2771 genes. By aligning the genes with the GO database, the analysis provided a more detailed functional interpretation framework for the genes. The genes in this study were identified as belonging to 45 subcategories across three main categories. The numbers of terms for Biological process (BP) and Molecular function (MF) were 22 and 21, respectively, while the Cellular component (CC) had the fewest subcategories with only 2. In the KEGG classification of genes, 29,332 genes completed functional annotation and classification. Further analysis revealed that these genes were categorized into 145 KEGG pathways. In addition to this, through the iTAK database prediction and identification, a total of 2580 transcription factors (TFs) were excavated and identified in the three different developmental stages of the flower organs of *C. petelotii*, including 2039 TFs and 541 transcriptional regulators (TRs). Among them, the top nine transcription families in terms of quantity were bHLH (149), AP2/ERF-ERF (148), MYB (128), C2H2 (118), NAC (106), MYB-related (97), WRKY (87), and C3H (80) (Figure 3C).

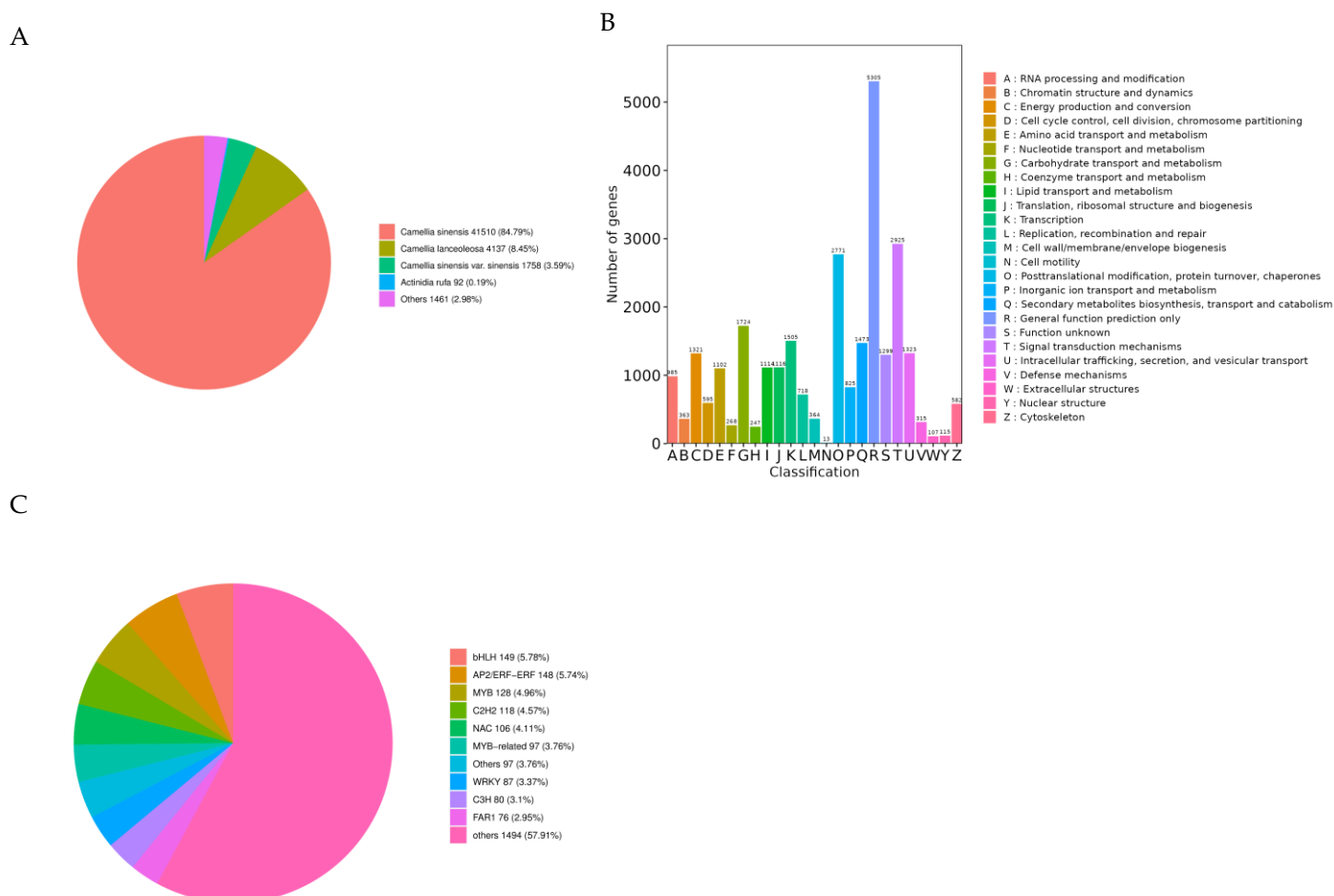


Figure 3. Functional annotation and classification of genes. (A). Results from the NR database (B). Functional classification of genes in the KOG database (C). Identification of the top nine transcription factor families.

3.3. DEGs in Three Developmental Stages of Flowers

To elucidate the expression patterns of DEGs associated with the three different developmental stages of flower organs in *C. petelotii*, we conducted comparative transcriptome analysis across the three developmental stages, S1, S2, and S3. In this study, we used $|\log_2\text{Fold Change}| \geq 1$ and $\text{FDR} < 0.05$ as the criteria for screening. A total of 18,732 DEGs (Supplementary Table S3) were detected across the S1, S2, and S3 stages, and the differences among the three developmental stages were visually represented by constructing a clustering heat map (Figure 4A). Specifically, in the S2 vs. S1 group, 9613 DEGs were detected, with 4819 upregulated genes and 4794 downregulated genes; in the S3 vs. S1 group, the highest number of DEGs (15,133) were detected, with 6957 upregulated genes and 8176 downregulated genes; the S3 vs. S2 group had the fewest DEGs among the three groups, with only 9228 DEGs, of which 3896 were upregulated genes and 5332 were downregulated genes (Figure 4B). The Venn plot of DEGs, as shown in Figure 4C, indicated that S2 vs. S1, S3 vs. S2, and S3 vs. S1 had 1107, 1086, and 3323 DEGs expressed specifically, respectively. There were 2026 DEGs that exhibited differential changes throughout the transition from S1 to S2 and finally to S3.

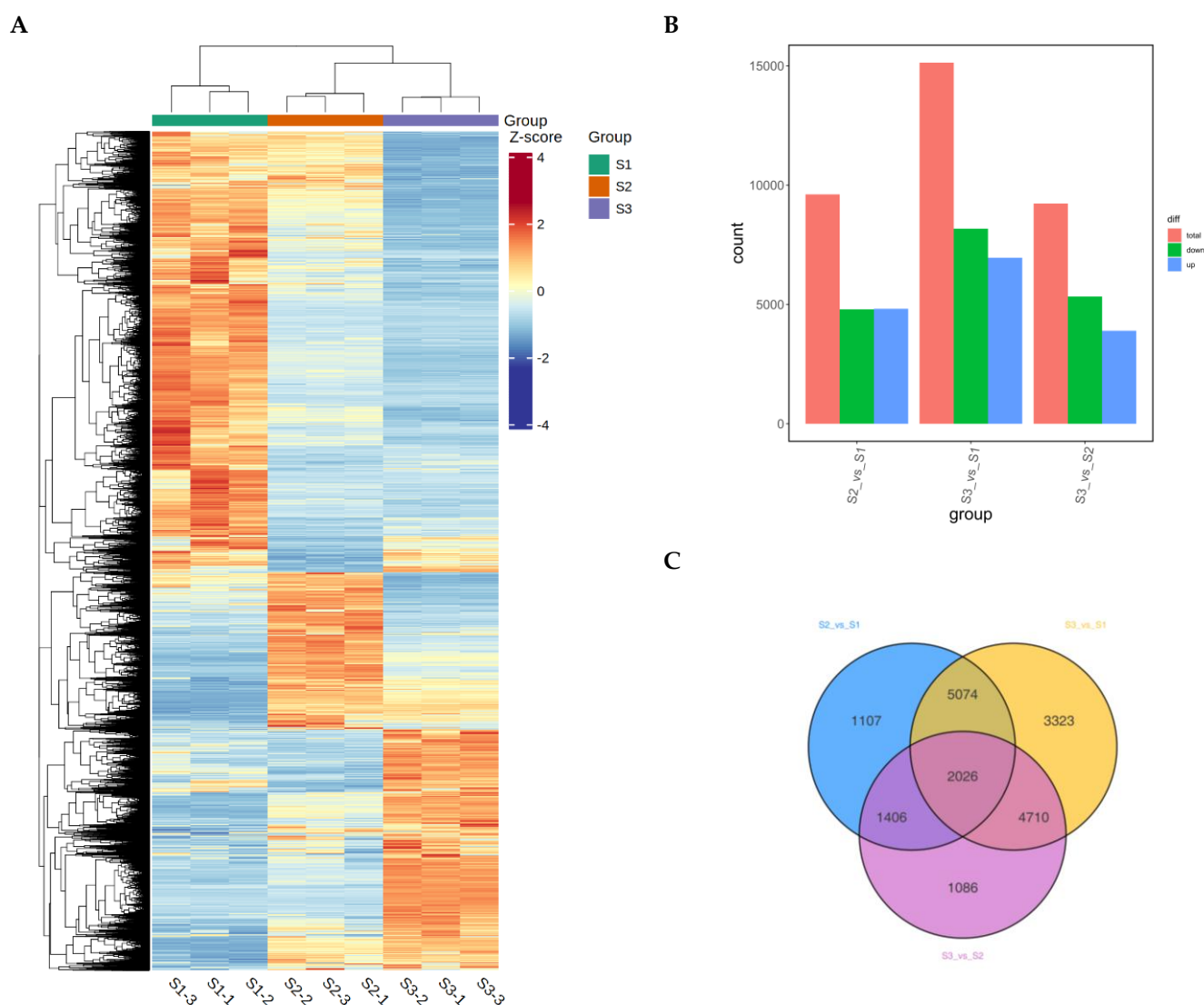


Figure 4. General data analysis of transcriptomics. (A). Cluster analysis of all DEGs across the three developmental stages. (B). The number of up- and downregulated genes at different stages, with the horizontal axis representing the groups and the vertical axis representing the number of genes. (C). Venn analysis of DEGs.

3.4. Comprehensive Transcriptome Analysis of DEGs

By conducting a K-means clustering analysis of all DEGs, we could more intuitively investigate the trends of gene expression changes during different developmental stages of plant flowering. As shown in Figure 5, based on the variation trends of DEGs, we categorized all DEGs into 7 subclasses. These were subclass 1 (3802), subclass 2 (949), subclass 3 (2144), subclass 4 (1439), subclass 5 (2077), subclass 6 (3505), and subclass 7 (4816). It was not difficult to observe from the figure that the relative expression levels of genes in subclass 1 and subclass 5 generally showed a trend of gradually increasing from S1 to S3, indicating that these genes might play a positive regulatory role in promoting plant flowering. In contrast, subclass 6 and subclass 7 showed a trend that was the opposite of subclass 1 and subclass 5; their overall pattern exhibited a gradual decrease from S1 to S3. These genes might have played an inhibitory role in plant flowering. The results of these clustering analyses further demonstrated the complexity of gene regulation during the development of *C. petelotii*.

To further clarify the specific roles of these DEGs during the three different stages of plant flowering, we analyzed the enrichment pathways of GO and KEGG to identify the changes in DEGs across various developmental stages. Firstly, the GO classification is shown in Figure 6. These DEGs were categorized into three categories: Biological process, Molecular function, and Cellular component. Overall, in the Biological process of S2 vs. S1 and S3 vs. S2, the majority of DEGs were identified as being related to cellular process, metabolic process, and response to stimulus, while in Cellular component, a significant number of DEGs were associated with cellular anatomical entities. In addition, in Molecular function, Binding, catalytic activity, and transcription regulator activity were all enriched, with a large number of DEGs across the three groups (Figure 6A,B). The KEGG pathway enrichment results indicated (Figure 7) that the Plant hormone signal transduction (ko04075) and Starch and sucrose metabolism (ko00500) pathways were significantly enriched with a large number of genes in the comparisons of S2 vs. S1, S3 vs. S2, and S3 vs. S1. The Circadian rhythm—plant (ko04712) pathway had a higher degree of enrichment in S2 vs. S1 than in S3 vs. S2. Through the annotation of KEGG pathways, we found that these five pathways might have significant associations with the flowering of *C. petelotii*. The discoveries from GO and KEGG pathways provided systematic theoretical data for the subsequent exploration and screening of DEGs related to plant flowering.

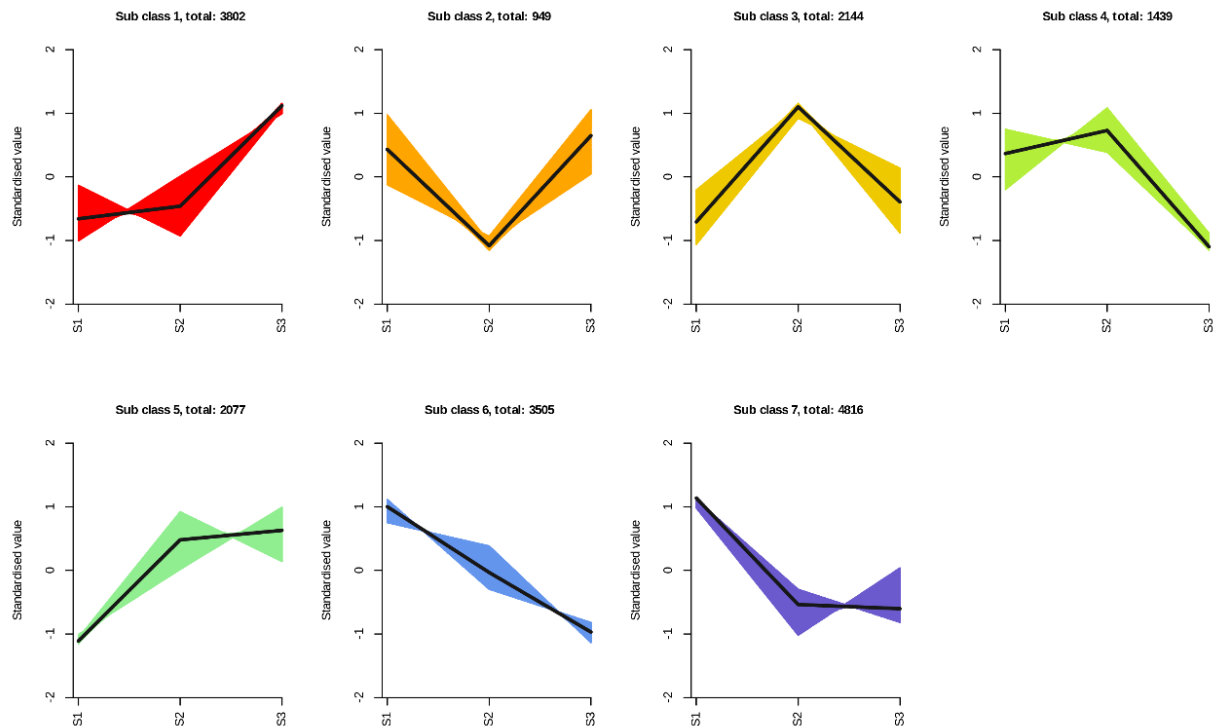
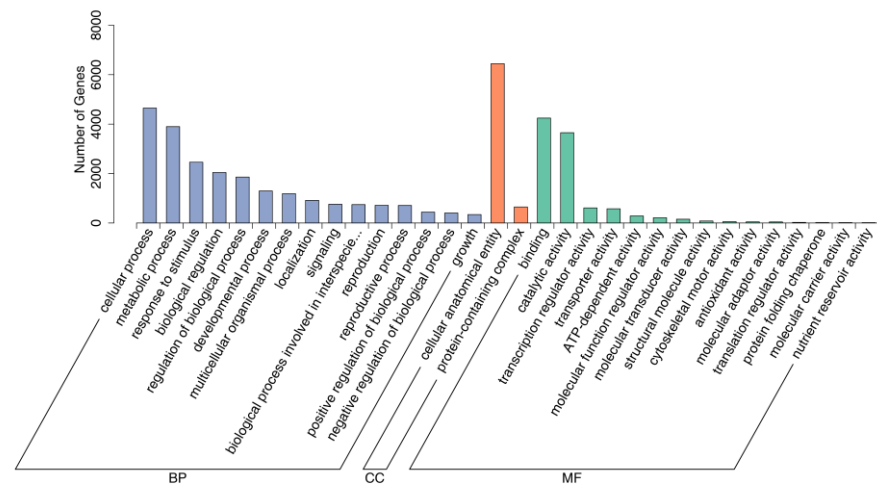


Figure 5. The K-means clustering analysis of DEGs is represented in the figure, where the horizontal axis indicates different developmental stages, and the vertical axis represents the normalized expression levels.

A



B

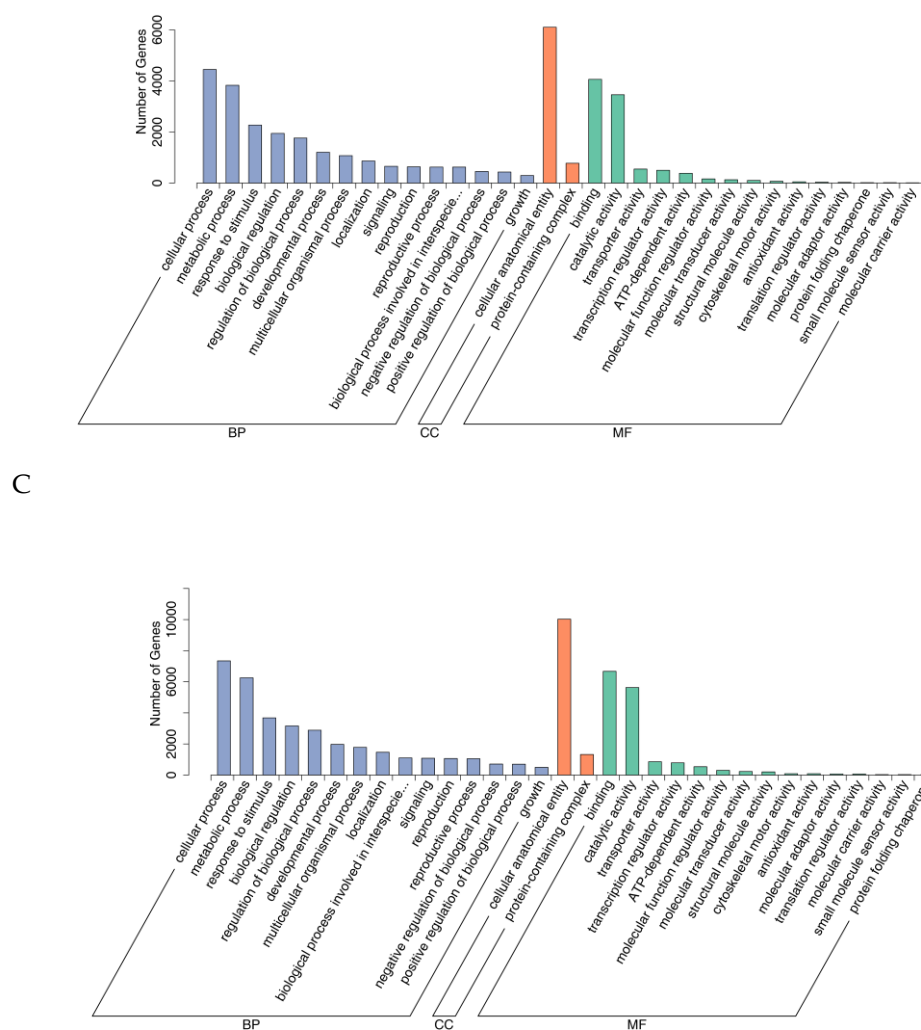


Figure 6. GO enrichment analysis of DEGs, with the horizontal axis representing functional categories and the vertical axis representing the number of enriched DEGs. (A). Enrichment results for S2 vs. S1; (B). Enrichment results for S3 vs. S2; (C). Enrichment results for S3 vs. S1.

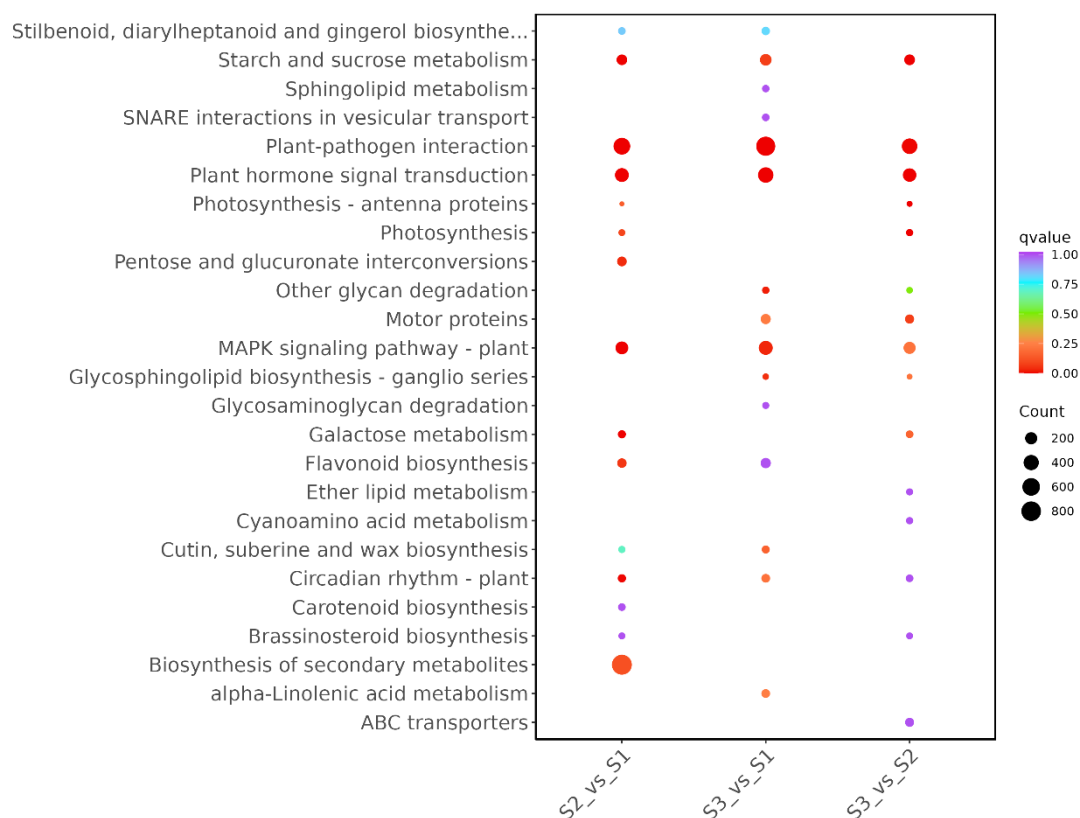


Figure 7. The display of the top 20 most significant pathway enrichment results across the three groups, with the horizontal axis representing the Rich factor. The larger the Rich factor, the greater the degree of enrichment. The vertical axis represents the KEGG pathways; the larger the points, the more differential genes are enriched in the pathways; the redder the points, the more significant the enrichment.

3.5. Analysis of DEGs Associated with Flowering in *C. petelotii*

In this study, we utilized the functional annotation results from databases and corroborating evidence from relevant literature to screen the 2026 common DEGs. We mined candidate genes for flowering from pathways closely related to plant flowering, such as plant hormone signal transduction, starch and sucrose metabolism, circadian rhythm—plants, and Autonomous pathway. Among them, there were 3 *AP2/ERF-ERF*, 7 *GID1*, 7 *SAUR*, 6 *BAK1*, 6 *BRI1*, 5 *AUX/IAA*, 4 *MYC2*, 4 *PR1*, 3 *CTR1*, 3 *PP2C*, 2 *ARR-B*, 2 *CYCD3*, 2 *GH3*, 2 *GRAS*, 2 *JAR4*, 2 *PYL*, 2 *TCH4*, 2 *TGA*, 4 *ARF*, 1 *ARR-A*, 1 *AUX1*, 1 *BK1*, 1 *BSK*, 1 *EBF*, 1 *TIFY*, and 1 *TIR1* related to plant hormone signal transduction. For starch and sucrose metabolism, 40 genes were identified, including 22 *GH*, 4 *GH32*, 3 *TPP*, 2 *BAM*, 2 *FrK*, 2 *GH5*, 1 *ENPP1*, 1 *HK*, 1 *SS*, 1 *SUS*, and 1 *UTP*. Regarding the photoperiod-circadian rhythm pathway, regulatory genes such as 4 *PRR*, 2 *PIF3*, 1 *CHS*, 1 *EF3* and 1 *LHY* were identified. Similarly, some typical regulatory genes of the autonomous pathway were also detected, including 3 *AP2/ERF-AP2*, 2 *AG*, 1 *SVF*, 1 *CO*, and 1 *SOC1* (Table 1). In addition to these, we also discovered 1 *FT* and 1 *LFY*. They were not among the 2026 common DEGs, yet they have been confirmed by numerous studies to play a key role in flowering; hence, they were also included in the list of flowering candidate genes for this study. Overall, a total of 134 flowering candidate genes were analyzed in depth during this research.

We combined K-means clustering analysis to investigate the trend of changes in candidate genes related to plant hormone signal transduction (Figures 5 and 8). We found that 5 gibberellin receptors (*GID1*), 3 SAUR family proteins (*SAUR*), 1 ethylene-responsive transcription factor (*AP2/ERF-ERF*), 2 auxin-responsive proteins IAA (*AUX/IAA*), 2 jasmonic acid-amino synthetases (*JAR4*), 1 jasmonate ZIM domain-containing protein (*TIFY*), 3 serine/threonine-protein kinases (*CTR1*), 1 abscisic acid receptor (*PYL*), 1 protein phosphatase 2C (*PP2C*), 1 pathogenesis-related protein 1 (*PR1*), and 1 two-component response regulator (*ARR-A*) all showed a continuous up-regulation trend from S1 to S3, and we speculated that these genes play a role in promoting signal transduction in plant hormone signaling. In stark contrast, 4 transcription factor MYC2s (*MYC2*), 4 protein brassinosteroid insensitive 1s (*BRI1*), 1 BRI1 kinase inhibitor 1 (*BKI1*), 4 brassinosteroid insensitive 1-associated receptor kinase 1s (*BAK1s*), 2 ethylene-responsive transcription factors (*AP2/ERF-ERFs*), 2 auxin response factors (*ARFs*), 1 two-component response regulator (*ARR-B*), 2 auxin-responsive protein IAAs (*AUX/IAAs*), 1 BR-signaling kinase (*BSK*), 2 cyclin D3s (*CYCD3*), 1 EIN3-binding F-box protein (*EBF*), 1 gibberellin receptor (*GID1*) and 2 transcription factors TGA (*TGA*) showed the highest expression at S1 and then decreased to the lowest value by S3, so we speculated that these genes play an inhibitory role in signal transduction in plant hormone signaling. In addition, we also found 16 genes that underwent a decrease followed by an increase in the hormone signal regulation process, such as 1 auxin-responsive protein IAA (*AUX/IAA*), 1 auxin influx carrier (*AUX1*), 2 DELLA proteins (*GRAS*) and 1 gibberellin receptor (*GID1*); there were also some genes that showed an increase followed by a decrease in the hormone signal regulation process. This result indicates that DEGs involved in hormone signal transduction have a complex network, with the up-regulation and down-regulation of a large number of regulatory genes collectively affecting the strength of hormone signal transduction, thereby influencing the synthesis of plant hormones.

In the starch and sucrose metabolism, there were 2 trehalose 6-phosphate phosphatases (TPSs), 2 glucan endo-1,3-beta-glucosidases (GH), 2 beta-glucosidases (GH), 1 beta-fructofuranosidase (GH32), 2 beta-amylases (BAMs), and 1 starch synthase (SS) that displayed similar up-regulation trends from S1 to S3, and positively regulated the transduction of *C. petilotii*. Regarding the negatively regulated genes, several key enzyme genes were identified, including: 5 glucan endo-1,3-beta-glucosidases (GH), 4 beta-glucosidases (GH), 2 endoglucanases (GH), 1 fructokinase (FrK), and 1 ectonucleotide pyrophosphatase (ENPP1). Additionally, other downregulated genes included 1 sucrose synthase (SUS), 1 hexokinase (HK), 1 UTP-glucose-1-phosphate uridylyltransferase (UGP), 1 fructokinase (FrK), and 1 trehalose 6-phosphate phosphatase (TPP). Furthermore, certain genes belonging to the glycoside hydrolase (GH) family and its subfamilies—specifically GH5 and GH32—were expressed at significantly higher levels at stage S2 compared to the other two developmental stages. In the circadian rhythm (photoperiod) pathway, two genes were identified as phytochrome-interacting factors (*PIF3*). Studies have shown that *PIF3* plays a key role in plant germination and flowering [37]. One of them consistently increased in expression as development progressed, while the other did the exact opposite, continuously decreasing as development proceeded. At the same time, 4 pseudo-response regulators (*PRRs*) and 1 MYB-related transcription factor (*LHY*) were found to reach their highest values in S3 across the three developmental stages. One chalcone synthase (*CHS*) belonging to Subclass 7 was identified, and we speculated that this gene is involved in the negative regulation of the flowering process. In addition to this, 1 protein EARLY FLOWERING (*EF3*) was found to be significantly expressed, which plays an important role in regulating the flowering time of plants. In the autonomous pathway, we discovered the core flowering gene *SOC1* in Subclass 1, which plays a role in promoting plant flowering. At the same time, there were 1 *CONSTANS-like CO3* (*CO*), 2 *Agamous* (*AGs*), 1 *SHORT VEGETATIVE PHASE* (*SVP*)

and 3 *AP2-like factors* (*AP2/ERF-AP2s*) that all showed significant changes across the three stages, leading us to speculate that these genes are involved in regulating the flowering time of plants.

Admittedly, plant flowering is an extremely complex process regulated by numerous genes. This study provided candidate genes associated with plant flowering for *C. petelotii*. At the same time, a large number of genes enriched in pathways related to flowering were identified and annotated. These results provided a substantial theoretical data foundation for further clarification of the flowering mechanism.

Table 1. Number of DEGs associated with *C. petelotii* flowering process.

Components	Count	Components	Count
Plant hormone signal transduction		Starch and sucrose metabolism	
<i>AP2/ERF-ERF</i>	3	<i>GH</i>	22
<i>GID1</i>	7	<i>GH32</i>	4
<i>SAUR</i>	7	<i>TPP</i>	3
<i>BAK1</i>	6	<i>BAM</i>	2
<i>BRI1</i>	6	<i>FrK</i>	2
<i>AUX/IAA</i>	5	<i>GH5</i>	2
<i>MYC2</i>	4	<i>ENPP1</i>	1
<i>PR1</i>	4	<i>HK</i>	1
<i>CTR1</i>	3	<i>SS</i>	1
<i>PP2C</i>	3	<i>SUS</i>	1
<i>ARR-B</i>	2	<i>UTP</i>	1
<i>CYCD3</i>	2	Photoperiod-circadian	
<i>GH3</i>	2	<i>PRR</i>	4
<i>GRAS</i>	2	<i>PIF3</i>	2
<i>JAR4</i>	2	<i>CHS</i>	1
<i>PYL</i>	2	<i>EF3</i>	1
<i>TCH4</i>	2	<i>LHY</i>	1
<i>TGA</i>	2	Flowering activators	
<i>ARF</i>	4	<i>AP2/ERF-AP2</i>	3
<i>ARR-A</i>	1	<i>SVP</i>	1
<i>AUX1</i>	1	<i>CO</i>	1
<i>BK11</i>	1	<i>SOC1</i>	1
<i>BSK</i>	1	<i>AG</i>	2
<i>EBF</i>	1	<i>FT</i>	1
<i>TIFY</i>	1	<i>LFY</i>	1
<i>TIR1</i>	1		

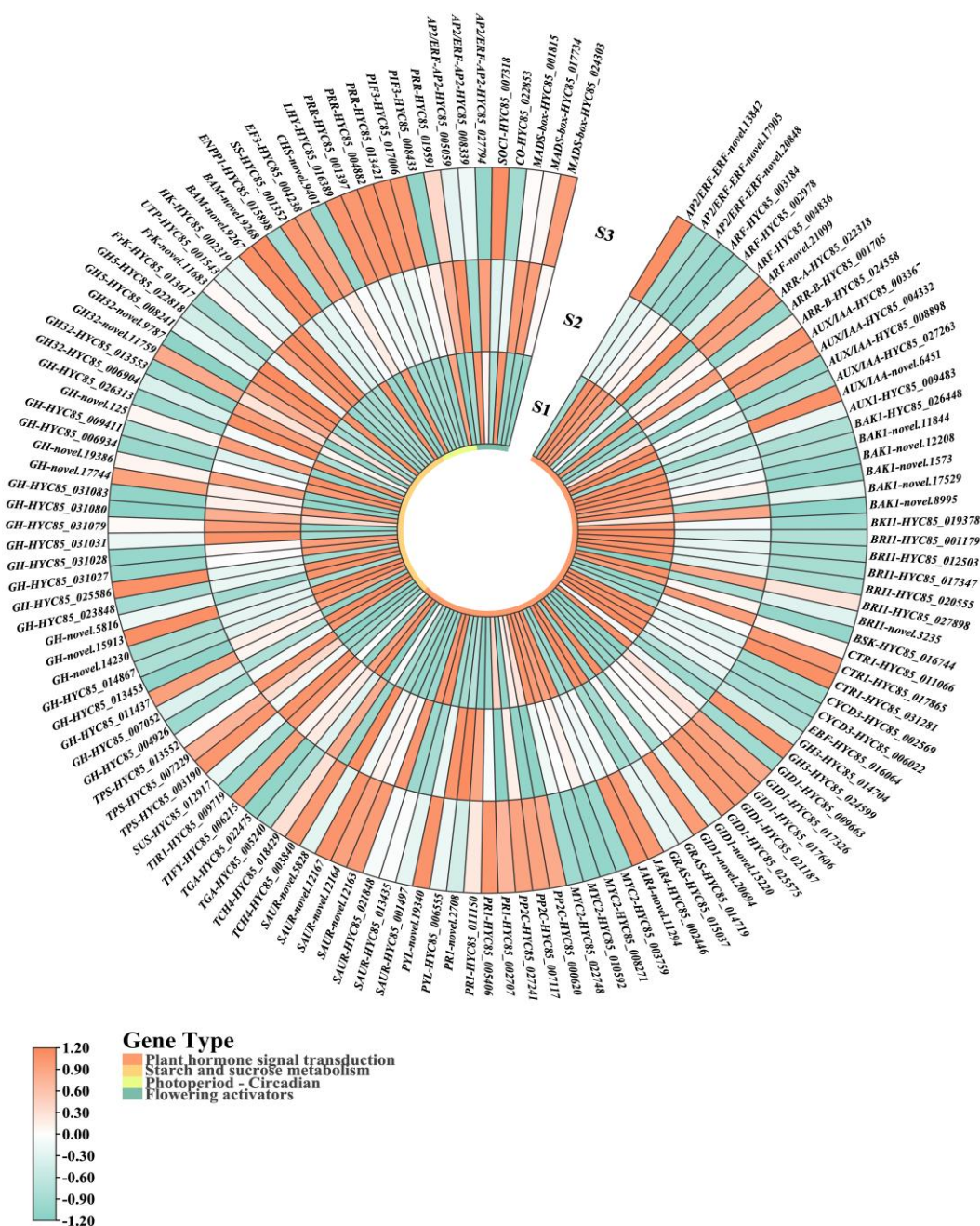


Figure 8. Differential expression analysis of candidate genes associated with flowering. The circular diagram features three concentric outer rings, each representing distinct developmental stages, while the innermost ring illustrates the functional classification of the identified genes.

3.6. qRT-PCR Qualification and Analysis

To assess the reliability of RNA-seq-derived transcriptome data for *C. petilotii*, a total of 11 DEGs associated with flowering were validated using qRT-PCR. The expression profiles of the 11 DEGs determined via qRT-PCR were highly consistent with those determined via RNA-seq (Figure 9), suggesting that the transcriptome-based analysis of the *C. petilotii* flower buds was highly accurate and reliable.

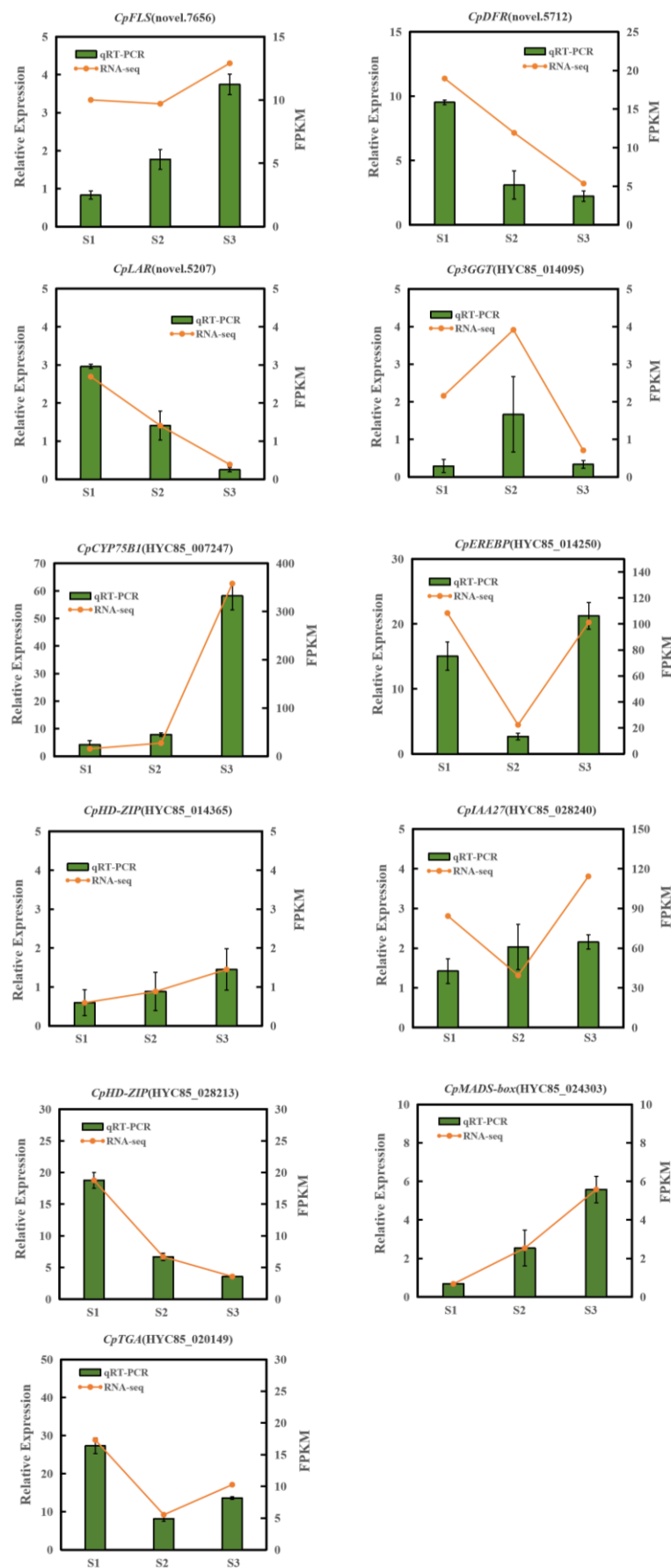


Figure 9. Relative expression profiles of 11 genes associated with flowering in *C. petelotii*, determined via qRT-PCR analysis. The data are presented as means $\pm$ standard deviations ($n = 3$).

4. Discussion

The transition from vegetative growth to reproductive development, marked by plant flowering, represents a pivotal phase in the plant life cycle and is a central focus in ornamental plant research due to its significant impact on floral aesthetics [6,7,38]. Among these plants, *C. petelotii*, a rare and highly ornamental species within the *Camellia* genus, remains poorly understood at the molecular level regarding its floral organ development, presenting a substantial challenge for its breeding and cultivation. To address this knowledge gap, we conducted a comprehensive transcriptomic analysis using RNA-Seq technology on *C. petelotii* floral organs at three developmental stages. Comprehensive functional annotation analyses identified several significantly enriched pathways associated with floral development, including plant hormone signal transduction, starch and sucrose metabolism, circadian rhythm regulation, and the autonomous pathway. Through subsequent refinement of our analysis, we pinpointed candidate DEGs potentially involved in the regulation of flowering processes.

4.1. Effects of Plant Hormone Signaling on *C. petelotii*

Plant hormone signal transduction plays an extremely crucial role in plant growth, development, and defense. It controls the levels of various hormones involved in every aspect of plant growth and reproduction through signal transduction, ultimately affecting plant flowering and development. Plant hormones mainly include auxins (IAAs), ethylene (ETH), gibberellins (GAs), and jasmonic acid (JA), among others [11]. In this study, the Plant hormone signal transduction pathway was significantly enriched during all three developmental stages, indicating that this pathway played a key role in the process of plant flowering. IAA is a key auxin that regulates cell division, elongation, and differentiation to control the flowering process in plants [39]. The *Aux/IAA* gene family encodes proteins that are key factors in responding to auxin signals, while *ARFs* are transcription factors that can interact with *Aux/IAA* proteins [40]. In our candidate genes for this study, we also observed a starkly opposite change in the *AUX/IAA* and *ARF* genes, indicating that *Aux/IAA* typically acts to inhibit *ARF* activation. This research outcome is largely consistent with Luo et al. [40]. In the article, there were 3, 2, and 2 *SAUR* genes that reached their peak values during stages S3, S2, and S1, respectively, demonstrating different expression patterns across various developmental stages of the plant. These results are essentially consistent with the findings of *SAUR* in *Impatiens uliginosa* Franch. In addition, the *GH3* gene was also involved in the synthesis and regulation of auxin [40], and the 2 annotated *GH3* genes both showed a trend of decreasing before increasing. Overall, the expression levels of *AUX1*, *ARF*, *SAUR*, and *AUX/IAA* varied significantly from S1 to S3, indicating that the expression profiles of genes involved in auxin signal transduction suggest that they may play an active role in determining and maintaining petal identity. This finding is consistent with previous transcriptomic studies on petal development in roses (*Rosa hybrida*) by Han et al. [41], as well as with studies on the adaxial and abaxial cell populations of petals [35].

In *Arabidopsis thaliana*, mutants with a deficiency in jasmonic acid synthesis and jasmonic acid receptor mutants exhibit floral organ deformities or sterility, indicating that jasmonic acid is essential for the normal development of floral organs [42]. Jasmonic acid-amino synthetase catalyzes the conjugation reaction between JA and amino acids in plants, forming jasmonoyl-L-isoleucine (JA-Ile), which is the active form of jasmonic acid [43]. The 2 *JAR4* genes identified in current study are persistently upregulated from stage S1 to S3, suggesting that they play a critical role in promoting jasmonic acid biosynthesis, which in turn facilitates the transition to flowering. *MYC2* and *TIFY* were also considered to be involved in the regulation of the jasmonic acid signaling pathway [44,45], and this study identified 4 *MYC2* genes and 1 *TIFY* that were significantly expressed at three stages. In

the differentiation of flower buds and the development of flowers, the role of GA is particularly important. For instance, gibberellins can promote flowering in long-day plants [46]. Several studies indicate that GAs promote flowering by increasing the expression of the *SOC1* gene, and this promotion is inhibited by DELLA proteins [12]. In this study, we observed similar trends, with 2 DELLA proteins (*GRAS*) being continuously downregulated during the flowering process. In contrast, the majority of genes involved in gibberellin signaling, such as *GID1* and *TF (PIF3)*, as well as the *SOC1* gene associated with promoting flowering, were predominantly upregulated, with only a minority being downregulated. These results suggest that gibberellin may play a crucial role in the flowering process of *C. petelotii*. Ethylene (ETH) typically plays a role in accelerating senescence and abscission in floral organs. In *Petunia*, altering the ethylene signal transduction can change the ethylene content, thereby extending the flowering period [47,48]. Serine/threonine-protein kinase *CTR1* (*CTR1*) serves as a key negative regulator in the ethylene signaling transduction pathway in plants [49]. In this study, we identified a continuous upregulation of 3 *CTR1* genes, which serve to inhibit ethylene synthesis. Similarly, the ethylene-responsive transcription factor (*AP2/ERF-ERF*) and EIN3-binding F-box protein (*EBF*), which are also important genes in ethylene signal transduction, showed a general trend of decreased expression as flowering progressed in this research. The specific regulatory processes of these genes could be further investigated in subsequent studies in conjunction with changes in ethylene levels.

Cytokinin (CTK) is involved in processes such as the growth of the shoot apical meristem [50] and the regulation of flowering induction [51]. In this study, we identified three *ARR-A* and *ARR-B* genes associated with the cytokinin signaling pathway that participate in the regulation of the flowering process. In contrast, for the abscisic acid signaling pathway, we screened for 2 *PYL* and 3 *PP2C* genes, which may play a significant role in abscisic acid synthesis. These gene specific regulatory mechanisms could be further investigated in subsequent studies in conjunction with changes in abscisic acid levels. In *A. thaliana*, fluctuations in salicylic acid (SA) levels have been found to regulate the timing of flowering and plants deficient in salicylic acid flower later [52]. In relation to the salicylic acid (SA) signaling pathway, we identified 4 *PR1* and 2 *TGA* genes as candidate flowering genes. In *A. thaliana*, mutants with impaired brassinosteroid (BR) biosynthesis (with levels below 10% of that in the wild type) display a flowering time that is delayed by at least 10 days compared to the wild type. This suggests that the endogenous levels of brassinosteroids influence the timing of flowering [53,54]. In this study, we found that the majority of genes associated with brassinosteroids signal transduction, including *BAK1*, *BRI1*, *BSK*, *BKI1*, and *TGA*, showed a continuous downregulation trend as flowering progressed. We propose that these genes play a negative role in the flowering transition of *C. petelotii*.

4.2. Effects of Starch and Sugar Signaling on *C. petelotii*

The starch and sucrose metabolism pathway plays an important role in plant flowering, primarily by providing the necessary energy for flowering and participating in signal transduction. The study by Hu et al. [12] indicates that during the flowering process of *Crocus sativus* L., there is a gradual decrease in starch content and a corresponding increase in the content of soluble sugars. This suggests the necessity of starch and sucrose in the transition to flowering. In this dataset, we identified 1 starch synthase with a continuously increasing expression level. We speculate that this is due to the need to convert starch into monosaccharides during the flowering process, which leads to the sustained overexpression of this *SS* to maintain the energy required for plant flowering. During the flowering period of *Sinapis alba* and *Xanthium strumarium*, there is a rapid accumulation of sucrose in the phloem and shoot tips of the plant, which plays a role in promoting flowering [55]. In this study, we observed a gradual accumulation of soluble sugars, which is crucial for

the flowering process. We identified key genes involved in sucrose metabolism, such as sucrose synthase (*SUS*) and beta-amylase (*BAM*), to be significantly important in the synthesis of sucrose. Notably, *BAM* is consistently upregulated throughout the flowering process. In *A. thaliana*, trehalose-6-phosphate (*T6P*) and sucrose work together to regulate flowering time, and they are considered signaling molecules for the adequate distribution of carbohydrates in plants, playing a pivotal role in the induction of flowering [55]. Our results indicated that *TPP*, involved in the degradation process of *T6P*, generally exhibited an upregulated trend throughout the flowering process. Glucose has been established as playing a critical role in regulating various developmental processes in plants. For instance, glucose signaling can interact with the signals of plant hormones such as auxin (IAA signal), cytokinin (CTK signal), and brassinosteroid (BR signal), thereby indirectly affecting plant flowering [56]. Through KEGG pathway annotation, the *GH* gene family and its subfamily (*GH5*) are identified as D-Glucose hydrolases that regulate gene expression, producing glucose units. In this study, a significant number of *GH* family genes were found to exhibit substantial changes between stages S1 and S3, leading us to hypothesize that glucose may play an indirect role in the flowering process of *C. petelotii*. In our study, we observed an increase in the expression of genes related to fructose metabolism, such as hexokinase (*HK*), fructokinase (*FrK*) and beta-fructofuranosidase (*GH32*). Fructose, often in conjunction with glucose, can be directly absorbed and metabolized by plants, providing the necessary energy and carbon skeletons for plant flowering. Based on our findings, we hypothesize that glucose may play an indirect role in the flowering process of *C. petelotii*. Overall, sugar metabolism typically exerts an indirect regulatory effect on plant flowering, and the specific regulatory roles of these genes require further investigation.

4.3. The Flowering Pathway in During the Floral Transition

Previous studies have indicated that the transition to flowering is actually a complex physiological and morphological response to internal plant signals, such as sugars, plant hormones, and autonomy, as well as environmental cues like photoperiod and vernalization [57]. For instance, in the model plant *A. thaliana*, genes have been discovered that rapidly induce plant growth under long-day conditions, and flowering time is shortened under high light intensity [13,58]. The circadian rhythm model is one of the key hypotheses explaining plant responses to photoperiodism. When the photoperiod is in basic alignment with the plant's internal rhythm, the overexpression of key flowering genes such as *CONSTANS* (*CO*) is induced, thereby promoting plant flowering [59]. In our study, we found that the expression of a *CONSTANS-like CO3* (*CO*) gene decreased from stage S1 to S3. We speculate that this downregulation may be due to the complex regulatory nature of *CO* in controlling the photoperiod, or the circadian rhythm, which in turn affects the transition to flowering. *PIF3* is a key signaling factor that interacts with light-activated phytochromes to transmit light signals to downstream circadian rhythm regulatory genes and activate their expression [37]. In our data, the expression levels of *PIF3*, *EF3*, *LHY* and *PRR* genes, which are related to the photoperiod (circadian rhythm), consistently increased from stage S1 to S3. This suggests that the photoperiod actively intervenes in the flowering transition of *C. petelotii*. Similar results have also been observed in *Crocus sativus* L. [12], *A. sinensis* [60] and *Juglans regia* [61]. In addition, we found that 1 *PIF3* and 1 *CHS* gene were downregulated during this process, which may play an inhibitory role.

The *SOC1* gene encodes an MADS-box transcription factor that serves as a signal integrator in the numerous pathways affecting plant flowering. It integrates multiple flowering signals from related signals such as photoperiod (circadian rhythm), plant hormones, sugars and age [62,63]. For instance, in *Litchi chinensis*, the activation of *SOC1* triggers the entry of lychee into the flowering phase, indicating that *SOC1* plays a key role in regulating the flowering process of lychee [64]. In this study, we also found that the expression level of the *SOC1* gene significantly increased during the flowering process of *C. petelotii*, which is consistent with the research by Shi et al. [64]. Therefore, we consider this gene to be a core gene in the flowering transition and highly worthy of further in-depth research and analysis. *Agamous* (*AG*), *SHORT VEGETATIVE PHASE* (*SVP*) and *AP2-like factors* (*AP2/ERF-AP2*) are present in this dataset. The *AP2* (*AP2/ERF-AP2*) gene plays a crucial role in determining the identity of floral organs and is directly involved in flower development [65]. In plant flowering, the *SVP* gene typically suppresses the expression of *SOC1* and *FT* genes, thereby inhibiting flowering [66]. *AG* also directly participates in the determination of floral organ identity, and an increase in its expression can lead to the transformation of floral organs [67]. In this dataset, the expression of the *AG* gene increased as flowering occurred. The *Flowering locus T* (*FT*) gene, referred to as the ‘florigen’, is directly activated by *CO*, which in turn activates the overexpression of the downstream gene *SOC1*, ultimately promoting flowering [68]. The primary pathways that induce flowering ultimately converge on the floral meristem [12]. *SOC1*, as a core gene in the transition to flowering, once activated, initiates the expression of numerous downstream floral organ development genes [63]. Among these, *LFY*, as one of the floral organ development genes, is a floral meristem identity gene and is very important in floral meristem formation [69]. Subsequently, the floral organ identity genes *AP2/ERF-AP2* and *AG*, under the regulation of *SOC1* and *LFY*, initiated the flowering transition in *C. petelotii*.

In general, the flowering process of *C. petelotii* is regulated by sugar metabolism, hormone signal transduction, photosynthesis, circadian rhythms, and the autonomous pathway. These pathways interact with each other; for example, plant hormones not only play a key role in plant flowering but also directly and indirectly regulate the plant’s response to photoperiod [70]. Sugars also have complex interactions with plant hormones, jointly affecting the flowering process [55]. Therefore, the flowering process of *C. petelotii* is a highly complex morphological and physiological transformation process. We preliminarily constructed a regulatory network for the flowering transition of *C. petelotii* (Figure 10), which included the candidate flowering genes from this study, laying the foundation for further elucidating the complex molecular mechanisms underlying the flowering of *C. petelotii*.

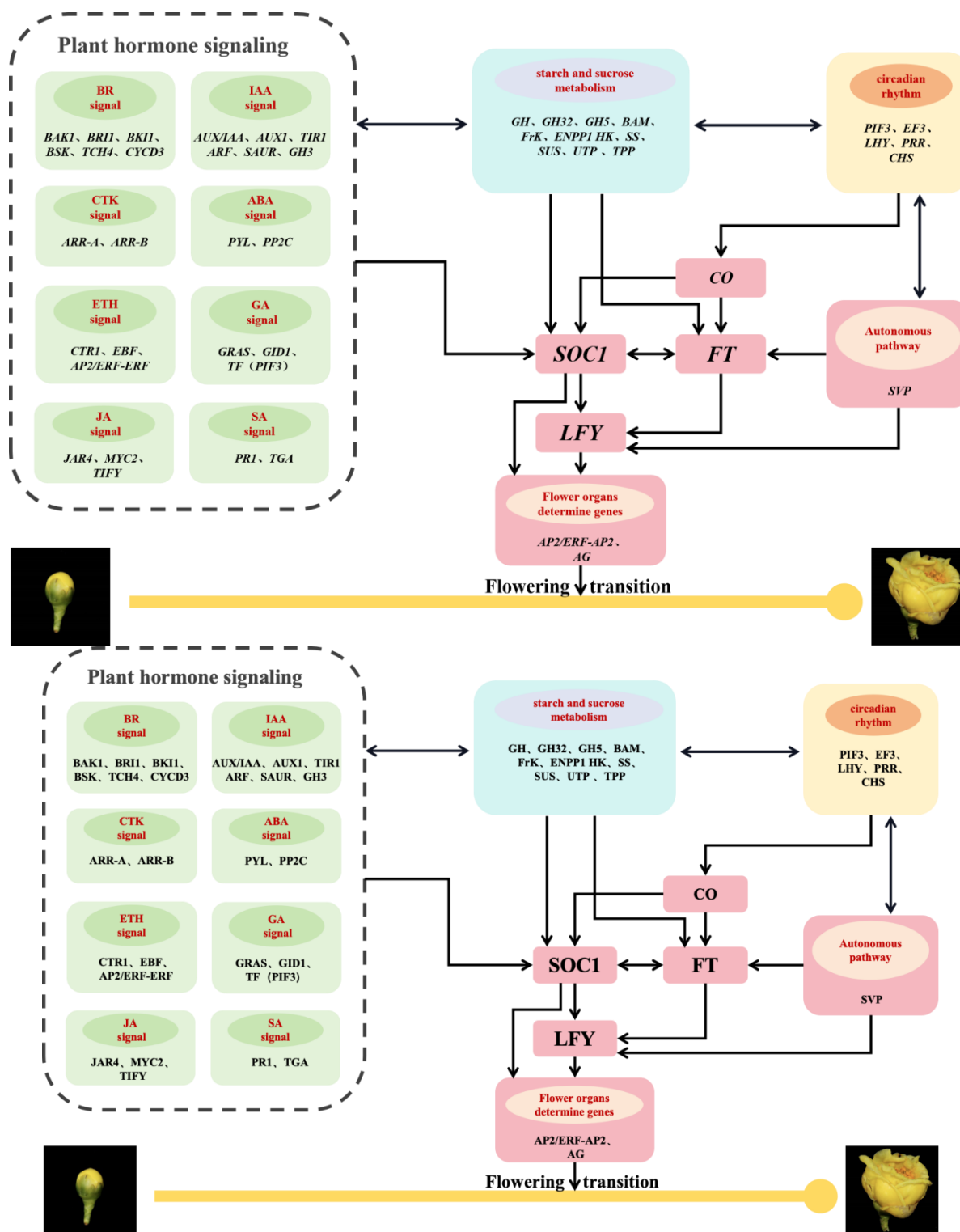


Figure 10. The hypothetical model of the molecular regulatory network for the flowering transition in *C. petelotii*. Unidirectional arrows represent promotional or inhibitory effects, and bidirectional arrows indicate interactive actions.

5. Conclusions

This study employed RNA-seq technology based on Illumina sequencing to investigate the molecular mechanisms underlying flowering in *C. petelotii*. A total of 67.05 Gb of clean data is obtained, with a mapping efficiency of clean reads ranging from 70.26% to 73.07%. The research findings indicate that the flowering process of *C. petelotii* is coordinately regulated by multiple signaling pathways. Hormones such as IAA, GA, JA, and

ETH regulate the flowering process through a complex signal transduction network. In particular, changes in the expression of genes such as *AUX/IAA*, *ARF*, *SAUR*, and *GH3* suggest that the auxin signaling pathway may play a significant regulatory role in the development of floral organs. Jasmonic acid promotes the flowering process through the continuous upregulation of the *JAR4* gene, while gibberellin promotes flowering through the upregulation of *GID1* and *SOC1* genes. Changes in the expression of genes such as starch synthase (*SS*) and sucrose synthase (*SUS*) indicate that starch and sucrose metabolism play an important role in energy supply during the flowering process. In particular, the continuous upregulation of β -amylase (*BAM*) suggests that starch degradation may provide the necessary sugars for flowering. Additionally, changes in the expression of genes related to glucose and fructose metabolism (such as *HK*, *FrK*, and *GH32*) also indicate that sugar signaling indirectly participates in the regulation of flowering. The expression of the *CONSTANS-like CO3* (*CO*) gene is downregulated during the flowering process, while the expression of photoperiod-related genes such as *PIF3*, *EF3*, *LHY*, and *PRR* is continuously upregulated, indicating that the photoperiod regulates the flowering of *C. petelotii* through the circadian rhythm. In summary, this study preliminarily constructed a regulatory network for the flowering transition of *C. petelotii* through transcriptome analysis, revealing the molecular mechanisms of its flowering process. Finally, a hypothetical model for the transition to flowering is proposed, offering significant references for the genetic breeding of *C. petelotii*. These findings provide an important theoretical basis for molecular breeding and genetic research of *C. petelotii* and lay the groundwork for further deciphering the complex molecular mechanisms of its flowering in the future.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/metabo16020119/s1>, Table S1: Primers sequences of genes used for quantitative RT-PCR analysis; Table S2: Overview of the sequencing and mapping results; Table S3: All_DEG.annot.

Author Contributions: Y.W. and W.G. collected the experimental material; W.G. and L.A. designed the experiments; Y.W., L.A., S.Z., X.L. and X.C. performed the experiments; L.A., S.Z., X.L. and X.C. analyzed the data; Y.W. wrote the article. All authors have read and agreed to the published version of the manuscript.

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