

Article

Molecular Mechanism Study on the Color of *Cosmos bipinnatus* and *Cosmos sulphureus*

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Abstract

Flavonoids are essential secondary metabolites that predominantly affect flower pigmentation in plants. Understanding the molecular mechanisms underlying flower color divergence is crucial for ornamental plant breeding. This study aimed to elucidate the factors responsible for the differences in color between white-petaled *Cosmos bipinnatus* and orange-petaled *Cosmos sulphureus*. We employed an integrated approach combining untargeted LC–MS/MS metabolomics and high-throughput transcriptome sequencing of fresh petals to analyze pigment composition and differential gene expression. Petal pigment extraction, total flavonoid quantification, and metabolomic profiling consistently revealed that differences in flavonoid abundance are responsible for flower color divergence between the two species. In contrast, carotenoids, previously considered potential contributors to flower coloration, were neither evident in the oil phase of the pigment extracts nor detected by metabolomic analysis. Flavonoid compounds accumulated at relatively high levels in the orange petals of *C. sulphureus*, reaching 11.36 times that of *C. bipinnatus*, contributing to its bright appearance. Transcriptomic analysis revealed differences in gene expression patterns between the two species, highlighting key candidate genes involved in the flavonoid biosynthesis pathway, such as chalcone synthase. These findings indicate that the orange coloration of *C. sulphureus* may be associated with CHS-regulated accumulation of naringenin chalcone and downstream compounds in the flavonoid metabolic pathway after CHS, providing valuable theoretical support for a deeper understanding of the causes underlying the differences in flower color between *C. bipinnatus* and the orange-petaled *C. sulphureus*.



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1. Introduction

Flavonoids are a class of phenolic secondary metabolites that are widely distributed throughout the plant kingdom. From bryophytes to angiosperms, flavonoids accumulate abundantly across diverse plant lineages [1]. Structurally, flavonoids share a characteristic

C6-C3-C6 carbon skeleton [2]. They are major secondary metabolites responsible for the coloration of most flowers [3–5], fruits, and seeds and are synthesized through branched pathways that yield both colorless compounds (e.g., flavonols) and colored pigments (e.g., anthocyanins, as well as polymeric phlobaphenes and proanthocyanidins) [6]. On the basis of structural features surrounding the heterocyclic C ring, flavonoids can be classified into six major groups: flavanones, flavones, flavonols, isoflavones, anthocyanins, and chalcones [7]. Accumulating evidence indicates that flavonoids regulate plant reproductive processes and exhibit pronounced organ-specific accumulation patterns in stems, leaves, fruits, and other tissues [8]. In addition, flavonoids have strong antioxidant activity and increase stress tolerance to adverse conditions such as ultraviolet radiation, drought, and flooding [9,10]. They can also promote the recruitment and activity of rhizosphere microorganisms and facilitate plant uptake of nitrogen after it is released into the soil [11–13].

To date, studies of the flavonoid biosynthetic pathway have relied heavily on model species such as *maize*, *Arabidopsis*, and *Petunia*. Through analyses of flavonoid-related mutants and characterization of numerous structural and regulatory genes [6–8], the core flavonoid biosynthetic pathway has been largely established. Flavonoid biosynthesis is a branch of the phenylpropanoid pathway, in which the C6-C3 backbone is derived from phenylalanine [14]. The first committed step of flavonoid biosynthesis is catalyzed by chalcone synthase (CHS), which condenses three molecules of malonyl-CoA with one molecule of 4-coumaroyl-CoA to produce naringenin chalcone. Chalcone isomerase (CHI) then converts naringenin chalcone to naringenin. Naringenin is subsequently hydroxylated by flavanone 3-hydroxylase (F3H) to form dihydrokaempferol, which is further converted to dihydroquercetin and dihydromyricetin by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) [15], respectively. Finally, sequential reactions catalyzed by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS) convert dihydroflavonols into colored anthocyanidin aglycones that are subsequently glycosylated [16]. Using molecular biological approaches, researchers have targeted key biosynthetic enzyme-encoding genes in the flavonoid pathway to modulate their expression and thereby alter flower color in plants [17–19].

The CHS gene was first successfully isolated from *Petroselinum hortense* [20]. CHS belongs to the type III polyketide synthase (PKS) superfamily and is one of its best-known representatives. This family also includes stilbene synthase, acridone synthase, pyrone synthase, bibenzyl synthase, and *p*-coumaroyl triacetic acid synthase [21,22] and catalyzes the formation of diverse scaffolds for plant secondary metabolites. Type III PKSs show high sequence similarity in amino acid alignment, and PKS-catalyzed reactions are initiated by the thiol group of the catalytic cysteine within the Cys–His–Asn catalytic triad [22]. Moreover, the active-site region of type III PKSs adopts a conserved three-dimensional structure such that substitutions at only a few amino acid residues can markedly reshape the substrate-binding pocket and redirect enzyme function [23]. CHS activity has been linked to pigmentation in multiple plant tissues and to flavonoid accumulation [24,25]. Loss of CHS enzymatic activity can cause albinism and prevent flavonoid accumulation [26,27]. In *Oncidium* spp., CHS loss of function results in a complete inability of floral organs to accumulate anthocyanins [27]. In *Clivia miniata*, the expression of multiple CHS genes is significantly greater in red leaves and fruit peels than in green tissues [24]. In *Malus spectabilis*, CHS has been identified as a key regulatory factor underlying red petal coloration, directly controlling the dynamics of anthocyanin biosynthesis [25]. Gutterson et al. introduced an antisense CHS construct into pink chrysanthemum via *Agrobacterium*-mediated transformation, resulting in complete flower-color whitening [28]. These findings further suggest that fine-tuned CHS expression patterns contribute to complex floral pig-

mentation and highlight CHS as an important target for the molecular design of flower color in ornamental plants.

C. bipinnatus is an annual or perennial herbaceous plant in the Asteraceae family (genus *Cosmos*). Its ray florets can be purplish red, pink, or white [29]. Another species in the genus, *C. sulphureus* (also known as sulfur *Cosmos*), differs from *C. bipinnatus* in that its ray florets are golden yellow or orange [30]. Because of its strong stress tolerance, short growth cycle, and diverse flower colors, *Cosmos* is widely cultivated as an ornamental plant [31].

Flower color is not only a key ornamental trait but also reflects ecological adaptation, pollinator attraction, and stress-response mechanisms [32,33]. Given these characteristics, *Cosmos* represents a suitable system for investigating the molecular mechanisms underlying floral coloration. However, the molecular basis for the rich color variation in *Cosmos* flowers remains unclear. Here, we used white flowers of *C. bipinnatus* and orange flowers of *C. sulphureus* as study materials. We first identified flavonoid compounds associated with flower color at the metabolite level. We subsequently screened genes related to flower color divergence at the genetic level and identified two key *CHS* genes, *CCHS2* and *CCHS1*. Although the metabolomic and transcriptomic differences between the two species may involve broader species-specific biological divergence beyond flower color, this study focuses primarily on resolving the changes in flavonoid metabolic pathways related to flower color. Finally, by characterizing the key *CHS* genes, we elucidated their specific roles in *Cosmos* flower color formation.

2. Materials and Methods

2.1. Collection of Plant Materials

Two *Cosmos* species (white flower *C. bipinnatus* and orange flower *C. sulphureus*) were used in this study. The plant materials used were taxonomically authenticated by Prof. Zhihua Liao. The plants were grown at Southwest University, Chongqing, China (29°82' N, 106°42' E). Flowers with two distinct petal colors, namely, orange (Cs) and white (Cb), were collected. Immediately after sampling, the sepals and reproductive organs were removed, and the petals were collected, snap-frozen in liquid nitrogen, transported to the laboratory, and stored at −80 °C. Each group included three biological replicates. Fresh petals were collected for subsequent experiments.

2.2. Petal Pigment Extraction and Determination of the Total Flavonoid Content

In plants, orange pigments can be classified into two main categories: lipid-soluble pigments, typically carotenoids, and water-soluble pigments, typically flavonoids [34]. To preliminarily identify the chemical basis underlying the difference in flower color between *C. bipinnatus* (white petals) and *C. sulphureus* (orange petals), pigments were extracted using a 1:1 (*v/v*) mixture of water and dichloromethane [35–37]. The total flavonoid content in the two sample types was subsequently determined by a colorimetric method, and the total flavonoid content was expressed as milligrams per gram fresh weight (mg/g FW). Briefly, 0.1 g of fresh petals was weighed, rapidly frozen in liquid nitrogen, and freeze-dried to constant weight. The dried material was ground into a fine powder. The powder was extracted with 1 mL of 70% (*v/v*) methanol in water, vortexed, and centrifuged. The supernatant was filtered through a 0.22-μm microporous membrane to obtain extracts for total flavonoid quantification.

Next, 1 mL of extract was mixed with 0.2 mL of 10% (*w/v*) AlCl_3 in methanol, 0.2 mL of 1 M potassium acetate, and 5.6 mL of distilled water. The mixture was incubated at room temperature for 30 min, after which the absorbance was measured at 415 nm. A quercetin standard curve was generated by substituting 1 mL of quercetin standard

solutions of graded concentrations for the sample extract and processing them in the same manner. The total flavonoid content in the petals of the two colors was calculated using the resulting linear regression equation. The results are expressed as mg/g quercetin equivalents, reported as milligrams per gram of dry extract (mg QE/g) [34].

2.3. Extraction, Identification, and Quantification of Total Flavonoids in Petals

The petals of the two flower colors were collected. For each sample, 0.1 g of fresh petals was weighed, snap-frozen in liquid nitrogen, and immediately ground into a powder. The powder was extracted with 1 mL of 70% (v/v) methanol in water, vortexed for 5 min, and centrifuged for 3 min (12,000 rpm, 4 °C). The supernatant was collected, and the extraction was repeated twice. The supernatants from the two extractions were combined, filtered through a 0.22 µm membrane, transferred to autosampler vials, and used for LC–MS/MS analysis.

The samples were analyzed using a Thermo Fisher LC–MS system consisting of a Vanquish™ Flex UHPLC coupled to an Orbitrap Exploris™ 120 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A 3 µL aliquot of extract was injected onto a C18 column (Waters ACQUITY UPLC HSS T3, 1.8 µm, 2.1 mm × 150 mm). Metabolite identification and quantification were performed using Compound Discoverer 3.0, with annotations supported by public metabolite databases, including MassBank (<http://www.massbank.jp/>, accessed on 22 January 2025), KNAPSACk (<https://www.knapsackfamily.com/KNAPSAcK/>, accessed on 22 January 2025), HMDB (<http://www.hmdb.ca/>, accessed on 22 January 2025), MoToDB (<http://www.ab.wur.nl/moto/>, accessed on 22 January 2025), ChemBank (<http://chembank.med.harvard.edu/compounds>, accessed on 22 January 2025), PubChem (<https://pubchemblog.ncbi.nlm.nih.gov/>, accessed on 22 January 2025), NIST (<http://www.nist.gov/>, accessed on 22 January 2025), Chemistry WebBook (<http://webbook.nist.gov/>, accessed on 22 January 2025), and METLIN (<http://metlin.scripps.edu/index.php>, accessed on 22 January 2025). Qualitative analysis of MS1 and MS2 data was carried out by comparing accurate precursor ions (Q1), product ions (Q3), and retention times (RT). All the identified flavonoids were putatively annotated, as no authentic reference standards were used in this study. Annotation confidence, mass error, MS/MS spectral matching quality, and absolute abundance were used as supporting evidence for data reliability.

The peak areas for the identified compounds were used for principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Differentially accumulated flavonoids were defined using the following criteria: a fold change ≥ 2 or ≤ 0.5 and a variable importance in projection (VIP) score ≥ 1 . OmicShare (<https://www.omicshare.com/>, accessed on 25 January 2025) was used to perform PCA clustering and volcano plot analysis across three replicates per sample type. TBtoolsV2.210 was used to generate a heatmap of metabolite abundances for the two samples, with both row-wise and column-wise normalization [38].

2.4. Transcriptome Analysis of Petals with Different Colors

Freeze-dried petal tissues were ground at low temperature, and total RNA was extracted using TRIzol reagent. RNA purity was assessed using an Allsheng NanoDrop spectrophotometer (Allsheng, Hangzhou, China, <https://www.allsheng.com.cn/products-detail-23>, accessed on 25 January 2025). The RNA concentration and total yield were quantified with a Qubit fluorometer. RNA fragment integrity was evaluated by RNase-free agarose gel electrophoresis. mRNA was enriched using mRNA capture beads (oligo dT beads). Fragmented mRNA was reverse transcribed into cDNA, followed by dA tailing for end repair and ligation to sequencing adapters. Magnetic beads were used for purification or size selection, and the libraries were amplified by PCR. Library concentration

and fragment size distributions were evaluated, and the libraries were pooled according to the required sequencing output. High-throughput sequencing was performed on a NovaSeq 6000 platform. All procedures were carried out by Benagen Technology Co., Ltd. (Wuhan, China).

Raw reads generated by the sequencing platform were filtered using fastp to obtain high-quality clean reads for downstream assembly and analysis. De novo transcriptome assembly was performed using Trinity v2.1.9. Transcript abundance was quantified with salmon v1.10.1. Redundant protein sequences were removed using CD-HIT v4.8.1. All transcripts were annotated against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Genes meeting the criteria of $p \leq 0.05$ and a fold change ≥ 2 or ≤ 0.5 were identified as differentially expressed genes (DEGs). OmicShare (<https://www.omicshare.com/>, accessed on 27 January 2025) was used for volcano plot analysis and correlation heatmap visualization of DEGs among the three sample groups, and KEGG enrichment analysis was performed for subsequent analyses.

Assembly statistics, including N50, N90, average length, total length, GC content, and the number of unigenes, were calculated for quality assessment. Transcript annotation was performed against the KEGG database using BlastKOALA, and the annotation rate was recorded. Detailed quality metrics are provided in the Supplementary Materials (Table S1).

2.5. Quantitative Real-Time PCR Analysis

cDNA prepared from *Cosmos* and sulfur *Cosmos* was used for qPCR analysis of *CHS2*, *CHS1*, *CHI1*, *CHI2*, *CYP758*, *F3H*, *FLS*, *FNSII*, and *KGLT* expression with Thermo Fisher's SYBR dye (<https://www.thermofisher.cn/order/catalog/product/cn/zh/S11494>, accessed on 30 January 2025). qPCR primers were designed using Primer-BLAST V2.5.0 (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>, accessed on 30 January 2025). The *Cbactin* gene was used as the internal reference control [39]. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, and statistical significance was determined by Student's *t*-test. Primer information, amplification efficiency, melting-curve validation, and amplification curves of the reference gene are available in the Supplementary Materials (Table S2, Figure S1).

2.6. Sequence Alignment and Phylogenetic Analysis of *CCHS2* and *CCHS1*

Reported CHS genes from filamentous fungi, diatoms, and higher plants retrieved from the NCBI database, together with *CCHS1* and *CCHS2* identified in *Cosmos* in this study, were used to construct a phylogenetic tree. Amino acid sequences were aligned using the E-INS-i algorithm in MAFFT v7.475. A phylogenetic tree was constructed with IQ-TREE v2.1.2 using a maximum-likelihood model (LG + F + G4). The tree was rooted with *CHS* from filamentous fungi (Clade 1). Visualization and annotation were performed using iTOL v6.

2.7. Enzyme Activity Assays for *CCHS2* and *CCHS1*

PCR products corresponding to the full-length open reading frames (ORFs) of *CCHS2* and *CCHS1* were cloned and inserted into the pET28a(+) expression vector, digested with *Sall* and *BamHI*, and transformed into the *Escherichia coli* strain BL21 (DE3). Cells were grown at 37 °C to an OD₆₀₀ of 0.4–0.6 and induced with 0.5 mM isopropyl beta-D-1-thiogalactopyranoside at 16 °C for 22 h. N-terminal 6xHis-tagged proteins were purified and desalted using a PD MiniTrap G-25 column.

Enzyme reactions (100 µL total volume) were conducted in buffer containing 0.1 M potassium phosphate (pH 7.2) with 30 µg of purified protein, 80 µM 4-coumaroyl-CoA, and 160 µM malonyl-CoA and incubated at 30 °C for 1 h. The reaction products were dissolved in 100 µL of methanol. As a negative control, 30 µg of purified *CCHS2* or *CCHS1* protein

was heat-inactivated by boiling for 15 min and processed in parallel. The reaction mixtures from both the experimental and control groups were analyzed by high-performance liquid chromatography (HPLC) [40,41].

3. Results

3.1. Pigment Extraction and Total Flavonoid Contents in Petals

The results of the extraction assays revealed that both the aqueous and organic phases of the extracts from *C. bipinnatus* white petals were colorless, whereas the extracts from *C. sulphureus* orange petals displayed an orange–yellow aqueous phase and a light-yellow organic phase. These results indicate that the yellow compounds in *C. sulphureus* are predominantly water-soluble and are therefore likely to be flavonoids (Figure 1B). The total flavonoid content was subsequently quantified via colorimetric assay. The total flavonoid content in *C. sulphureus* orange petals was 37.05 mg/g FW, whereas that in *C. bipinnatus* white petals was 3.26 mg/g FW, indicating that the total flavonoid content in the orange petals is 11.36 times that in the white petals (Figure 1C). Together, these findings support a dominant role for flavonoids in the formation of orange petals. Accordingly, subsequent analyses focused on the flavonoid biosynthetic pathway to elucidate the molecular mechanisms underlying flower color formation.

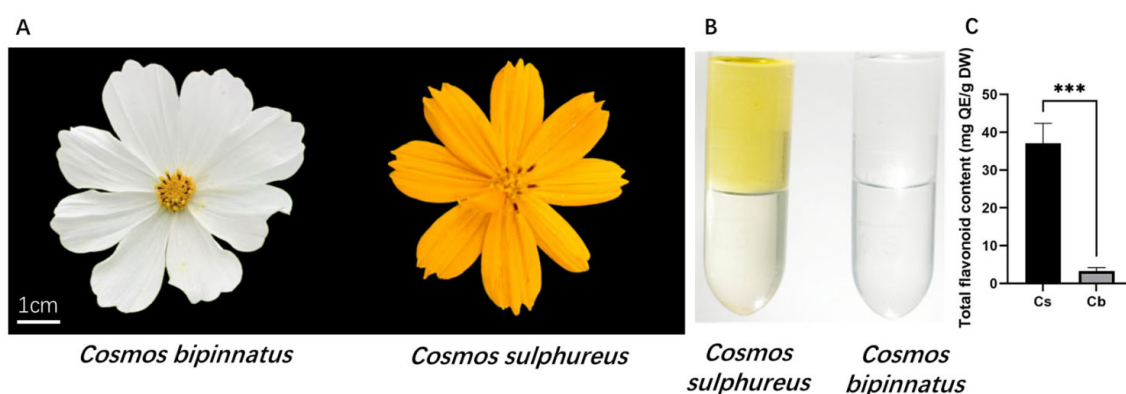


Figure 1. Analysis of the total flavonoid content: (A) Flower color phenotypes of *C. sulphureus* and *C. bipinnatus*. (B) Pigment extraction from *C. sulphureus* and *C. bipinnatus*. (C) Total flavonoid contents in *C. sulphureus* and *C. bipinnatus*. QE, quercetin equivalents; Cs, *C. sulphureus*; Cb, *C. bipinnatus*. Three biological replicates were used for each group. Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3.2. Untargeted Metabolomic Analysis of Petals

UPLC–MS/MS profiling was performed to characterize the composition and abundance of metabolites in the petals of *C. sulphureus* and *C. bipinnatus*, yielding a total of 3845 metabolites identified across the two petal colors. Principal component analysis (PCA) revealed that the first principal component (PC1) and the second principal component (PC2) explained 92.1% and 4.0% of the total variance, respectively, accounting for 96.1% of the overall variation. These findings indicate that PC1 and PC2 capture the major information contained in the original dataset (Figure 2A). Sample clustering further revealed clear separation between orange *C. sulphureus* petals and white *C. bipinnatus* petals, while the differences among the biological replicates within each group were small, indicating good reproducibility (Figure 2C).

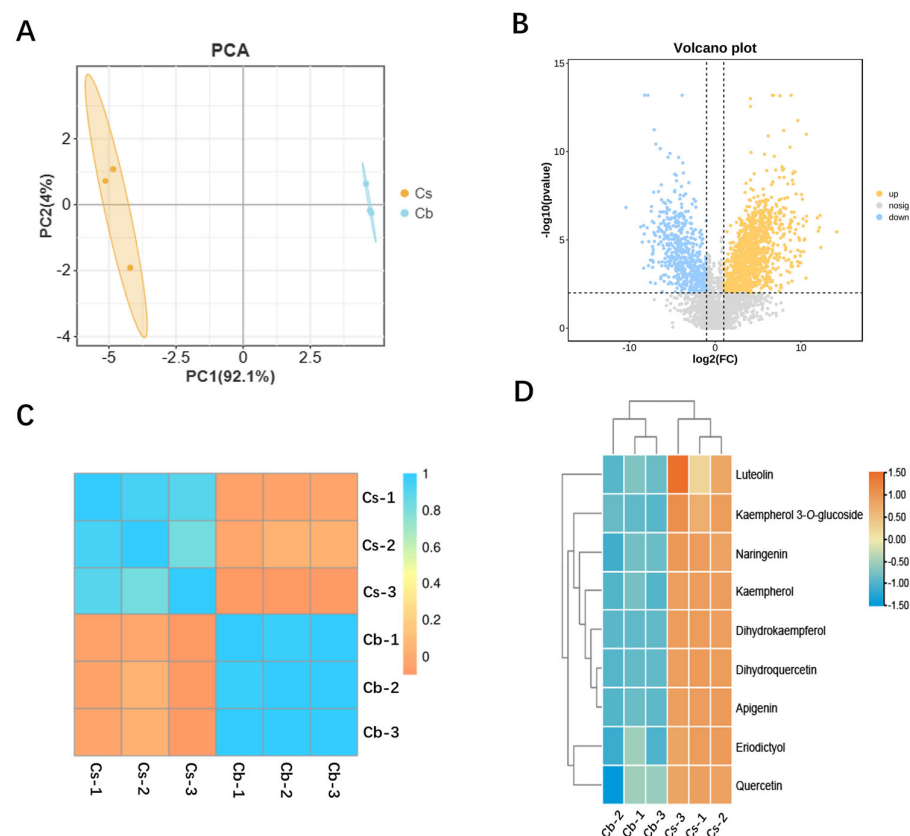


Figure 2. Metabolomic analysis of petals from *C. sulphureus* and *C. bipinnatus*: **(A)** PCA of untargeted metabolomic profiles of *C. sulphureus* and *C. bipinnatus*. Orange dots (Cs) indicate *C. sulphureus*, and blue dots (Cb) indicate *C. bipinnatus*; three biological replicates per group. **(B)** Differentially abundant metabolites between Cs and Cb petals. Cs, *C. sulphureus*; Cb, *C. bipinnatus*; orange dots represent compounds enriched in *C. sulphureus*, and blue dots represent compounds enriched in *C. bipinnatus*. **(C)** Hierarchical clustering of metabolomic samples. Three biological replicates per group were used. **(D)** Heatmap of the abundances of nine flavonoid compounds in *C. sulphureus* and *C. bipinnatus*.

To further identify the metabolites contributing to the color difference between *C. bipinnatus* (Cb) and *C. sulphureus* (Cs) petals, a volcano plot was generated with the Log2 fold change (Cs/Cb) on the x-axis and the $-\log_{10}(p\text{ value})$ on the y-axis. This analysis revealed 602 metabolites that accumulated at relatively high levels in *C. bipinnatus* white petals and 1135 metabolites that accumulated at relatively high levels in *C. sulphureus* petals (Figure 2B). Notably, nine flavonoids were highly abundant in *C. sulphureus* (Table 1, Figure S2, and Table S3). These compounds are yellow pigments, and their high accumulation in *C. sulphureus* petals likely underlies yellow coloration, whereas they were barely detectable in *C. bipinnatus* petals, which appear white (Figure 2D).

Table 1. Differentially accumulated flavonoid compounds in petals with different flower colors.

Name	Class	Formula	Reference Ion	m/z	RT [min]
Naringenin	Flavonone	C ₁₅ H ₁₂ O ₅	[M+H] ⁺ ¹	273.0765	8.873
Eriodictyol	Flavonone	C ₁₅ H ₁₂ O ₆	[M+H] ⁺ ¹	289.0713	0.885
Dihydrokaempferol	Flavanonol	C ₁₅ H ₁₂ O ₆	[M+H] ⁺ ¹	289.0713	1.809
Dihydroquercetin	Flavanonol	C ₁₅ H ₁₂ O ₇	[M+H] ⁺ ¹	305.0663	3.883
Apigenin	Flavone	C ₁₅ H ₁₀ O ₅	[M+H] ⁺ ¹	271.0606	6.852
Luteolin	Flavone	C ₁₅ H ₁₀ O ₆	[M+H] ⁺ ¹	287.0552	6.315
Kaempferol	Flavonol	C ₁₅ H ₁₀ O ₆	[M+H] ⁺ ¹	287.0555	6.477
Kaempferol 3-O-glucoside	Flavonol	C ₂₁ H ₂₀ O ₁₁	[M+H] ⁺ ¹	449.1092	6.671
Quercetin	Flavonol	C ₁₅ H ₁₀ O ₇	[M+H] ⁺ ¹	303.0506	6.127

3.3. Transcriptome Sequencing and Differential Expression Analysis

Fresh petals from *C. sulphureus* and *C. bipinnatus* were subjected to transcriptome sequencing, with three biological replicates per group. Each replicate yielded more than 6 Gb of data, and the Q30 values exceeded 80% for all the samples. After expression normalization, PCA revealed that PC1 and PC2 explained 69.9% and 11.7% of the total variance, respectively, accounting for 81.6% of the overall variation, indicating that these two components capture the major structure of the dataset (Figure 3C). Sample clustering indicated good reproducibility among the three independent replicates of each species (Figure 3B). The two species were clearly separated, suggesting substantial divergence in gene expression patterns. Differential expression analysis using DESeq2, with thresholds of $|\log_2\text{FoldChange}| > 1$ and $p < 0.05$, revealed 13,763 differentially expressed genes (DEGs), including 8073 genes whose expression was upregulated and 5690 genes whose expression was downregulated in *C. sulphureus* petals (Figure 3A).

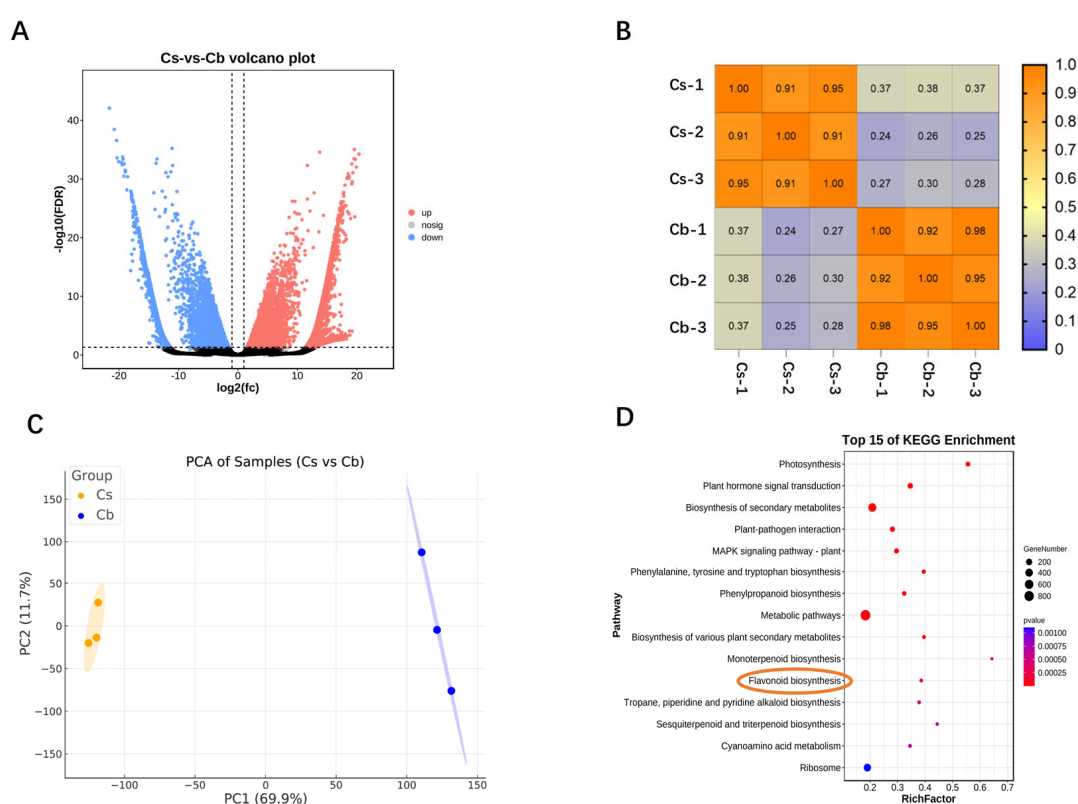


Figure 3. Transcriptome analysis of petals from *C. sulphureus* and *C. bipinnatus*: (A) Differentially expressed genes between *C. sulphureus* and *C. bipinnatus* petals. (B) Sample clustering analysis of transcriptomic data. Cs, *C. sulphureus*; Cb, *C. bipinnatus*. (C) PCA of the transcriptomes of *C. sulphureus* and *C. bipinnatus*. Orange dots indicate *C. sulphureus*; blue dots indicate *C. bipinnatus*. (D) KEGG enrichment analysis of DEGs between *C. sulphureus* and *C. bipinnatus* petals.

In addition, we assessed the quality of the transcriptome assembly and RNA-seq data. The co-assembly generated 760,881 unigenes with an N50 of 1057 bp and an average length of 708.7 bp (Supplementary Table S1-1). For each of the six samples, the clean reads ranged from X to Y million, with Q30 values exceeding 93% and mapping rates to the co-assembled transcriptome ranging from 93.6% to 96.2% (Supplementary Table S1-2). Furthermore, 21.61% of the unigenes were successfully annotated against the KEGG database (Supplementary Table S1-1). These results demonstrate that the transcriptome assembly and RNA-seq data are of high quality and suitable for subsequent differential expression and functional analyses.

KEGG enrichment analysis of the DEGs revealed that the flavonoid biosynthesis pathway was significantly enriched (Figure 3D). These results suggest that the difference in the expression of genes involved in flavonoid biosynthesis contributes to the differences in flower color between *C. bipinnatus* and *C. sulphureus*.

3.4. Analysis of the Flavonoid Biosynthetic Pathway

To further elucidate the molecular basis of flower color formation in *C. bipinnatus* and *C. sulphureus*, we integrated metabolite accumulation data with transcriptomic expression profiles to systematically analyze key metabolites and enzyme-encoding genes in the flavonoid biosynthetic pathway. The findings delineate the metabolic route from the precursor *p*-coumaroyl-CoA to multiple flavonoid products, and a heatmap of the expression levels of the corresponding biosynthetic genes is presented in Figure 4.

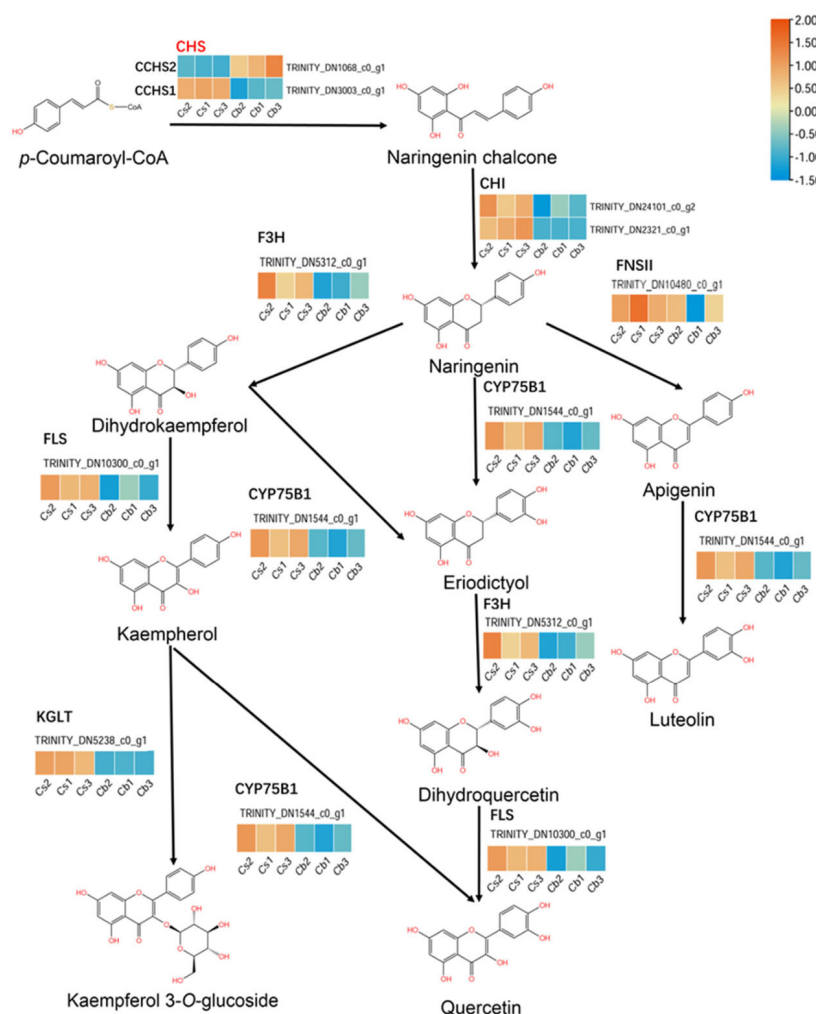


Figure 4. Transcriptional expression patterns of flavonoid biosynthetic genes in *C. sulphureus* and *C. bipinnatus*.

First, chalcone synthase (CHS), the entry-point enzyme of flavonoid biosynthesis, catalyzes the conversion of *p*-coumaroyl-CoA to naringenin chalcone. Two *CHS* transcripts were identified in the two species: *TRINITY_DN3003_c0_g1* (*CCHS1*) and *TRINITY_DN1068_c0_g1* (*CCHS2*). *CCHS1* was highly expressed in *C. sulphureus* petals but nearly undetectable in *C. bipinnatus* petals, whereas *CCHS2* showed the opposite pattern, being highly expressed in *C. bipinnatus* petals but barely expressed in *C. sulphureus* petals (Figure 5).

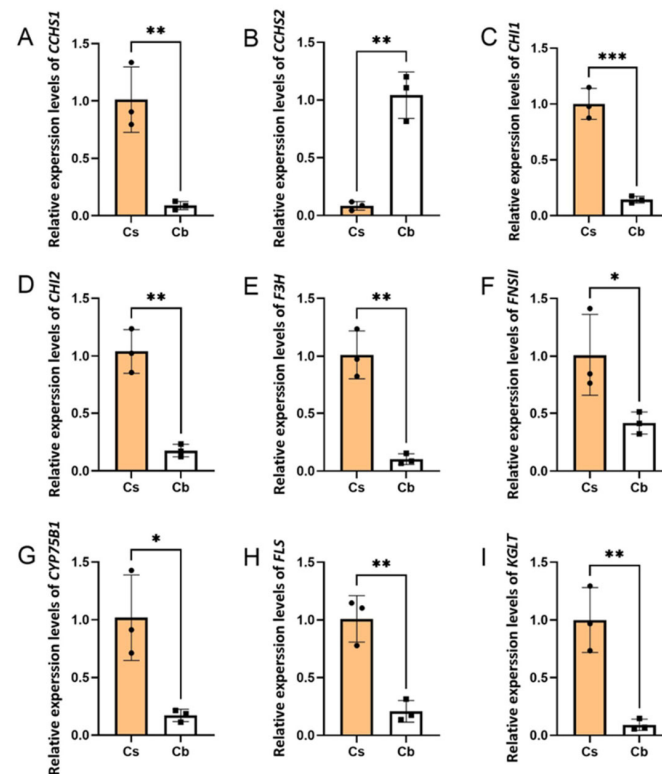


Figure 5. qRT-PCR validation of flavonoid biosynthesis pathway gene expression: (A) CCHS1 (*TRINITY_DN3003_c0_g1*); (B) CCHS2 (*TRINITY_DN1068_c0_g1*); (C) CHI1 (*TRINITY_DN24101_c0_g2*); (D) CHI2 (*TRINITY_DN2321_c0_g1*); (E) F3H (*TRINITY_DN5312_c0_g1*); (F) FNSII (*TRINITY_DN10480_c0_g1*); (G) CYP75B1 (*TRINITY_DN1544_c0_g1*); (H) FLS (*TRINITY_DN10300_c0_g1*); (I) KGLT (*TRINITY_DN5238_c0_g1*). Cs, *C. sulphureus* (orange bars); Cb, *C. bipinnatus* (white bars). The error bars represent the standard deviation ($n = 3$). Asterisks denote statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Chalcone isomerase (CHI), a key enzyme that catalyzes the isomerization of naringenin chalcone to naringenin, was represented by two transcripts (*TRINITY_DN24101_c0_g2* and *TRINITY_DN2321_c0_g1*), both of which were expressed at significantly higher levels in *C. sulphureus* petals than in *C. bipinnatus* petals (Figure 5).

During the conversion of naringenin into multiple downstream branches, F3H (*TRINITY_DN5312_c0_g1*), FLS (*TRINITY_DN10300_c0_g1*), CYP75B1 (*TRINITY_DN1544_c0_g1*), and KGLT (*TRINITY_DN5238_c0_g1*) play important roles, and all four genes were expressed at much higher levels in *C. sulphureus* petals than in *C. bipinnatus* petals (Figure 5). F3H catalyzes the formation of dihydrokaempferol from naringenin. Dihydrokaempferol is then further converted by FLS and CYP75B1 to flavonols such as kaempferol and quercetin. KGLT catalyzes the glycosylation of kaempferol to form astragalin. In addition, FNSII (*TRINITY_DN10480_c0_g1*) was more highly expressed in *C. sulphureus* petals than in *C. bipinnatus* petals; FNSII mediates the formation of apigenin from naringenin, and apigenin is subsequently converted to luteolin by CYP75B1. These expression patterns were further validated by qRT-PCR.

3.5. Phylogenetic Analysis

A phylogenetic tree was constructed using CCHS1 and CCHS2 together with previously reported CHS family members from diverse species. These results indicate that CHS phylogeny is closely associated with species evolution. Using CHS from the filamentous fungus *Stemphylium lycopersici* as the outgroup (Clade 1), Clade 2 contained two CHSs from the green alga *Coccomyxa subellipsoidea*, Clade 3 contained maize-specific CHSs from

monocots, and Clade 4 included numerous CHSs from core angiosperms as well as several maize CHSs (Figure 6).

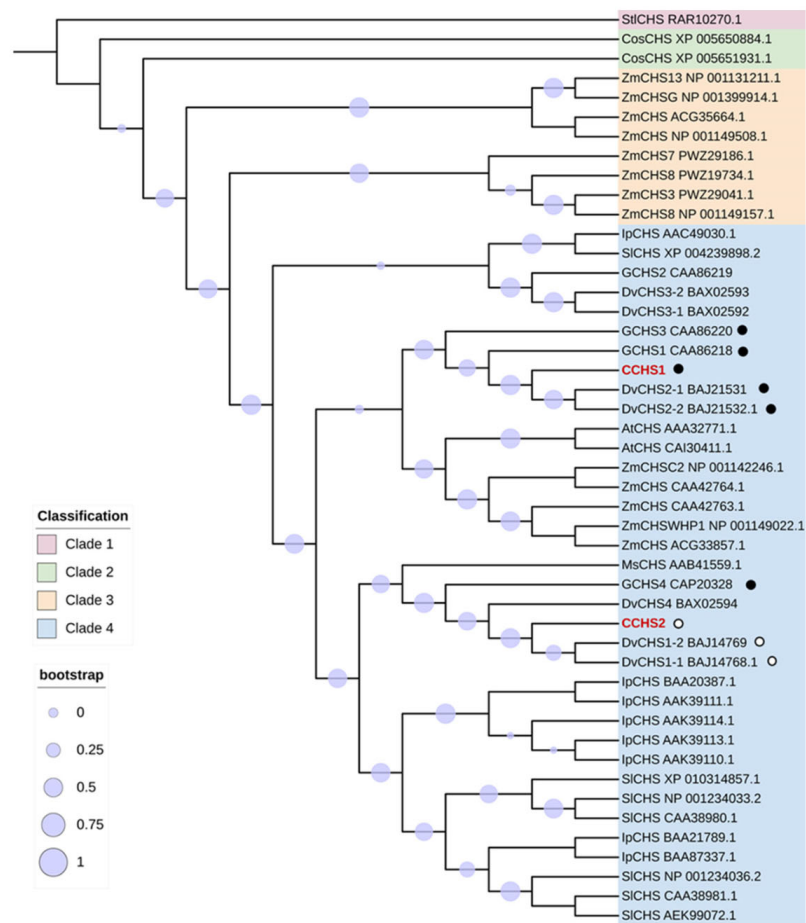


Figure 6. Phylogenetic analysis of CHS proteins. Filled black circles indicate *Asteraceae* CHSs experimentally validated to catalyze naringenin chalcone formation; open circles indicate *Asteraceae* CHSs experimentally validated to negatively regulate naringenin chalcone formation, as well as CCHS2 identified in this study as nonfunctional.

Both CCHS1 and CCHS2 clustered within Clade 4, and they shared 80.2% amino acid identity. This clade also contained functionally validated CHSs, including the alfalfa chalcone synthase MsCHS and the gerbera chalcone synthases GCHS1, GCHS3, and GCHS4 (Figure 6), which have been demonstrated to function as catalytic CHSs involved in flavonoid biosynthesis in alfalfa and gerbera petals [41]. The proteins most closely related to CCHS1 and CCHS2 were derived from the bicolored dahlia (*Dahlia variabilis*), which also belongs to *Asteraceae* (Figure 6). The closest homolog of CCHS1 was DvCHS2 (Figure 6), with 95.5% amino acid identity. Previous work has shown that DvCHS2 is expressed in the red regions of dahlia petals and acts as a functional CHS contributing to flavonoid biosynthesis [42]. In contrast, the closest homolog of CCHS2 was DvCHS1 (Figure 6), sharing 96.4% amino acid identity. DvCHS1 has been reported to be expressed in the white regions of dahlia petals and to suppress the expression of both itself and DvCHS2 via posttranscriptional gene silencing (PTGS) [43].

Taken together with the expression patterns observed in *Cosmos* and in bicolored dahlia, CCHS1 and DvCHS2 appear to retain canonical CHS function, catalyzing the formation of the key flavonoid precursor naringenin chalcone. Nevertheless, whether CCHS2, similar to DvCHS1, acts as a negative regulator of flavonoid biosynthesis remains unknown.

3.6. Prokaryotic Recombinant Expression and Functional Characterization of CCHS2 and CCHS1

The engineered plasmids were subsequently transformed into competent *Escherichia coli* BL21 (DE3) cells. Positive clones were screened by colony PCR and induced with IPTG, and the target proteins were purified using Ni-NTA affinity chromatography. The predicted molecular masses of His-tagged CCHS1 and His-tagged CCHS2 were 44.4 kDa and 46.8 kDa, respectively. SDS-PAGE analysis revealed distinct bands between 40 and 50 kDa in the 100 mM imidazole elution fractions in both purification experiments, which is consistent with the expected sizes and indicates successful purification of the recombinant proteins (Figure 7A).

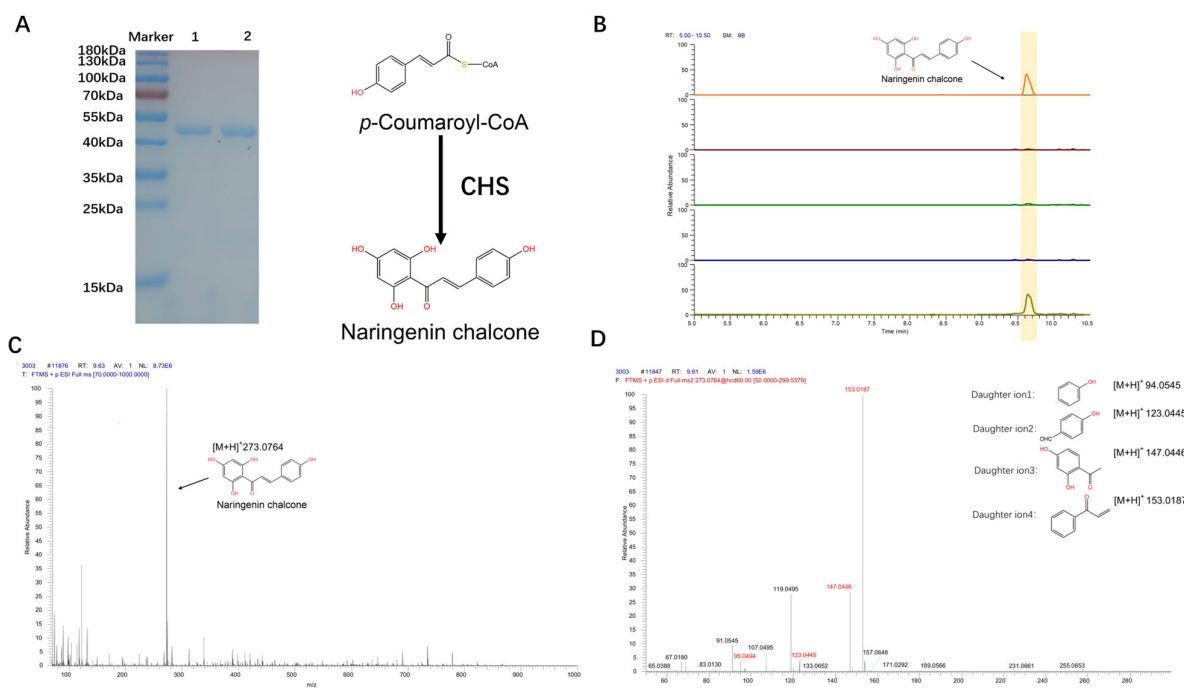


Figure 7. Expression and functional identification of CCHS2 and CCHS1: (A) SDS-PAGE analysis of His-tagged CCHS1 and His-tagged CCHS2. Lane 1, 100 mM imidazole elution for His-tagged CCHS1 purification; Lane 2, 100 mM imidazole elution for His-tagged CCHS2 purification. (B) Enzymatic activity analysis of His-tagged CCHS1 and His-tagged CCHS2. (C) MS1 spectrum of the product catalyzed by His-tagged CCHS1. (D) MS2 spectrum of the product catalyzed by His-tagged CCHS1.

Freshly purified His-tagged CCHS1 and His-tagged CCHS2 were immediately used for *in vitro* activity assays, and the reaction products were identified by LC-MS/MS. When malonyl-CoA and coumaroyl-CoA were used as substrates, His-tagged CCHS1 produced a characteristic compound with an [M+H]⁺ m/z of 273.0764. Its diagnostic fragment ions [M+H]⁺ at m/z 94.0545, 123.0445, 147.0446, and 153.0187 matched those of the naringenin chalcone standard (Figure 7D). This product eluted at 9.61 min, which is consistent with the retention time of the naringenin chalcone standard (Figure 7B). In contrast, neither His-tagged CCHS2 nor heat-inactivated protein produced this product (Figure 7B). These results demonstrate that CCHS1, which is highly expressed in *C. sulphureus*, catalyzes the formation of naringenin chalcone from malonyl-CoA and coumaroyl-CoA. Naringenin chalcone can then be converted by downstream enzymes into multiple yellow flavonoid compounds. In contrast, CCHS2, which is highly expressed in *C. bipinnatus*, lacks detectable catalytic activity for naringenin chalcone formation, thereby limiting flavonoid biosynthesis and resulting in white petals.

4. Discussion

In addition to their diverse biological functions in plants, flavonoids are among the key determinants of pigmentation in flowers and fruits. Here, we used *C. bipinnatus* (white petals) and *C. sulphureus* (orange petals) as study systems to investigate the molecular mechanisms underlying flower color formation. By integrating metabolomics, transcriptomics, and molecular approaches with a focus on flavonoids, we identified factors responsible for the color divergence between these two species.

By pigment extraction followed by untargeted LC–MS/MS metabolomics, we identified a set of flavonoid compounds associated with flower color. These compounds accumulated at high levels in *C. sulphureus* petals. Their conjugated double-bond systems exhibit absorption maxima in the UV–visible region (380–450 nm), resulting in a bright yellow appearance [44]. Previous studies have further shown that the position of the hydroxyl groups in flavonoids from orange flowers can increase molecular conjugation and yellow hues [45] and that flavonoid accumulation in petal epidermal cells is positively correlated with increased yellow intensity [46]. Together, these findings underscore the importance of flavonoid accumulation for yellow pigmentation. In contrast, flavonoid levels are markedly lower in white petals than in orange petals, which is consistent with the white phenotype. Notably, flower color is shaped not only by the abundance and composition of flavonoids but also by the vacuolar microenvironment, metal ions, and copigmentation effects [47,48]. Although these factors have been most thoroughly characterized in anthocyanin-based coloration, variations in cellular pH may also influence the stability and visual expression of flavonoid-derived yellow pigments, thereby modulating color hue and intensity. Moreover, flavonoids may interact with other phenolic compounds, including flavonols, flavones, and hydroxycinnamic acid derivatives, altering their light absorption properties and enhancing or stabilizing petal pigmentation [49]. In orange flowers, carotenoids may further contribute to visible coloration, either independently or in concert with flavonoids [50,51]. However, in this study, carotenoids were barely visible in the oil phase during the extraction experiment, and no clear presence of carotenoids was detected in the metabolomic analysis. Thus, the orange phenotype of *C. sulphureus* is likely not determined by flavonoid accumulation alone but may arise from the combined effects of the vacuolar environment, pigment copigmentation, and carotenoids or other auxiliary pigments. Future studies combining targeted carotenoid profiling, vacuolar pH measurements, and pigment interaction analyses will help clarify the relative contributions of these factors to flower color formation in *Cosmos*.

Comparative transcriptomic analyses of the flavonoid biosynthetic pathway in the petals of *C. sulphureus* and *C. bipinnatus* revealed significant upregulation of genes encoding chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavone synthase II (FNSII), flavonoid hydroxylase (CYP75B1), flavonol synthase (FLS), and a glycosyltransferase (KGLT) in *C. sulphureus*. High CHI expression promotes the conversion of chalcones to flavanones, thereby establishing a stable flavonoid backbone [52]. Elevated F3H activity drives the conversion of flavanones to dihydroflavonols, which supply substrates for downstream flavone/flavonol branches [53]. In contrast, reduced expression or silencing of these genes in *C. bipinnatus* petals likely constrains flux through the pathway and limits the production of pigmented flavonoid derivatives [54]. Moreover, differential expression of FNSII can directly affect flavone biosynthetic efficiency; loss of FNSII expression has been associated with sharply reduced apigenin accumulation [55]. In addition, CYP75B1-mediated B-ring hydroxylation can increase absorption in the visible range and thereby increase coloration [56]. The high expression of FLS in *C. sulphureus* may also promote the accumulation of flavonols (e.g., quercetin), which can stabilize yellow tones via copigmentation with chalcones [57]. Finally, KGLT-mediated glycosylation alters flavonoid solubility

and vacuolar deposition patterns; highly glycosylated flavonoids often display weaker coloration because of increased dispersion [58]. Collectively, these expression differences likely drive pronounced differences in flavonoid composition and abundance, resulting in yellow–orange versus white phenotypes [59].

On the basis of the transcriptomic data and the results of the *in vitro* enzymatic activity assays of CCHS1 and CCHS2, we hypothesize that the white/orange flower color differentiation between *C. bipinnatus* and *C. sulphureus* is closely associated with the allocation of CHS-mediated flavonoid metabolic flux. This phenomenon may be attributable to the role of the “metabolic bottleneck effect” in flower pigmentation. In the present study, the expression level of CHS, the rate-limiting enzyme that initiates flavonoid biosynthesis, directly affected the accumulation threshold of naringenin chalcone, thereby controlling the flux through downstream metabolic branches [22,60]. In *C. sulphureus*, CCHS1 ensures an abundant supply of chalcone precursors by efficiently catalyzing the condensation of 4-coumaroyl-CoA and malonyl-CoA, maintaining the continuous synthesis of flavones and flavonols [61,62]. Conversely, the deficiency of chalcone synthesis in white *C. bipinnatus* [63] forces the metabolic flux toward nonchromogenic compounds [64], a metabolic remodeling mechanism highly similar to that observed in white-flowered mutants of *Brassicaceae* [65].

In addition, we hypothesize that the impact of CHS functional loss on the floral phenotype may have evolutionary adaptive significance. For instance, the UV absorption properties of chalcones facilitate photoprotection under intense light [66]; *C. sulphureus* achieves both pigmentation and stress resistance through high CHS expression, whereas white *C. bipinnatus* might sacrifice pigmentation for other adaptive advantages, such as reduced metabolic costs [67]. Furthermore, the impact of genetic mutations on CHS activity warrants attention: in *Chrysanthemum*, mutations in the CHS gene trigger petal bleaching [68], which is a possible mechanism for the variation in flower color in *Cosmos*. Recent evidence indicated that genotypic variation in environmental response modulates resource allocation and secondary metabolism, suggesting that *C. sulphureus* may employ high CHS expression for pigmentation and photoprotection, whereas *C. bipinnatus* may divert resources to alternative adaptive traits [69]. These hypotheses have not yet been validated in *Cosmos* species and currently remain speculative. Nevertheless, they provide new directions for subsequent experimental investigations.

5. Conclusions

In this study, white-petaled *C. bipinnatus* and orange-petaled *C. sulphureus* were used as research materials. By combining pigment extraction, untargeted LC–MS/MS metabolomics, comparative transcriptomics, and related molecular analyses, we systematically investigated the causes of flower color differentiation in the genus *Cosmos*. The results revealed that the differences in flower color between the two species were closely related to the accumulation levels and compositional differences of flavonoids, as well as changes in the expression of key structural genes in the flavonoid biosynthesis pathway.

Metabolomic analysis revealed that compared with white petals, orange petals of *C. sulphureus* were significantly enriched in multiple flavonoid compounds related to yellow-to-orange coloration. These compounds have strongly conjugated structures and can absorb in the ultraviolet–visible region, thereby promoting the formation of yellow or orange tones in petals. In contrast, the accumulation levels of related flavonoid metabolites in the white petals of *C. bipinnatus* were relatively low, indicating that insufficient synthesis of flavonoid pigments is one of the important reasons for its white color. Follow-up experiments should investigate other factors in addition to flavonoids, such as vacuolar pH and carotenoids, to address the limitations of the current study.

Transcriptome analysis further revealed that genes related to the flavonoid biosynthesis pathway were generally expressed at relatively high levels in *C. sulphureus* petals. In particular, the upregulation of genes such as CHI, F3H, FNSII, CYP75B1, FLS, and KGLT may promote flavonoid skeleton formation, hydroxylation modification, flavone/flavonol branch metabolism, and glycosylation, thereby increasing the synthesis and accumulation of colored flavonoid derivatives. In contrast, the lower expression levels of these genes in *C. bipinnatus* may restrict flavonoid metabolic flux, resulting in insufficient production of colored compounds.

In addition, this study emphasized the central regulatory role of CHS in flower color formation in *Cosmos*. As the initial rate-limiting enzyme in the flavonoid biosynthesis pathway, the expression level and functional status of CHS directly affect the synthesis of chalcone precursors and further determine the metabolic flux distribution of downstream flavonoids. In *C. sulphureus*, the high expression of CCHS1 may ensure a continuous supply of chalcone precursors, thereby promoting the accumulation of flavones and flavonol pigments. In *C. bipinnatus*, weakened CHS-related function may cause a “metabolic bottleneck” in the flavonoid biosynthesis pathway, directing metabolic flux toward colorless or weakly colored branches and ultimately leading to the formation of a white-petal phenotype. According to the results of the phylogenetic tree analysis, the amino acid sequence of CCHS2 in white flowers was highly similar to that of the other two types of CHS genes. Therefore, it is speculated that the expression of CCHS2 may negatively regulate CCHS1, and follow-up experiments will further investigate this possibility.

In summary, the white/orange flower color differentiation between *C. bipinnatus* and *C. sulphureus* is the result of the combined effects of multilayered regulation, among which differences in flavonoid metabolite accumulation and CHS-mediated flavonoid metabolic flux distribution are important factors.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae12070771/s1>, Figure S1. qPCR melting curve; Figure S2. MSMS spectra of nine flavonoids. Table S1. Transcriptome quality assessment; Table S2. qPCR primer information; Table S3. Other detection data for the nine flavonoids. Text S1. Sequences involved in phylogenetic tree construction.

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Data Availability Statement: The transcriptome data and gene sequences have been uploaded to NCBI. *C. sulphureus*’s transcriptome is PRJNA1451823, and *C. bipinnatus*’s transcriptome is PRJNA1451726. *C. sulphureus*’s gene sequence is PZ310414, and *C. bipinnatus*’s gene sequence is PZ310415. The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding authors.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

CHS	Chalcone synthase
CHI	Chalcone isomerase
F3H	Flavanone 3-hydroxylase
F3'H	Flavanone 3'-hydroxylase
DFR	Dihydroflavonol 4-reductase
ANS	Anthocyanin synthase
UFGT	Flavonoid-3-O-glucosyltransferase
PSY	Phytoene synthase
PDS	Phytoene desaturase
ZDS	ζ-carotene desaturase
LCYB	Lycopene β cyclase
LC-MS/MS	Liquid chromatograph mass spectrometer
HPLC	High-performance liquid chromatography
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
CCHS2	<i>Cosmos bipinnatus</i> chalcone synthase
CCHS1	<i>Cosmos sulphureus</i> chalcone synthase
CYP75B1	Cytochrome75B1
FNSII	Flavone synthase II
KGLT	Kaempferol 3-O-β-D-galactosyltransferase
StlCHS	<i>Stemphylium lycopersici</i> Chalcone synthase
CosCHS	<i>Coccomyxa subellipsoidea</i> Chalcone synthase
ZmCHS	<i>Zea mays</i> chalcone synthase
IpCHS	<i>Ipomoea nil</i> chalcone synthase
SlCHS	<i>Solanum lycopersicum</i> chalcone synthase
GCHS	<i>Gerbera jamesonii</i> chalcone synthase
DvCHS	<i>Dahlia variabilis</i> chalcone synthase

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