

# Integrated analysis of transcriptomes and metabolomes reveals the mechanism underlying the flower color transition from green to yellow in *Paphiopedilum* 'GXAAS Prince'

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## Research Article

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**Integrated analysis of transcriptomes and metabolomes reveals the mechanism underlying the flower color transition from green to yellow in *Paphiopedilum* ‘GXAAS Prince’**

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**Abstract**

**Background:** *Paphiopedilum* (Venus slipper) is of considerable ornamental value, yet pure-colored cultivars remain scarce. *Paphiopedilum* ‘GXAAS Prince’, a yellow-green hybrid, exhibits a progressive color transition from green to faint yellow during its three floral development stages, waiting to bloom (S1), blooming (S2), and late flowering (S3). The regulatory mechanisms underlying this transition remain largely unexplored. Elucidating this mechanism is significant for advancing the molecular breeding of pure-colored *Paphiopedilum* cultivars.

**Results:** Pigments content determination, colorimetric values determination, flavonoid metabolomic profiling, and transcriptome sequencing were conducted to investigate the mechanism of color change across three floral developmental stages. Results showed that total chlorophyll content and *a*\* values gradually decreased across the three stages,

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while total flavonoid content and  $b^*$  value first declined and then increased. Carotenoid-targeted metabolome analysis showed that the content of carotenoids decreased across the three stages, and flavonoid-targeted metabolome analysis showed that flavones, flavonols, and chalcones were identified as the possible key metabolites contributing to the yellow coloration of *P. 'GXAAS Prince'* flowers. The down-regulation of genes related to chlorophyll biosynthesis and the up-regulation of genes related to chlorophyll degradation led to the petal color transition from green to yellow. Genes encoding 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), and flavonol synthase (FLS), likely led to the increased accumulation of flavonoid metabolites, driving the faint yellow coloration of *P. 'GXAAS Prince'* flowers. Moreover, the down-regulation of genes related to carotenoid biosynthesis resulted in a decrease in carotenoid content, limiting the deepening of yellow color, serving as an important factor in floral coloration.

**Conclusions:** Our results demonstrate that the green-to-faint-yellow color transition of *Paphiopedilum 'GXAAS Prince'* is driven by chlorophyll degradation coupled with flavonoid accumulation. The down-regulation of chlorophyll biosynthesis genes and the up-regulation of chlorophyll degradation genes mediate the green-to-yellow transition, while the up-regulation of key flavonoid biosynthesis genes (*4CL*, *CHS*, *CHI*, *F3H*, and *FLS*), which promote the accumulation of flavones, flavonols, and chalcones, establishes the faint yellow hue. The down-regulation of carotenoid biosynthesis genes constrains yellow color intensity. These findings provide a theoretical basis for the molecular breeding of pure-colored *Paphiopedilum* cultivars.

**Keywords:** *Paphiopedilum 'GXAAS Prince'*, Flower color transition, Chlorophyll degradation, Flavonoid biosynthesis, Carotenoid, Transcriptome, Metabolome

## Introduction

Flower color is an important ornamental trait in flowering plants, with petal hue and coloration patterns serving as critical ornamental features (Gao et al., 2016). Orchids possess high ornamental value due to the diverse floral color combinations

exhibited by their sepals, petals, and lips, which arise from variations in the types and distribution patterns of pigments (Li et al., 2020). *Paphiopedilum* Pfitzer (Venus slipper), the largest genus in the subfamily Cypripedioideae of the Orchidaceae family, has become a new favorite in the flower industry owing to its unique flower shape, rich colors, and long flowering period (Zhang et al., 2018; Li et al., 2021). However, solid yellow, green, and white cultivars remain highly prized yet scarce in the current market.

*Paphiopedilum* hybrid ‘GXAAS Prince’, derived from a cross between *P. concolor* ((female)) and *P. ‘Cocoa Oliver’* (male) (Fig. 1A), is the first new variety granted protection under the Chinese Plant Variety Rights (Li et al., 2019; Bai, 2025). The flowers of this cultivar are elegant and delicate, featuring a highly waxy texture and a well-proportioned shape, with a distinctive yellow-green hue of high color purity that has gained considerable market recognition. We observed that the flower color of *P. ‘GXAAS Prince’* undergoes a progressive transition across three developmental stages: waiting to bloom (green), blooming (yellow-green), and late flowering (faint yellow), demonstrating significant ornamental potential (Fig. 1B).

The formation of floral color in plants is influenced by many factors, including the type and content of pigments, as well as environmental conditions. The type and content of pigments are considered the most critical, widely distributed in plant tissues and cells, showing diverse colors (Li et al., 2024). Chlorophyll, carotenoids, flavonoids, and betalains are the primary pigment categories that determine flower coloration in *Paphiopedilum* (Deng et al., 2023). The green color of petals originates from chlorophyll within chloroplasts. Unopened flower buds in many plants exhibit green color due to the presence of these chloroplasts (Pyke and Page, 1998). Chlorophyll metabolism involves biosynthesis, the chlorophyll cycle, and degradation. In chlorophyll biosynthesis, glutamyl-tRNA<sup>Glu</sup> will be converted into chlorophyllide *a* through a series of reactions catalyzed by enzymes such as glutamyl-tRNA reductase (GluTR), glutamate 1-aldehyde aminotransferase (GSA), magnesium chelatase (Mg-Ch), magnesium protoporphyrin IX methyltransferase (CHLM), protochlorophyllide oxidoreductase (POR), and divinyl reductase (DVR) (Ilag et al., 1994; Tottey et al.,

2003; Tanaka and Tanaka, 2007). During the chlorophyll cycle, chlorophyllide *a*, chlorophyllide *b*, chlorophyll *a* (Chl *a*), and chlorophyll *b* (Chl *b*) can be interconverted under the catalysis of chlorophyll *a* oxygenase (CAO), chlorophyll synthase (CHLG), chlorophyllase (CLH), chlorophyll *b* reductase (NYC/NOL), and HCAR (Tanaka et al., 1998; Tanaka and Tanaka, 2011). The degradation of chl *b*, on the other hand, begins with its conversion to chl *a*, which undergoes magnesium removal by magnesium dechelatase (STAY GREEN, SGR), leading to subsequent degradation steps (Tanaka and Ito, 2025).

Carotenoids and flavonoids are two major categories of yellow pigments in nature. In many plants, flavonoids have been reported to contribute to the yellow coloration of petals (Zhu et al., 2019). The carotenoid biosynthetic pathway has been characterized in numerous species (Rezende et al., 2025; Zhao et al., 2023). Geranylgeranyl pyrophosphate (GGPP) is sequentially converted to lycopene through the actions of phytoene synthase (PSY), phytoene desaturase, and  $\zeta$ -carotene desaturase. Lycopene is then converted to  $\alpha$ -carotene and  $\beta$ -carotene by lycopene  $\epsilon$ -cyclase (LCYE) and lycopene  $\beta$ -cyclase (LCYB), respectively.  $\alpha$ -Carotene is further converted to lutein by  $\beta$ -carotene hydroxylase (BCH) and  $\epsilon$ -carotene hydroxylase (ECH), whereas  $\beta$ -carotene is converted to zeaxanthin by BCH. Zeaxanthin is converted to violaxanthin and neoxanthin by zeaxanthin epoxidase (ZEP).

Flavonoids, classified into chalcones, anthocyanins, flavonols, flavones, flavanones, flavanols, and isoflavones based on their structures (Zhao et al., 2012), are synthesized via a branched pathway derived from phenylalanine metabolism. Phenylalanine is first converted to dihydroflavonols through the successive reactions by phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavanone 3'-hydroxylase (F3'H), and flavanone 3',5'-hydroxylase (F3'5'H) (Wohl and Petersen, 2020). Dihydroflavonols are then converted into flavonols under the catalysis of flavonol synthase (FLS) (Nakatsuka et al., 2014). Chalcones, flavones, and flavonols synthesized in this process play a critical role in the

development of yellow coloration. Finally, dihydroflavonols are converted into anthocyanins and anthocyanidins through the catalysis of dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose flavonoid 3-O-glucosyltransferase (UFGT) (Koes et al., 2005).

Although *P. 'GXAAS Prince'* exhibits significant potential, the specific genes and metabolites underlying its color transition remain unexplored. Metabolomics, a powerful approach for profiling the complete metabolic landscape of an organism, has been widely used to identify and characterize metabolites across different tissues or species (Takpah et al., 2023). The integration of high-throughput metabolomics and RNA sequencing (RNA-seq) has become a reliable strategy for examining notable metabolic alterations and identifying differentially expressed genes involved in metabolite biosynthesis (Ren et al., 2024; Zhang et al., 2023).

In this study, we aimed to investigate the key differentially expressed genes (DEGs) and differentially expressed metabolites (DEMs) underlying the color transition of *P. 'GXAAS Prince'* by conducting a joint transcriptome and metabolome analysis across three developmental stages: waiting to bloom (S1), blooming (S2), and late flowering (S3). This investigation will provide a foundation for mapping the metabolic pathways of key metabolites and identifying the major functional genes and metabolites governing the flower color gradient in *Paphiopedilum*, thereby offering a scientific basis for the molecular breeding of *Paphiopedilum* cultivars with specific flower colors.

## **Materials and methods**

### **Plant materials and sample preparation**

*P. 'GXAAS Prince'* plants were cultivated at the Orchid Germplasm Nursery (22°50'N, 108°14'E) of the Flower Research Institute, Guangxi Academy of Agricultural Sciences, Nanning, China. Petals were harvested from three developmental stages (S1, S2, and S3; Fig. 1B) in the morning under sunny greenhouse conditions. Three biological replicates were collected per stage, with 20 g of petals per replicate. From each 13 g sample, 5 g was used for pigment content determination, 7.5

g for metabolomic analysis, and the remaining 0.5 g for transcriptomic analysis. In parallel, colorimetric values were measured on petals of intact plants at the same three stages.

All harvested samples were immediately frozen in liquid nitrogen for pigment content determination, transcriptomic analysis, and metabolomic analysis, and then stored at -80 °C. For transcriptomic analysis, the frozen tissues were ground into a fine powder using a Retsch-MM 400 grinder (30 Hz for 1.5 min) and stored at -80 °C for subsequent RNA extraction. For metabolomic analysis, the frozen samples were vacuum freeze-dried using a Scientz-100F lyophilizer and then ground into a powder. Approximately 100 mg of the lyophilized powder was weighed using a digital balance (MS105DM). Subsequently, 1.2 mL of pre-cooled (-20 °C) 70% aqueous methanol containing internal standards was added for extraction. The mixture was vortexed and extracted overnight at 4 °C. Following centrifugation at 12,000 rpm for 10 min, the supernatant was collected and filtered through a 0.22 µm microporous membrane (SCAA-104, ANPEL, Shanghai, China). The filtrate was transferred into an injection vial for subsequent UPLC-MS/MS analysis.

#### **Determination of pigment content and colorimetric values**

Approximately 5 g of collected petals were ground in liquid nitrogen. Afterwards, the total chlorophyll content was determined using a Plant Chlorophyll Content Assay Kit (Solarbio, Beijing, China). The total carotenoid content was determined using a Plant Carotenoid Content Assay Kit (Solarbio, Beijing, China). And the total flavonoid content was determined using a Plant Flavonoid Content Assay Kit (Solarbio, Beijing, China).

Anthocyanin extraction and determination were performed according to Chen et al. (2021b), with minor modifications. Approximately 0.1 g of petal tissue was mixed with 10 mL of 0.1 mol·L<sup>-1</sup> HCl-ethanol solution and incubated in a 60 °C water bath for 30 min. The mixture was transferred to a 25 mL volumetric flask, and the extraction was repeated twice with 5 mL of the extraction solvent (15 min each). The volume was

then adjusted to 25 mL with the extraction solvent. The absorbance of the extract was measured at 530, 620, and 650 nm using a spectrophotometer, with the 0.1 mol·L<sup>-1</sup> HCl-ethanol solution as the blank. Anthocyanin content was calculated using the following formula:

$$OD_{\lambda} = (OD_{530} - OD_{620}) - 0.1(OD_{650} - OD_{620})$$

$$\text{Anthocyanin content (nmol/g)} = \frac{OD_{\lambda}}{\epsilon} \times \frac{V}{m} \times 10^6,$$

where  $OD_{\lambda}$  is the corrected optical density of anthocyanin,  $OD_{530}$ ,  $OD_{620}$ , and  $OD_{650}$  are the optical densities of the extract at 530 nm, 620 nm, and 650 nm, respectively;  $\epsilon$  is the molar extinction coefficient of anthocyanins ( $4.62 \times 10^6 \text{ L} \cdot \text{nmol}^{-1}$ ),  $V$  is the total volume of the extraction mixture (mL), and  $m$  is the mass of the sample (g).

Colorimetric values were measured on the central portions of the middle petals of intact plants using a CM-5 spectrophotometer (Konica Minolta, Japan) under uniform indoor lighting conditions. Measurements were taken from three biological replicates per stage. To explore the changes in floral color, two color parameters from the CIE 1976 color space,  $a^*$  and  $b^*$ , were used for further analysis, where  $a^*$  represents the red-green axis (positive values indicate redness, negative values indicate greenness) and  $b^*$  represents the yellow-blue axis (positive values indicate yellowness, negative values indicate blueness).

#### **Metabolite detection via UPLC-MS/MS analysis**

Metabolite assessments were performed using a UPLC-ESI-MS/MS system, consisting of a Nexera X2 UPLC system (Shimadzu, Japan) coupled to a 4500 QTRAP mass spectrometer (AB Sciex, USA). Chromatographic separation was achieved on an Agilent SB-C18 column (1.8  $\mu\text{m}$ , 2.1 mm  $\times$  100 mm). The mobile phases consisted of solvent A (ultrapure water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). A gradient elution program was applied with initial conditions of 95% A and 5% B. A linear gradient was programmed to reach 5% A and

95% B within 9 minutes, and this composition was maintained for 1 minute. Subsequently, the mobile phase was returned to the initial composition (95% A and 5.0% B) between 10.00 and 11.10 min, and held until 14 min to allow for column re-equilibration. The flow rate and injection volume were fixed at  $0.35 \text{ mL} \cdot \text{min}^{-1}$  and  $4 \text{ } \mu\text{L}$ , respectively. To minimize the impact of instrument variation, samples were analyzed in randomized order.

Data were acquired in both LIT and QQQ scan modes using an ESI Turbo Ion-Spray interface. The system was controlled by Analyst 1.6.3 software (AB Sciex) and operated in both positive and negative ion modes. The ESI source parameters were set as follows: ion source gases GS1 and GS2 were set to 50 and 60 psi, respectively; the curtain gas (CUR) was set to 25.0 psi; the source temperature was maintained at  $550 \text{ }^{\circ}\text{C}$ ; and the ion spray voltage (IS) was set to  $5500 \text{ V}$  in positive mode and  $-4500 \text{ V}$  in negative mode. The collision-activated dissociation (CAD) gas was set to medium. Mass calibration and instrument tuning were performed in QQQ and LIT modes using polypropylene glycol (PPG) solutions at concentrations of  $10$  and  $100 \text{ } \mu\text{mol} \cdot \text{L}^{-1}$ , respectively. For multiple reaction monitoring (MRM) experiments, nitrogen was used as the collision gas. The declustering potential (DP) and collision energy (CE) for individual MRM transitions were optimized via direct infusion or flow injection analysis. A scheduled MRM (s-MRM) approach was employed to monitor specific transitions based on the retention times of the target metabolites.

### **Data processing and metabolite identification**

Raw mass spectrometry data were converted to mzXML format using ProteoWizard. Peak extraction, peak alignment, and retention time correction were performed using the XCMS package. Peaks with a missing rate exceeding 50% in any sample group were filtered out. Missing values were imputed using the k-nearest neighbor (KNN) method, and peak area correction was performed using support vector regression (SVR). Metabolite identification was carried out by searching against a combination of public databases, predicted spectral libraries, and an in-house database constructed in this laboratory, supplemented by the metDNA approach. Metabolites

were retained if they met the following criteria: coefficient of variation (CV) < 0.5 in QC samples and a comprehensive identification score  $\geq 0.5$ . Data from positive and negative ion modes were merged, with duplicate metabolites resolved by retaining the entry with the lowest CV and highest identification confidence level. The final dataset was exported as Excel files.

### **Quantification of metabolites**

In flavonoid metabolomics, the relative abundance of metabolites was represented by their corresponding chromatographic peak areas. For carotenoid metabolomics, absolute quantification was performed using external calibration. Carotenoid standard solutions were prepared at concentrations of 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5, 10, 50, 100, 250, and 400  $\mu\text{g/mL}$ , and the corresponding mass spectral peak intensities were recorded (Table S2). Calibration curves for each compound were constructed by plotting the concentration ratio of the external standard to the internal standard on the x-axis against the peak area ratio on the y-axis. The integrated peak area ratios from all samples were then substituted into the linear regression equations to calculate the concentration of each carotenoid in the actual samples.

### **RNA extraction and transcriptome sequencing**

Total RNA was extracted using the RNAPrep Pure Plant kit (Tiangen, Beijing, China). RNA quality was assessed by agarose gel electrophoresis, and RNA purity was evaluated by measuring the absorbance ratios at OD<sub>260</sub>/OD<sub>280</sub> and OD<sub>260</sub>/OD<sub>230</sub> using a NanoPhotometer spectrophotometer (Thermo Fisher Scientific, USA). RNA integrity was further assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, USA).

Sequencing libraries were constructed from qualified RNA samples and pooled according to the required sequencing depth and library concentration. Paired-end sequencing (2 $\times$ 150 bp) was performed on an Illumina platform. Raw sequencing reads were quality-filtered using fastp v0.19.3. Paired-end reads were discarded if either read

contained more than 10% ambiguous bases (N), contained untrimmed adapter sequences, or had more than 50% of bases with a quality score of  $Q \leq 20$ .

Due to the absence of a reference genome for *Paphiopedilum*, a de novo transcriptome assembly was performed. High-quality clean reads were assembled into transcripts using Trinity. These transcripts were subsequently clustered using Corset, and the longest transcript from each cluster was designated as the representative unigene. The assembled unigenes were annotated by sequence similarity searches against the NR, Swiss-Prot, TrEMBL, KEGG, and COG/KOG databases using BLAST. Gene Ontology (GO) annotations were assigned based on the corresponding functional annotations of the homologous sequences. Predicted protein sequences encoded by the unigenes were further searched against the Pfam database using HMMER to identify conserved protein domains. Gene expression levels were quantified using the fragments per kilobase of transcript per million mapped reads (FPKM) method. For transcriptomic analysis, DEGs were identified using a threshold of  $|\text{Log}_2(\text{Fold Change})| \geq 1$  and  $\text{FDR} < 0.05$ .

#### **Real-time quantitative PCR (qRT-PCR) analysis**

Six candidate genes (three from the porphyrin metabolism pathway and three from the flavonoid biosynthesis pathway), considered key regulators of flower coloration in *P. 'GXAAS Prince'*, were selected for qRT-PCR validation. A unigene (*Cluster-7592.66983*), annotated as encoding the Actin-1 protein, was selected as the reference gene, which exhibited relatively stable and moderate expression levels among all Actin and Actin-like proteins, with  $|\text{Log}_2(\text{Fold Change})| < 0.5$  between any two developmental stages (Fig. S1). Gene-specific primers were designed using the NCBI Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Primer sequences are listed in Supplementary Table S1 and were synthesized by Tsingke Biotechnology Co., Ltd. (Beijing, China).

qRT-PCR was performed using the same RNA samples as the transcriptomic analysis, as described above. cDNA was synthesized by reverse transcription using the

PrimeScript™ FAST RT Reagent Kit with gDNA Eraser (Takara, Beijing, China). A 10 µL qRT-PCR reaction mixture consisted of 1 µL template cDNA, 0.4 µL each of forward and reverse primers, 5 µL of 2 × Q5 SYBR qPCR Master Mix (TOLOBIO, Shanghai, China), and 3.2 µL of ddH<sub>2</sub>O. The reactions were then run on a Roche LightCycler® 480 Instrument (Roche, Switzerland) using a three-step PCR program: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, 56 °C for 30 s, and 72 °C for 30 s. Three technical replicates were performed for each gene-sample combination.

## Statistical analysis

All experiments were performed in triplicate. Data were presented as mean ± standard deviation (SD). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) post hoc test using IBM SPSS Statistics (version 26; IBM Corp., Armonk, NY, USA). Statistical significance was defined as  $P < 0.05$ . Bar and pie charts were constructed using GraphPad Prism 9.5 (GraphPad Software, San Diego, CA, USA) and Origin 2022 (OriginLab Corporation, Northampton, MA, USA). Figures were further assembled and formatted using Adobe Illustrator 2024 (Adobe Systems, San Jose, CA, USA).

## Results

### Pigment content analysis

To identify the primary pigments involved in the floral color formation of *P. 'GXAAS Prince'*, we measured the contents of chl *a*, chl *b*, total chlorophyll, total carotenoids, total flavonoids, and total anthocyanins in petals at three floral developmental stages, S1, S2, and S3 (Fig. 1C). Results showed that the contents of chl *a* (chlorophyll *a*), chl *b* (chlorophyll *b*), and total chlorophyll gradually decreased across the three developmental stages. Specifically, chl *b* and total chlorophyll content at S2 were significantly lower than those at S1. Similarly, the total carotenoid content also progressively decreased during flower development. The carotenoid content at S2 was significantly lower than that at S1. And at S3, it was significantly lower than at both S2 and S1. The total flavonoid content decreased significantly from S1 to S2, followed by

a significant increase at S3. Although total anthocyanin content showed a gradual decreasing trend during flower development, the differences among stages were not statistically significant.

Subsequently, to accurately quantify the changes in the petal color of *P. 'GXAAS Prince'*, we measured the colorimetric values, including  $a^*$  value, and  $b^*$  value of the petals during the three floral developmental stages. The results showed that the  $a^*$  value gradually decreased throughout flower development, whereas the  $b^*$  value declined from S1 to S2 and then increased at S3 (Fig. 1D). This indicates that the intensity of green coloration gradually decreases during flower development, while the intensity of yellow coloration first decreases and then increases.

To clarify the contribution of various pigments to floral coloration, we established linear regression equations between total chlorophyll content and the  $a^*$  value for green, as well as between the yellow-related  $b^*$  value and the contents of total carotenoids and total flavonoids (Table 1). The results showed that the  $a^*$  value was significantly positively correlated with total chlorophyll content. In contrast, the  $b^*$  value showed no significant correlation with total carotenoid content but was significantly positively correlated with total flavonoid content. Additionally, the total carotenoids content in petal tissues was relatively low. Therefore, flavonoids, rather than carotenoids, are more likely to contribute to the faint yellow coloration of the petals in *P. 'GXAAS Prince'* at the S3 stage.

Table 1 The linear equation between pigments and the color indices in *P. 'GXAAS Prince'*.

| Equations                                                                   |
|-----------------------------------------------------------------------------|
| $a^* = 55.91 \text{ Chl} - 15.60 \text{ (R}^2 = 0.901, P < 0.001\text{)}$   |
| $b^* = -37.35 \text{ Car} + 37.54 \text{ (R}^2 = -0.097, P = 0.604\text{)}$ |
| $b^* = 4.14 \text{ Fla} + 19.60 \text{ (R}^2 = 0.75, P = 0.001\text{)}$     |

$a^*$  represents the red-green axis (positive values indicate redness, negative values indicate greenness) and  $b^*$  represents the yellow-blue axis (positive values indicate

yellowness, negative values indicate blueness), the same below. Chl, total chlorophyll content. Car, total carotenoids content. Fla, total flavonoid content. Adjusted  $R^2$ s were applied for these linear equations.

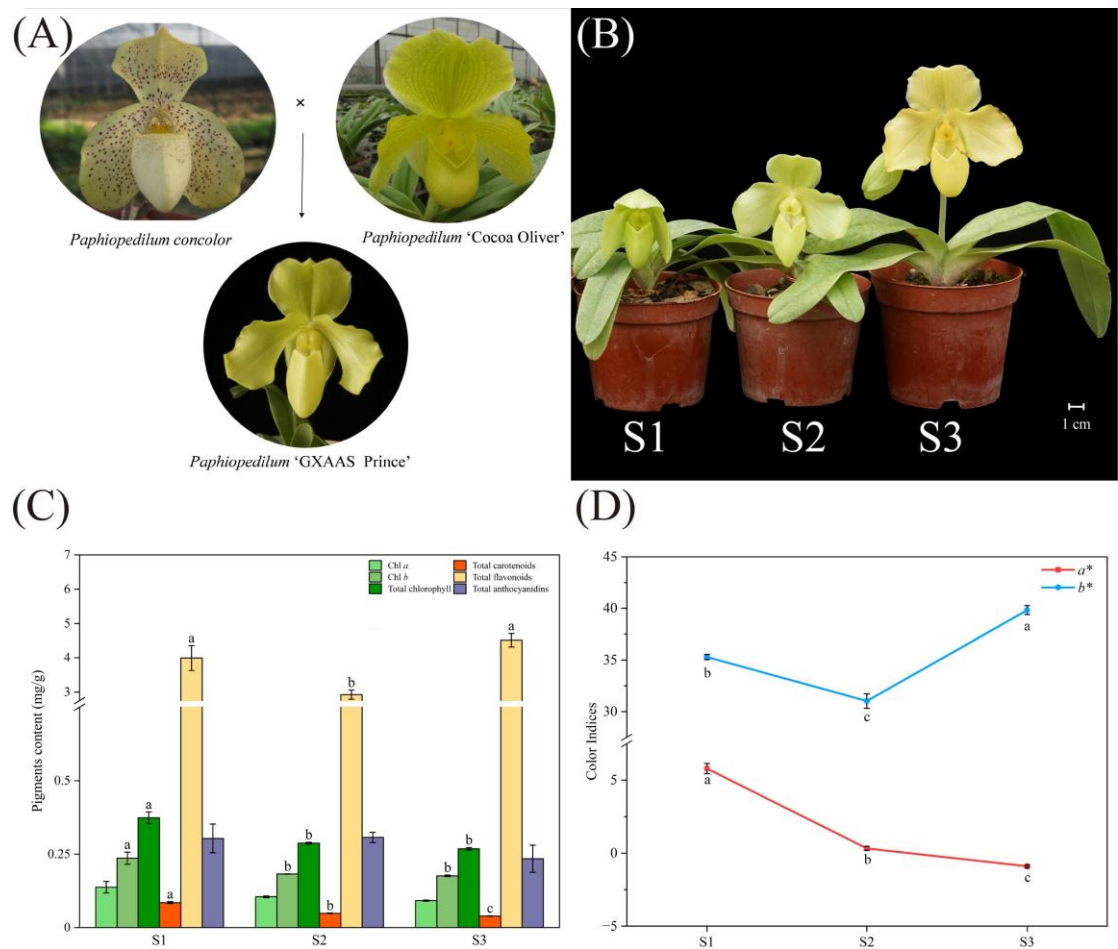


Figure 1. (A) The new cultivar *Paphiopedilum* 'GXAAS Prince' and its parents, *P. concolor* (female) and *P. 'Cocoa Oliver'* (male). (B) *P. 'GXAAS Prince'* at three floral development stages. S1, waiting to bloom. S2, blooming. S3, late flowering. (C) Pigment content of three stages in *P. 'GXAAS Prince'*. Bar charts and error bars represent the means  $\pm$  standard deviation, and different letters indicate significant differences,  $P < 0.05$ . Chl *a*, chlorophyll *a*. Chl *b*, chlorophyll *b*. (D) Changes in floral color indices across three stages in *P. 'GXAAS Prince'*.

### Key metabolite identification

To further investigate the key components underlying the faint yellow coloration in the petals of *P. 'GXAAS Prince'*, we performed targeted metabolomic analyses of

both carotenoids and flavonoids on petal samples collected from the three developmental stages. The results of the carotenoid-targeted metabolomics revealed that a total of 40 carotenoids were detected, which could be classified into carotenes, xanthophylls, and their esterified forms (Fig. 2A).

Differential expression analysis of metabolites was performed to identify key metabolites. DEMs were identified using a variable importance in projection (VIP) score  $> 1$ , a  $|\text{Log}_2(\text{Fold Change})| \geq 1$ , and a  $P\text{-value} < 0.05$ . DEM analysis showed that there were 12 significantly differential metabolites between S1 and S2, with 12 up-regulated and none down-regulated; 18 between S2 and S3, with 14 up-regulated and 4 down-regulated; and 26 between S1 and S3, with 19 up-regulated and 7 down-regulated (Fig. 2B). We noticed that the contents of various compounds, including  $\alpha$ -carotene,  $\beta$ -carotene, lutein, neoxanthin, and zeaxanthin, were lower at S3 than that at S1 and S2, whereas many carotenoid esters showed increased contents at S3 relative to S1 and S2. Given that the decline in carotenes and xanthophylls might be attributable to their esterification, we calculated the sum content of carotenes and xanthophylls along with their esters across the three stages, to roughly estimate the changes in total carotenoid metabolite content during floral development (Fig. 2C, D; Fig. S2). The results showed that no esters of carotenes were detected, and the sum content of carotenoid metabolites and xanthophyll metabolites in petals at S2 and S3 was significantly lower than that at S1. Moreover, the sum content of various xanthophylls, including lutein, neoxanthin, violaxanthin, and zeaxanthin, was also significantly reduced at S2 and S3 compared to S1. These findings are consistent with the pigment content determination, indicating that carotenoid metabolite levels gradually decreased and did not accumulate during the floral development of *P. 'GXAAS Prince'*.

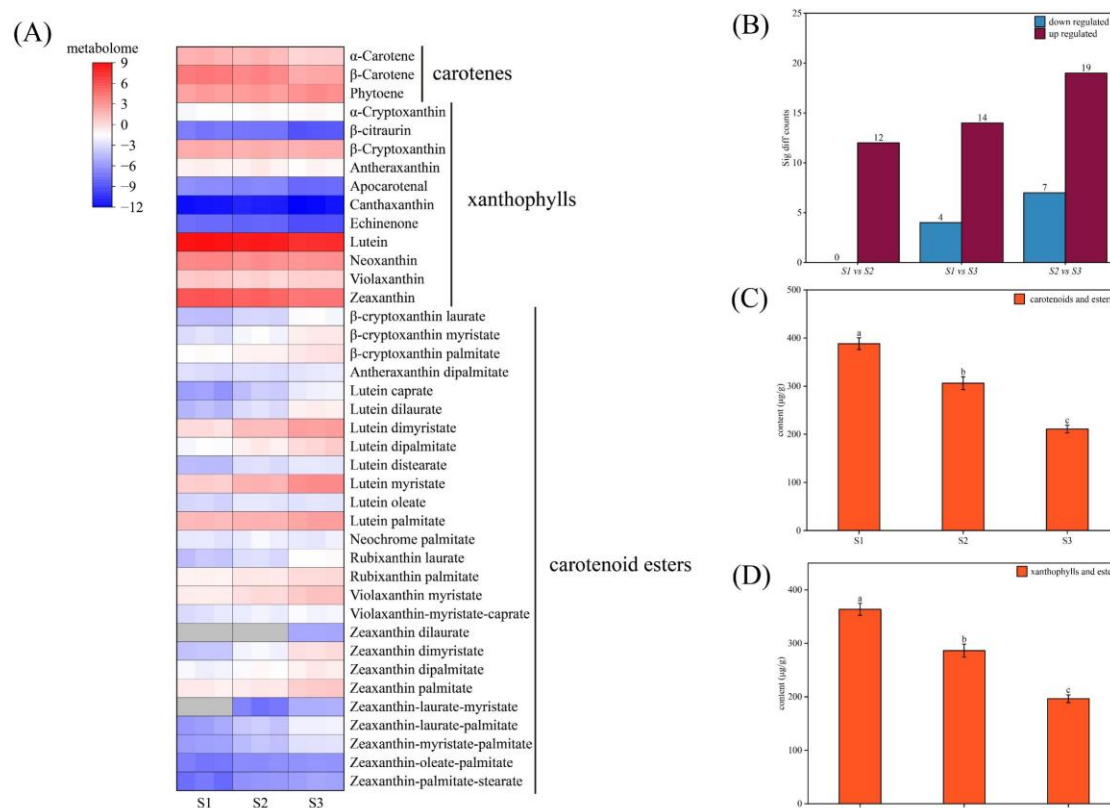


Figure 2. (A) Expression profiles of carotenoid metabolites in *P. 'GXAAS Prince'*. The heatmap was generated using Log<sub>2</sub> (Relative Content). (B) Numbers of differentially expressed carotenoid metabolites in *P. 'GXAAS Prince'*. (C) Sum content of all carotenoid metabolites and their esters. (D) Sum content of all xanthophyll metabolites and their esters.

On the other hand, the flavonoid-targeted metabolomic results showed that a total of 285 flavonoid metabolites were identified across the nine samples from the three floral development stages, which were classified into 15 subcategories, including 20 chalcones, 54 dihydroflavones, 14 dihydroflavonols, 49 flavones, 87 flavonols, 8 flavanols, and 12 anthocyanins (Fig. 3A). Among these, flavonols constituted the largest proportion. DEM analysis showed that there were 120 significantly differentially expressed metabolites between S1 and S2, with 49 up-regulated and 71 down-regulated; 135 between S2 and S3, with 119 up-regulated and 16 down-regulated; and 108 between S1 and S3, with 85 up-regulated and 23 down-regulated (Fig. 3B).

Since both total flavonoid content and the *b*\* value were significantly higher at S3

than at S2, we focused on the differential metabolites significantly up-regulated in the S2 vs. S3 comparison. The criteria of  $\text{Log}_2$  (relative metabolome content)  $> 20$  and  $\text{Log}_2$  (Fold Change S2 vs S3)  $> 2$  were used to further identify the potential key differential metabolites. We first identified 14 metabolites and then excluded two anthocyanins previously reported to be unrelated to yellow coloration. Finally, 12 key flavonoid metabolites were identified, including isoorientin-7-O-(6"-p-coumaroyl) glucoside, kaempferol-3-O-sophorotrioside, kaempferol-3,7-di-O-glucoside, amoenin (kaempferol-3-O-beta-D-mannoside), kaempferol-3-O-sambubioside, luteolin-7-O-gentiobioside, luteolin-7,3'-di-O-glucoside, luteolin-7-O-glucoside, cryptostrobin, 4,4'-dihydroxy-2,6-dimethoxydihydrochalcone, cardamonin, and (E)-cardamonin (Fig. 3C).

Among these, kaempferol-3-O-sophorotrioside, kaempferol-3,7-di-O-glucoside, amoenin, and kaempferol-3-O-sambubioside are kaempferol derivatives; luteolin-7-O-gentiobioside, luteolin-7,3'-di-O-glucoside, and luteolin-7-O-glucoside are luteolin derivatives; and 4,4'-dihydroxy-2,6-dimethoxydihydrochalcone, cardamonin, and (E)-cardamonin are chalcone derivatives. Kaempferol derivatives belong to flavonols, luteolin derivatives to flavones, and the chalcone derivatives to chalcones. We then calculated the correlations between the relative contents of the 12 identified metabolites and the  $b^*$  value across the three floral developmental stages. The results showed that the correlation coefficients ranged from 0.81 to 0.96. Among these, kaempferol-3-O-sophorotrioside exhibited the strongest and significant correlation with the  $b^*$  value. These findings suggest that flavonoids are likely the key substances responsible for the faint-yellow coloration of the petals in *P. 'GXAAS Prince'* at the S3 stage, with flavones, flavonols, and chalcones serving as the main flavonoid categories.

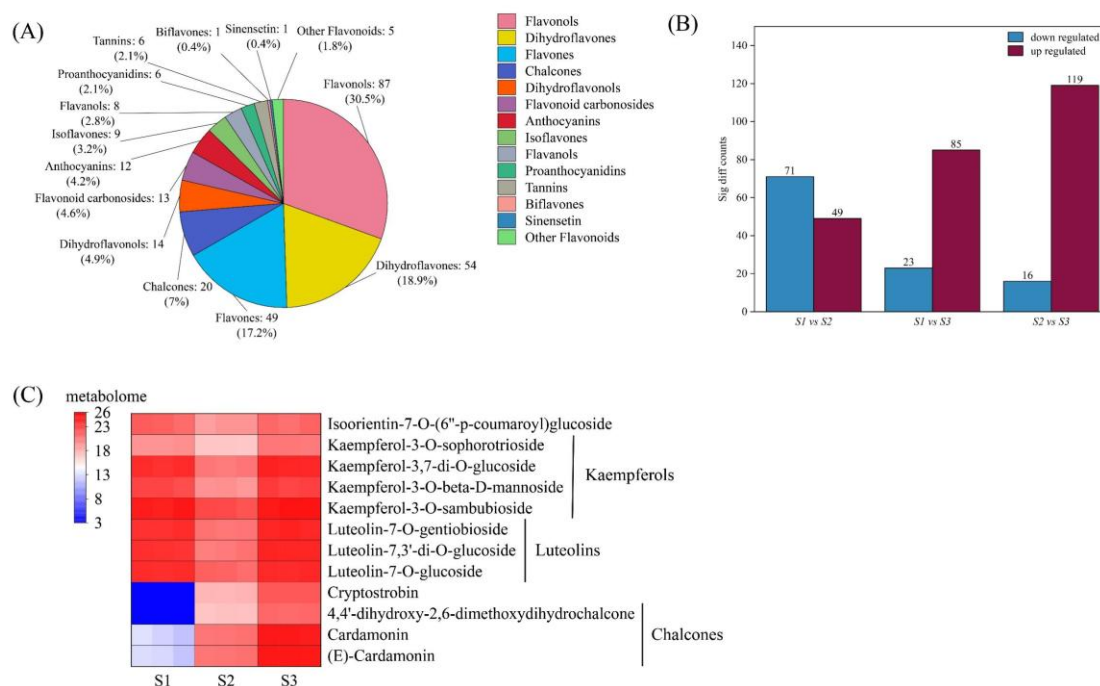


Figure 3. (A) Sub-classification of flavonoid metabolites. (B) Numbers of differentially expressed flavonoid metabolites in *P. 'GXAAS Prince'*. (C) Expression profiles of key flavonoid metabolites. Heatmap was generated using Log<sub>2</sub> (Relative Content).

Table 2. Correlation between 12 flavonoid metabolites and the *b*\* value

| Substances                                        | r        | P      |
|---------------------------------------------------|----------|--------|
| Cryptostrobin (8-C-Methyl-5,7-Dihydroxyflavanone) | 0.825652 | 0.006  |
| Cardamonin                                        | 0.816257 | 0.007  |
| (E)-Cardamonin                                    | 0.816036 | 0.006  |
| 4,4'-dihydroxy-2,6-dimethoxydihydrochalcone       | 0.822754 | 0.007  |
| Amoenin                                           | 0.875907 | 0.002  |
| Luteolin-7-O-glucoside (Cynaroside)               | 0.885317 | 0.002  |
| Kaempferol-3-O-sambubioside                       | 0.901157 | 0.001  |
| Luteolin-7,3'-di-O-glucoside                      | 0.933891 | <0.001 |
| Luteolin-7-O-gentiobioside                        | 0.92746  | <0.001 |
| Kaempferol-3,7-di-O-glucoside*                    | 0.901191 | <0.001 |
| Isoorientin-7-O-(6"-p-coumaroyl) glucoside        | 0.629846 | 0.069  |
| Kaempferol-3-O-sophorotrioside                    | 0.961341 | <0.001 |

## Transcriptome analysis results

To investigate the molecular mechanisms underlying the floral color transition in *P. 'GXAAS Prince'*, we performed comparative transcriptome analysis on petal

samples collected from three developmental stages. Transcriptome sequencing of nine samples yielded a total of 75.85 Gb of clean data, with approximately 8.43 Gb of clean data generated for each sample. Raw reads were generated using the Illumina HiSeq platform. After removing low-quality reads, adapters sequences, and poly-N tails, high-quality clean reads were obtained, with Q30 base proportions ranging from 92.24% to 93.86% and GC content between 46.95% and 48.64%.

The assembled sequences generated 276,014 transcripts and 182,412 unigenes. These unigenes were functionally annotated against seven databases, including NR, GO, KEGG, SwissProt, KOG, Pfam, and TrEMBL. A total of 76,324 unigenes (41.84%) were successfully annotated to at least one database (Fig. 4A). Among these, 75,419 unigenes (41.35%) were annotated to the NR database, 41,437 unigenes (22.72%) to the GO database, 50,650 unigenes (27.77%) to the KEGG database, and 34,672 unigenes (19.01%) to the SwissProt database.

The DEG analysis identified 3095 DEGs (1285 up-regulated and 1810 down-regulated) in the S1 vs. S2 comparison group, 4070 DEGs (2462 up-regulated and 1608 down-regulated) in S1 vs. S3, and 3584 DEGs (1681 up-regulated and 1903 down-regulated) in S2 vs. S3 (Fig. 4B). The horizontal axis represents the average gene expression levels. The data distribution demonstrates that variations in expression intensity do not skew the trend of expression differences (Fig. 4C–E).

Since the green coloration was significantly reduced and the yellow coloration was significantly increased in the petals at S3 compared to S1, we performed KEGG enrichment analyses on the up-regulated and down-regulated DEGs in the S1 vs. S3 comparison group (Fig. 5A, B). The top 20 enriched pathways are displayed, ranked by enrichment factor and sorted in descending order by the number of enriched DEGs. We observed that down-regulated DEGs were significantly enriched in the “porphyrin metabolism” pathway and “carotenoid biosynthesis” pathway, and up-regulated DEGs were significantly enriched in the “flavonoid biosynthesis” pathway. These results provided initial insights into the petal color transition in *P. ‘GXAAS Prince’*.

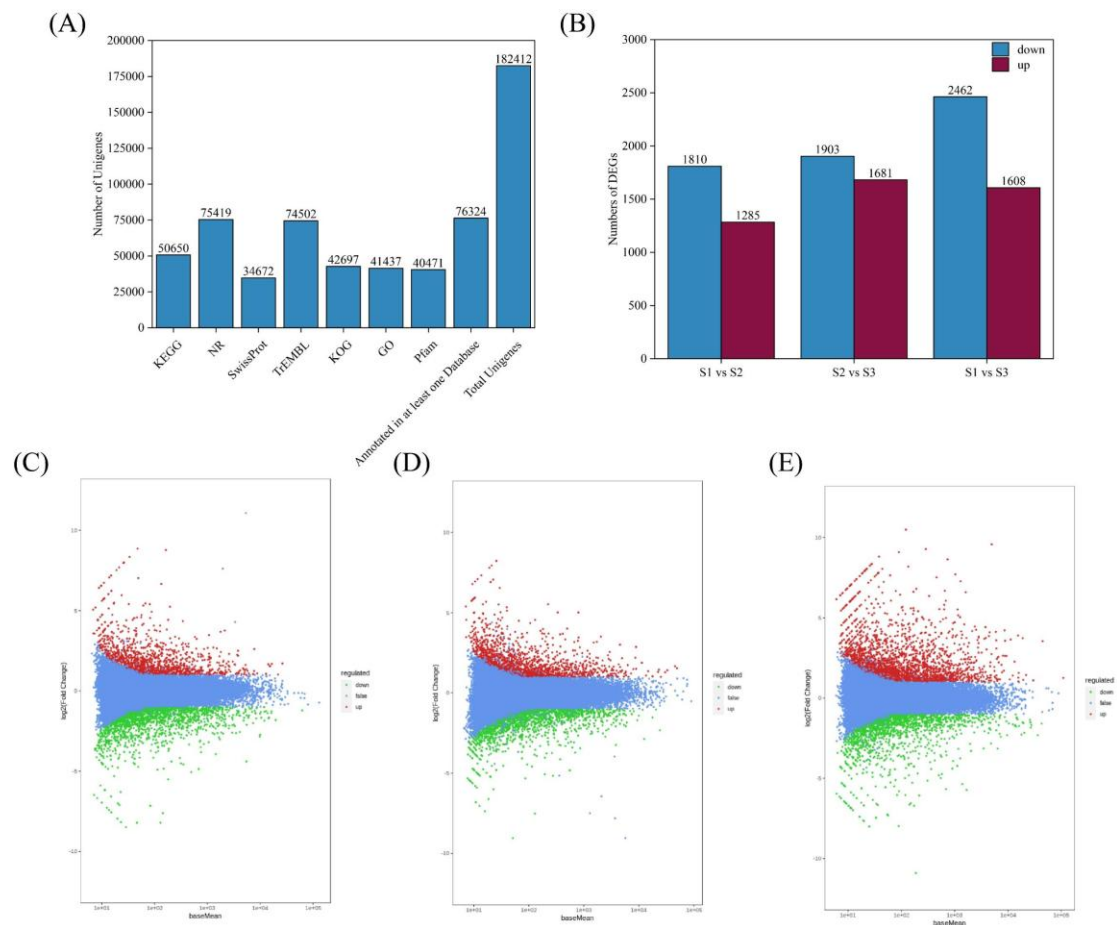


Figure 4. (A) Numbers of unigenes annotated to different databases. (B) Numbers of DEGs identified in *P. 'GXAAS Prince'*. (C) The MA plot of DEGs of S1 vs. S2 comparison group. (D) The MA plot of DEGs of S2 vs. S3 comparison group. (E) The MA plot of DEGs of S1 vs. S3 comparison group.

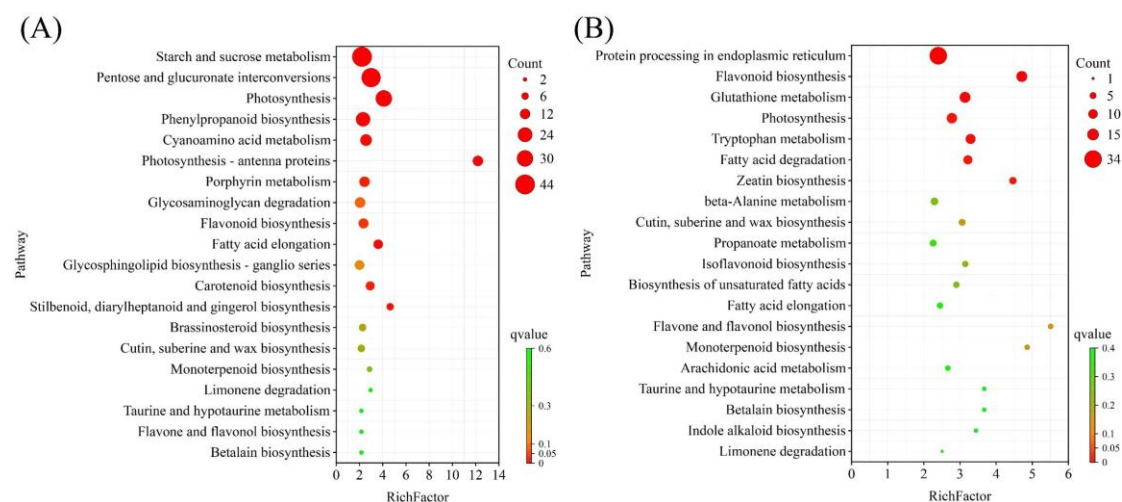


Figure 5. KEGG enrichment analyses of the DEGs in S1 vs. S3 comparison group.

(A) Down-regulated DEGs. (B) Up-regulated DEGs.

#### The expression of genes in the porphyrin metabolism pathway

Based on the KEGG enrichment analysis results, we mapped the possible pathway of chlorophyll biosynthesis and degradation based on the “porphyrin metabolism (map00860)” pathway on the KEGG database (<https://www.genome.jp/kegg>) (Fig. 6A). We also identified unigenes annotated in this KEGG pathway from the transcriptome data and displayed their expression levels using a heatmap, following previous studies (Li et al., 2025; Tanaka and Ito, 2025) (Fig. 6B). One *HEMA* gene (*Cluster-7592.60757*), one *GSA* gene (*Cluster-7592.70222*), three *ChlH* genes (*Cluster-7592.59737*, *Cluster-7592.61673*, *Cluster-7592.67286*), one *ChlI* gene (*Cluster-7592.71618*), three *ChlD* genes (*Cluster-7592.116921*, *Cluster-7592.59206*, *Cluster-7592.67308*), one *CHLM* gene (*Cluster-7592.76045*), one *POR* gene (*Cluster-7592.55916*), two *DVR* genes (*Cluster-7592.104371*, *Cluster-7592.89698*), one *CAO* gene (*Cluster-7592.59697*), one *CHLG* gene (*Cluster-7592.67601*), two *CLH* genes (*Cluster-7592.115026*, *Cluster-7592.68783*), two *NOL* genes (*Cluster-7592.21581*, *Cluster-7592.73778*), three *NYCI* genes (*Cluster-7592.63788*, *Cluster-7592.67761*, *Cluster-7592.74448*), two *HCAR* genes (*Cluster-7592.34210*, *Cluster-7592.64231*), and one *SGR* (*Cluster-7592.66701*) gene were identified. Except for the *HEMA* (*Cluster-7592.60757*), *CAO* (*Cluster-7592.59697*), and one *ChlH* gene (*Cluster-7592.59737*), all other unigenes related to chlorophyll biosynthesis showed down-regulation at S2 compared to S1. Moreover, all unigenes related to chlorophyll biosynthesis were down-regulated at S3 compared to S1. On the other hand, with the exception of two *NOLs*, unigenes related to chlorophyll recycling and chlorophyll degradation were up-regulated at S2 compared to S1. The expression levels of unigenes related to chlorophyll recycling, such as *NYCI* (*Cluster-7592.63788*), may represent preparation for chlorophyll degradation. These findings were consistent with the gradual decrease in both  $a^*$  value and chlorophyll content during the three floral developmental stages.

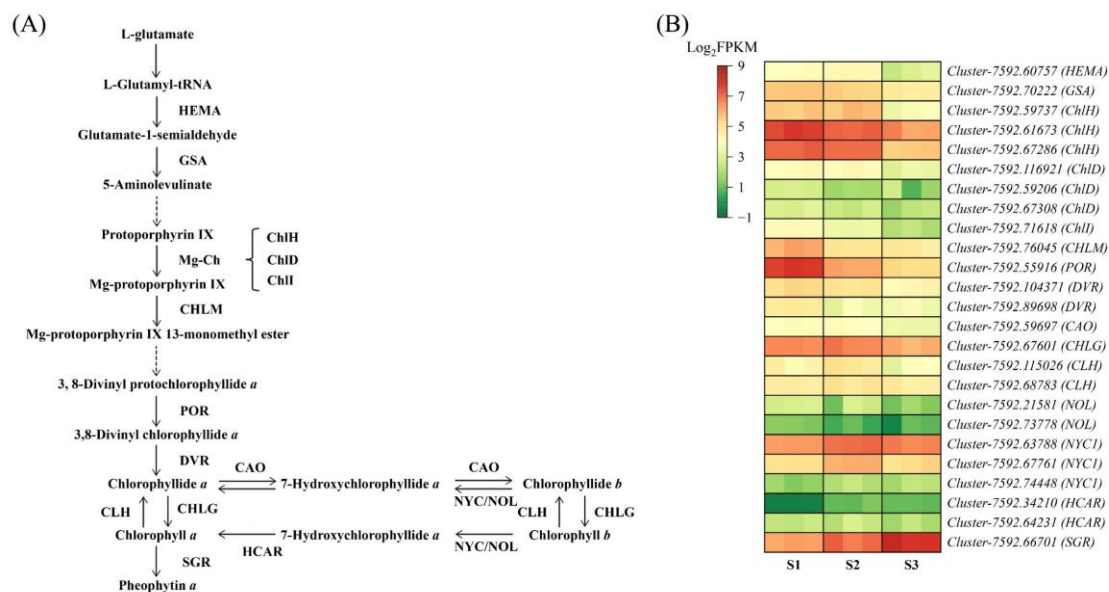


Figure 6. The expression profiles of genes in the porphyrin metabolism pathway in *P. 'GXAAS Prince'*. (A) The porphyrin metabolism pathway. (B) Expression profiles of structural genes in the porphyrin metabolism pathway. Heatmaps were generated using Log<sub>2</sub> (FPKM values).

### The expression of genes in the flavonoid biosynthesis pathway

To investigate the mechanism underlying the faint-yellow coloration at the S3 stage, we mapped the possible flavonoid biosynthesis pathway in *P. 'GXAAS Prince'* based on previous studies and the “Phenylpropanoid biosynthesis (map00940)”, “Flavonoid biosynthesis (map00941)”, and “Anthocyanin biosynthesis (map00942)” pathways in the KEGG database (Zhao et al., 2023). We then identified unigenes annotated to these KEGG pathways from the transcriptome data and integrated the relative contents of related metabolites from the metabolomic data. The results are visualized as heatmaps overlaid on the KEGG pathway map (Fig. 7).

Although the overall expression levels of genes in the flavonoid biosynthesis pathway did not show a uniform trend, several key genes were significantly up-regulated at S3 compared to S2, which is consistent with the higher total flavonoid content observed at the S3 stage. Specifically, the expression levels of several *4CL* genes (*Cluster-7592.122705*, *Cluster-7592.56421*, *Cluster-7592.64206*, *Cluster-*

7592.68019, Cluster-7592.68717, Cluster-7592.96170), two *CHI* genes (Cluster-7592.102149, Cluster-7592.62559), one *F3H* gene (Cluster-7592.56559), and one *FLS* gene (Cluster-7592.67410) were up-regulated at the S3 stage. Conversely, although the expression levels of a *C4H* gene (Cluster-7592.66457) and a *CHS* gene (Cluster-7592.64920) slightly decreased at S3 compared to S2, they remained relatively high across all three floral development stages, suggesting that they are also important to the flavonoid biosynthesis process. Consistent with these transcriptomic changes, the contents of naringenin chalcone, naringenin, and kaempferol derivatives were founded to increase at the S3 stage.

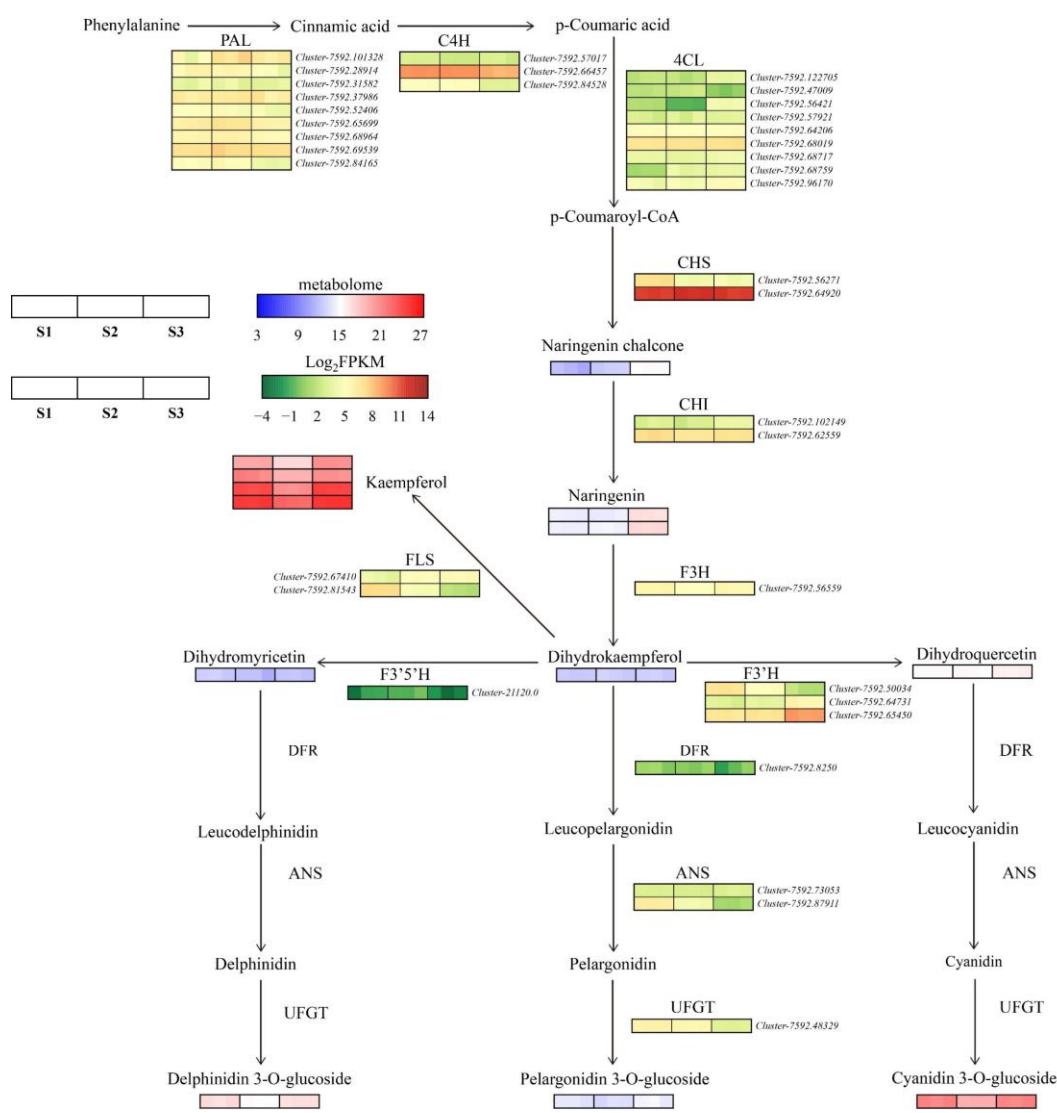


Figure 7. Expression profiles of unigenes and metabolites in the flavonoid biosynthesis pathway. Heatmaps were generated using Log<sub>2</sub> (FPKM values) for

511 unigenes and Log<sub>2</sub> (Relative Content) for metabolites.

## 512 **The expression of genes in the carotenoid biosynthesis pathway**

513 All structural genes annotated to the “carotenoid biosynthesis (map00906)”  
514 pathway, along with their expression levels, and the absolute contents of related  
515 carotenoid metabolites, were also mapped to the possible carotenoid biosynthesis  
516 pathway as heatmaps (Fig. 8). At the S3 stage of floral development in *P. ‘GXAAS*  
517 *Prince’*, the expression levels of most genes involved in carotenoid biosynthesis were  
518 down-regulated, and the contents of related metabolites did not show significant  
519 accumulation but rather decreased. Notably, two *BCH* genes (*Cluster-7592.62988* and  
520 *Cluster-7592.64047*) exhibited high expression levels throughout all floral  
521 development stages in *P. ‘GXAAS Prince’*. In contrast, the expression of *PSY* (*Cluster-*  
522 *7592.74958*), which acts at the entry point of the carotenoid biosynthetic pathway, was  
523 down-regulated at S3, consistent with the lack of carotenoid metabolite accumulation.

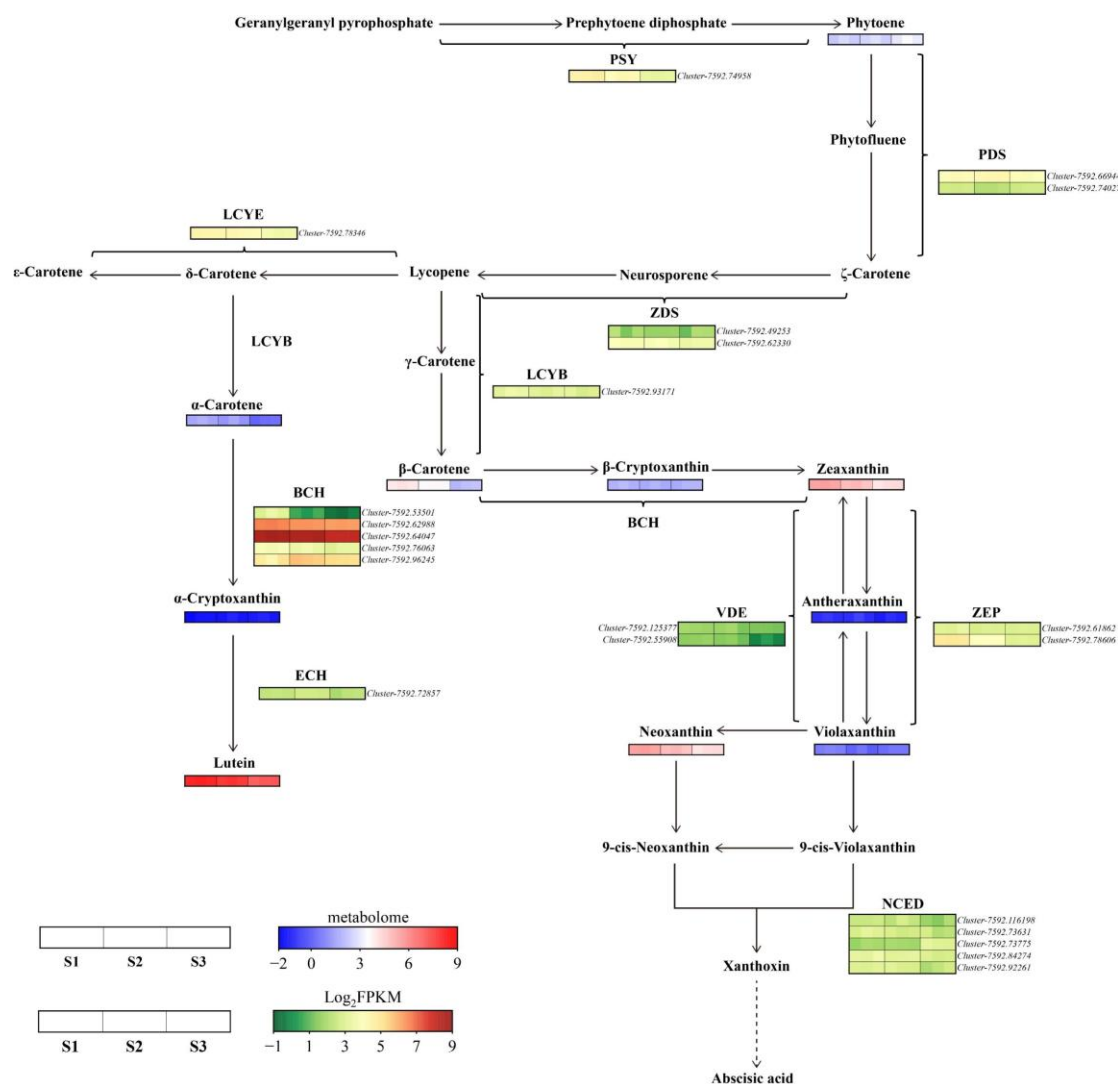
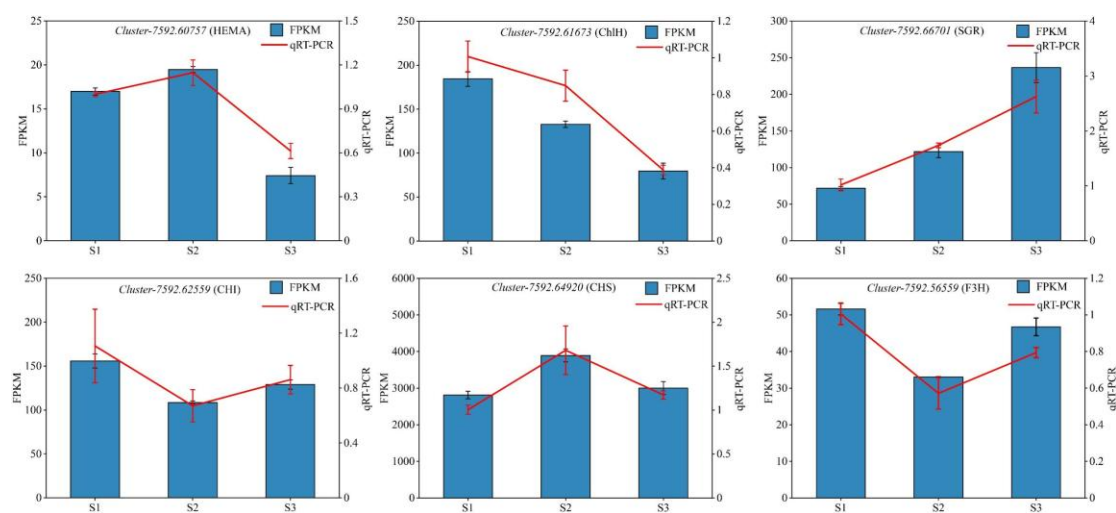


Figure 8. Expression profiles of unigenes and metabolites in the carotenoid biosynthesis pathway. Heatmaps were generated using Log<sub>2</sub> (FPKM values) for unigenes and Log<sub>2</sub> (Absolute Content) for metabolites.

## Validation of RNA-seq data using qRT-PCR

To validate the reliability of RNA-Seq data, six unigenes, including *Cluster-7592.60757* (*HEMA*), *Cluster-7592.61673* (*ChlH*), *Cluster-7592.66701* (*SGR*), *Cluster-7592.64920* (*CHS*), *Cluster-7592.62559* (*CHI*), *Cluster-7592.56559* (*F3H*), considered as key genes in the pathway “porphyrin metabolism” and “flavonoid biosynthesis”, were selected for qRT-PCR analysis (Fig. 9). Although the qRT-PCR results were not exactly identical to the FPKM values obtained from the RNA-Seq data, the expression trends were highly consistent, thereby confirming the reliability of the

536 transcriptomic analysis..



538 Figure 9. Validation of RNA-seq data by qRT-PCR analyses for 6 selected unigenes  
539 during floral development in *P. 'GXAAS Prince'*. Bar charts represent the mean of  
540 FPKM values, while line charts represent the mean of relative expression levels in  
541 qRT-PCR results. Error bars represent the standard deviation.

## 542 Integrated analysis of transcriptome and metabolome

543 To investigate the correlations between flavonoid metabolites and genes involved  
544 in flavonoid biosynthesis, Pearson correlation analysis was performed between the  
545 relative contents of flavonoid metabolites and the expression levels of all structural  
546 genes related to flavonoid biosynthesis across the three floral development stages (as  
547 presented in Section 3.5) (Fig. S4). Considering that the 12 flavonoid metabolites  
548 identified in Section 3.2 exhibited the most pronounced changes during floral color  
549 transition and were likely closely associated with faint-yellow coloration, their  
550 correlations with the structural genes were particularly presented (Fig. 10A). The results  
551 showed that genes such as *4CL*, *CHS*, *CHI*, *FLS*, and *F3H* were significantly positively  
552 correlated with multiple flavonoid metabolites ( $r > 0.8$ ,  $P < 0.05$ ). A network  
553 constructed based on these significant correlations further revealed that several *4CL*  
554 genes were primarily associated with chalcone metabolites, whereas *F3H* was mainly  
555 correlated with flavone and flavonol metabolites, consistent with their catalytic roles in

the flavonoid biosynthetic pathway (Fig. 10B). These findings suggest that 4CL may play an important role in establishing the flavonoid metabolic flux in the petals of *P. 'GXAAS Prince'*, while F3H may be involved in promoting the accumulation of downstream flavones and flavonols.

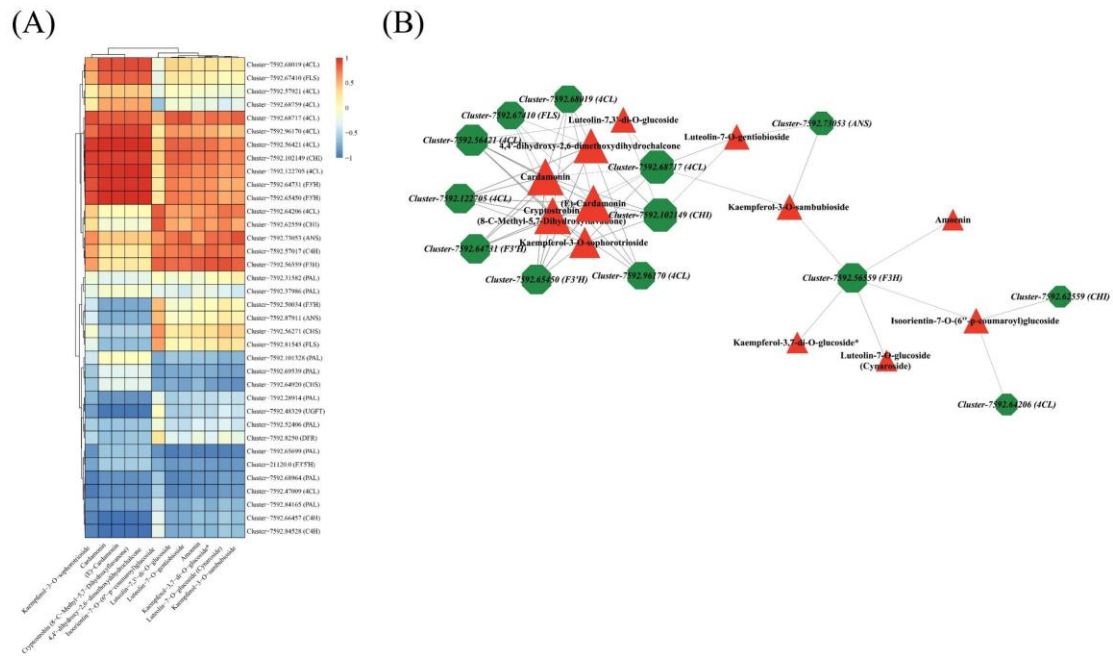


Figure 10. (A) Heatmap of correlation between 12 flavonoid metabolites and structural genes in the flavonoid biosynthesis pathway. (B) Network of genes and metabolites. Green octagons represent genes, and red triangles represent metabolites.

Similarly, Pearson correlation analysis between the expression levels of structural genes involved in carotenoid biosynthesis across the three floral development stages and the contents of major carotenoid metabolites was presented (Fig. 11A, B). The correlations between carotenoids and structural genes in Section 3.6 were presented. The results showed that multiple carotenoid metabolites exhibited significant positive correlations with several genes, including *BCH*, *PSY*, *ZDS*, *ZEP*, and violaxanthin de-epoxidase (*VDE*) ( $r > 0.8$ ,  $P < 0.05$ ). Considering the positions of genes and metabolites in the biosynthetic pathway, the decrease in carotene content may be attributed to the downregulation of genes such as *PSY* and *ZDS*. Similarly, the reduction in xanthophyll content may be due to the downregulation of genes such as *BCH*, *ZEP*, and *VDE*. These correlations between metabolites and genes showed consistency with their respective

positions within the pathway.

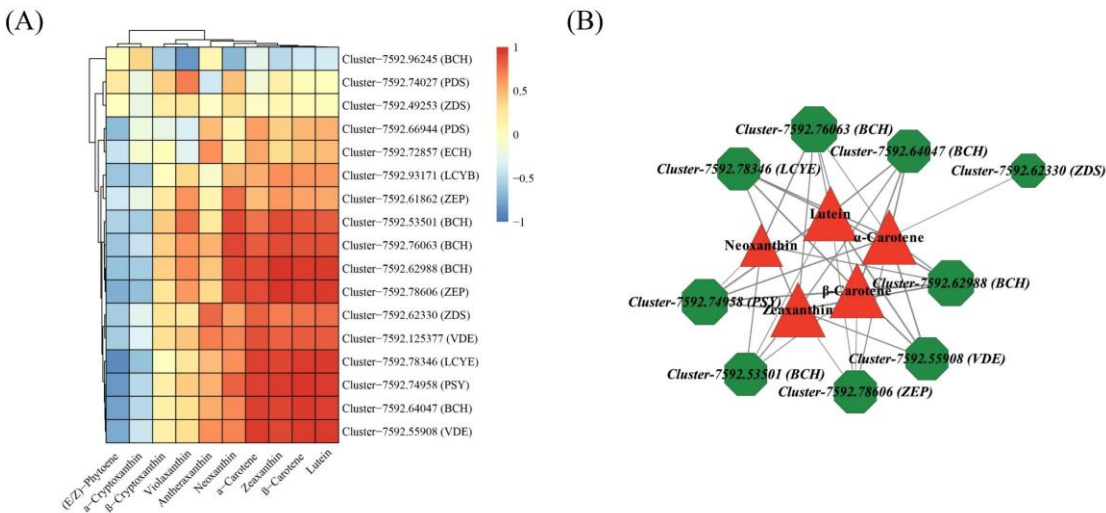


Figure 11. (A) Heatmap of correlation between major carotenoid metabolites and structural genes in the carotenoid biosynthesis pathway. (B) Network of genes and metabolites. Green octagons represent genes, and red triangles represent metabolites.

Overall, the biosynthesis of flavonoid and carotenoid metabolites is more likely attributable to the coordinated expression of multiple structural genes rather than to the regulation of a single key gene. Moreover, the highly consistent variation patterns between gene expression and metabolite accumulation further support the reliability of the integrated transcriptomic and metabolomic analyses.

## Discussion

Floral color is a trait of significant value in ornamental plants. Infertility frequently arises when breeding complex white *Paphiopedilums*, and to date it remains extremely difficult to produce albino flowers (without dots), let alone eliminate the green or yellow tint (Liu, 2000). The maternal parent of *P. 'GXAAS Prince'* is *P. concolor* (subgenus *Brachypetalum* sect. *Concoloria*), and the paternal parent is the large-flower complex type *P. 'Cocoa Oliver'*. The six species within sect. *Concoloria* exhibit uniform coloration across all floral organs and include albino individuals, making them highly valuable for flower color breeding (Liu, 2000; Culbertson, 2014). The emergence of *P. 'GXAAS Prince'*, a cultivar with a pure yellow-green hue, provides a

valuable reference for future breeding of solid-colored *Paphiopedilum*. Compared with regular *Paphiopedilum*, we observed that during flower development, the petal color of *P. 'GXAAS Prince'* shifts from green to faint yellow, both of which are relatively pure and distinct hues. Therefore, these two colors may serve as key focuses for our future breeding efforts. In this study, we proposed a possible mechanism underlying the floral color transition in *P. 'GXAAS Prince'* by integrating transcriptomic and metabolomic analyses.

### **The degradation of chlorophyll led to the decline in green color**

Flowers of many plant species appear green at the unopened bud stage, which is generally considered to result from the presence of chlorophyll (Pyke and Page, 1998). As flowers develop, petals of most species gradually change to other colors. In this study, the greenness of the petals of *P. 'GXAAS Prince'* was characterized by the  $a^*$  value. As the flowers developed, the  $a^*$  value gradually decreased, while the contents of total chlorophyll, chl  $a$ , and chl  $b$  also exhibited a consistent decline. Correlation analysis revealed that the reduction in green coloration was directly associated with the decrease in chlorophyll content. Zheng et al. (2022) suggested that suppression of chlorophyll biosynthesis genes is a primary factor leading to decreased chlorophyll content in petals. A study showed that in carnation cultivars with faint green petals, the expression levels of chlorophyll biosynthesis genes such as *HEMA*, *CHLH*, *CHLI*, and *CHLM* are higher than those in red-petaled cultivars, whereas chlorophyll degradation genes like *NYC*, *NOL*, and *HCAR* are more highly expressed in red-petaled varieties (Ohmiya et al., 2014). Similarly, Liu et al. (2025) observed that during the color transition from green to white in a peony cultivar, the expression of *CHLH* and *CHLG* was higher in the green stage than in the white stage, while *SGR* expression was elevated in the white stage. RNA-seq revealed that genes related to chlorophyll biosynthesis, such as *HEMA*, *GSA*, *CHLD*, *CHLH*, *CHLI*, *CHLM*, *POR*, *DVR*, and *CHLG*, were down-regulated at S3, while genes related to chlorophyll degradation, including *NYC*, *NOL*, *CLH*, and *SGR*, were up-regulated at the same period. These findings are consistent with the previous studies and show a consistent trend with the

decrease in the  $a^*$  value and chlorophyll content. Therefore, we suggest that the down-regulation of genes related to chlorophyll biosynthesis and the up-regulation of genes related to chlorophyll degradation collectively contribute to the decrease in chlorophyll content, thereby resulting in the reduction of green coloration in *P. 'GXAAS Prince'* petals.

### **The accumulation of flavonoids drove the faint-yellow coloration of *P. 'GXAAS Prince'***

Studies have also suggested that flavonoids such as kaempferol and quercetin may play a significant role in the coloration of yellow flowers (Luo et al., 2021). In this study, the total flavonoid content in the petals of *P. 'GXAAS Prince'* showed a significant positive correlation with the  $b^*$  value during flower development. Our targeted flavonoid metabolomic analysis revealed that the petals of *P. 'GXAAS Prince'* contained a variety of flavonoid metabolites, among which flavones, flavonols, and chalcones accumulated at the S3 stage. Previous studies on *Paeonia lactiflora* and *Camellia nitidissima* have demonstrated that flavones, flavonols, and chalcones accumulate during yellow coloration of the petals (Feng et al., 2024a; Wu et al., 2016). Notably, kaempferol and luteolin derivatives consistently showed accumulation trends in these species, and such metabolites were also identified as key differential metabolites in our study, indicating that flavonol and flavone metabolites play a conserved role in yellow flower coloration across different species, and suggesting that they may play a critical role in the faint-yellow coloration of *P. 'GXAAS Prince'*.

The color variation is regulated by the expression of genes encoding key enzymes of flavonoid biosynthesis, such as CHS, CHI, DFR, and FLS, which synthesize different types of flavonoid compounds (Saigo et al., 2020). In this study, the integrated metabolomic and transcriptomic analyses supported the coordinated accumulation of these metabolites. Correlation analysis revealed that multiple structural genes exhibited significant positive correlations with chalcone derivatives, suggesting that genes related to flavonoid biosynthesis collectively enhanced the metabolic flux through the entire flavonoid biosynthetic pathway by coordinated regulation during flower development,

thereby promoting the accumulation of metabolites such as chalcones, flavones, and flavonols in the petals of *P. 'GXAAS Prince'*.

4CL serves as the starting point of the flavonoid biosynthesis pathway by synthesizing p-Coumaroyl-CoA. Increased levels of kaempferol derivatives were observed compared to the wild type upon overexpression of the *4CL* gene (Li et al., 2022). In the flavonoid biosynthetic pathway, CHS catalyzes the synthesis of naringenin chalcone. Feng et al. (2024b)'s research revealed the consistency between *CHS* expression levels and flavonoid content. In a yellow-flowered cultivar of pansy (*Viola × wittrockiana* Gams), high expression levels of a *CHS* gene were observed (Wang et al., 2025). In this study, we observed that one *CHS* gene (*Cluster-7592.64920*) exhibited high expression levels throughout all three stages of floral development, which may sustain the catalytic capacity required for flavonoid biosynthesis during floral development. In the petals of *P. 'GXAAS Prince'*, multiple *4CL* genes were up-regulated at the S3 stage and showed strong positive correlations with the relative contents of several chalcone metabolites. Combined with the consistently high expression of *CHS* throughout the floral development period, these findings suggest that enhanced supply of upstream substrates promotes the accumulation of chalcone metabolites. Therefore, the increased accumulation of downstream chalcone compounds and other flavonoids at the S3 stage is more likely due to enhanced metabolic flux driven by the coordinated regulation of *4CL* and *CHS* genes, rather than to changes in *CHS* expression levels alone.

In the flavonoid biosynthetic pathway, CHI catalyzes the reaction of naringenin chalcone to naringenin, while F3H is responsible for the conversion of naringenin to dihydroflavonols, which are also essential for the biosynthesis of flavones and flavonols (Shi et al., 2015). Overexpression of *CHI* led to an increase in total flavonoid content (Yu et al., 2023). Similarly, overexpression of *F3H* also led to increased levels of flavonoid metabolites, including kaempferol derivatives (Han et al., 2017). In a *Nelumbo nucifera* cultivar with yellow flowers, the expression level of *F3H* was observed to be gradually up-regulated, while the expression level of *CHI* was high at

the early stages of flower development (Wang et al., 2016). In the present study, the expression level of one *CHI* gene (*Cluster-7592.62559*) in *P. 'GXAAS Prince'* remained consistently high across all three floral development stages, which was consistent with the increased accumulation of its downstream product, naringenin. Furthermore, the expression level of *F3H* was up-regulated at S3 and exhibited strong correlations with multiple flavone and flavonol metabolites, suggesting that its catalytic synthesis of dihydroflavonols is a critical step for the subsequent accumulation of flavones and flavonols. These results indicate that CHI and F3H sequentially catalyze the conversion of chalcones to dihydroflavonols, thereby providing substrates for the subsequent biosynthesis of flavones and flavonols.

FLS is the key rate-limiting enzyme in the flavonoid synthesis pathway, directly associated with the biosynthesis of flavonols (Liu et al., 2021). Overexpression of *FLS* has been demonstrated to increase the accumulation of flavonols (Yu et al., 2021). Luo et al. (2021)'s research observed the up-regulation of *FLS* gene in the yellow coloration of *Paeonia suffruticosa*. In our study, up-regulation in the expression of one *FLS* gene (*Cluster-7592.67410*) was also observed at S3, and its expression level exhibited strong correlations with the relative contents of various kaempferol derivatives, suggesting that it may contribute to the high accumulation of kaempferol derivatives in *P. 'GXAAS Prince'*. It is reported that FLS and DFR exhibit a competitive relationship in utilizing dihydroflavonol as a substrate, mediating flavonoid biosynthesis into different branch pathways (Chen et al., 2021). Similarly, lower expression levels of *DFR*, *ANS*, and *UFGT* were observed to inhibit anthocyanin production, resulting in yellow pigment formation in *Paeonia lactiflora* (Zhao et al., 2014). In this study, the expression of *DFR*, *ANS*, and *UFGT* also showed a down-regulated trend. Since FLS and DFR share dihydroflavonol substrates, the enhanced expression of *FLS* may promote more metabolic flux into the flavonol biosynthetic branch, while the down-regulation of *DFR*, *ANS*, and *UFGT* further reduces the flux through the anthocyanin biosynthesis pathway. The relative contents of some anthocyanins increased at the S3 stage, but were lower than those of flavones and flavonols, and the total anthocyanin content did not change

significantly, suggesting that the contribution of anthocyanin to the final petal coloration may be relatively limited. These results further indicate that *FLS* may be crucial for flavonol synthesis in *P. 'GXAAS Prince'*, thereby contributing to the faint-yellow coloration of the petals.

Overall, the coordinated regulation of structural genes such as *4CL*, *CHS*, *CHI*, *F3H*, and *FLS* promotes the metabolic flux through the flavonoid biosynthesis pathway, leading to the accumulation of chalcone, flavone, and flavonol derivatives while simultaneously suppressing anthocyanin biosynthesis. It is also noteworthy that in this study, chalcones, flavones, and flavonols all showed significant accumulation, rather than a single class of flavonoids, suggesting that the faint-yellow coloration of the flowers is not driven by a single class of flavonoid metabolite, but is more likely to result from an overall enhancement of metabolic flux through the entire flavonoid biosynthetic pathway.

#### **The carotenoid accumulation deficiency limited the yellow deepening of flowers in *P. 'GXAAS Prince'***

Carotenoids and flavonoids are two major categories of yellow pigments in nature. Studies have shown that carotenoids such as violaxanthin and zeaxanthin, along with small amounts of neoxanthin, lutein, and  $\beta$ -carotene, contribute to the yellow coloration of flowers (Suzuki et al., 2007). In the present study, however, we observed that the total carotenoid content remained low and continuously declined with flower development of *P. 'GXAAS Prince'*, and showed a negative correlation with the  $b^*$  value. Carotenoid-targeted metabolomics further revealed that the contents of various carotenes and xanthophylls progressively decreased, while the contents of their esterified forms increased. It has been reported that carotenoid esters exhibit absorption peaks and colors identical to those of their corresponding free carotenoids. Thus, the increase in carotenoid esters is unlikely to lead to the faint-yellow coloration of the petals in *P. 'GXAAS Prince'*. Moreover, we found that the sum content of carotenoid metabolites together with their esters also continued to decrease across the three floral development stages. Therefore, we suggest that carotenoids are unlikely to be the

primary driver of the faint-yellow coloration of the petals in *P. 'GXAAS Prince'* during the S3 stage.

As discussed above, we have addressed the contribution of flavonoids to the faint yellow coloration of *Paphiopedilum* petals and excluded the involvement of carotenoids in the coloration in *P. 'GXAAS Prince'*. However, a study also observed a color transition from green to yellow in *P. armeniacum*, where carotenoids such as zeaxanthin and violaxanthin were considered key pigments responsible for the yellow coloration of the petals (Bai et al., 2024). Similarly, a study on orchids demonstrated that genes involved in carotenoid biosynthesis contribute to yellow petal coloration by promoting carotenoid accumulation (Li et al., 2020). These studies indicate that the accumulation of carotenoids generally results in yellow petal coloration. Therefore, the role of carotenoids in the flower coloration of *P. 'GXAAS Prince'* requires further discussion. From another perspective, although carotenoids are not the primary factor driving the faint-yellow coloration of the petals, their decreased content may limit the further deepening of the yellow color, thereby causing the petals to remain faint-yellow.

In the carotenoid biosynthetic pathway, genes such as *PSY*, *PDS*, and *ZDS* are responsible for the synthesis of carotenes, while *BCH*, *VDE*, and *ZEP* are responsible for the reactions of carotenes to xanthophylls. In this study, we observed that the expression levels of *PSY*, *PDS*, and *ZDS* were relatively low and even down-regulated at the S3 stage. Among these, *PSY* is considered a key rate-limiting enzyme in the carotenoid biosynthetic pathway (Zhou et al., 2022). The down-regulated expression of the *PSY* gene may limit the accumulation of carotenes from the source, which is consistent with the metabolomic results, showing no sustained accumulation of carotenoids. Meanwhile, *BCH* maintained high expression levels, and given the continuous decline of  $\alpha$ -carotene and  $\beta$ -carotene, we suggest that it may promote the reactions of carotenes to xanthophylls such as lutein and zeaxanthin, resulting in the content of xanthophylls remaining higher than that of carotenes. Since carotenoid levels continued to decline at S2 and S3, along with downregulation of *ZEP* and *VDE*, xanthophyll levels also decreased accordingly. The changing trend of metabolites in the

entire pathway exhibited consistency. Therefore, we suggest that carotenoids are not the primary pigments driving yellow coloration in the petals of *P. 'GXAAS Prince'*, but they may serve as an important factor in the faint-yellow coloration. In other words, in this study, the formation of faint-yellow coloration is mainly dependent on flavonoid accumulation, whereas carotenoid deficiency limited the deepening of yellow color, thereby determine the overall flower color.

## **Conclusion**

This study revealed a possible mechanism underlying the color change from green to faint-yellow during the floral development of *P. 'GXAAS Prince'* by conducting metabolomics and transcriptomics analyses. During the floral development of *P. 'GXAAS Prince'*, the down-regulation of genes associated with chlorophyll synthesis and the up-regulation of genes related to chlorophyll degradation led to the decline in total chlorophyll content and in the  $a^*$  value, indicating their key roles in the transition of flower color from green to yellow. Flavonoid metabolites, including flavones, flavonols, and chalcones, may be the primary metabolites contributing to the yellow coloration of *P. 'GXAAS Prince'* petals. The high expression of *CHI* and *CHS*, together with the up-regulation of *4CL*, *F3H*, and *FLS*, enhanced flavonoid accumulation, thereby driving the faint-yellow coloration in *P. 'GXAAS Prince'*. The down-regulation of genes related to carotenoid biosynthesis resulted in the decrease of carotenoid content, thereby limiting the deepening of yellow color and serving as an important factor in floral coloration.

## **Abbreviation**

4CL: 4-coumarate: CoA ligase

ALA: 5-aminoacyl-L-alanine

$a^*$ : red to green ratio

ANOVA: analysis of variance

ANS: anthocyanidin synthase

796  $b^*$ : yellow to blue ratio

797 BCH:  $\beta$ -carotene hydroxylase

798 BLAST: basic local alignment search tool

799 C4H: cinnamic acid 4-hydroxylase

800 CAD: collision-activated dissociation

801 CAO: chlorophyll *a* oxygenase

802 cDNA: complementary deoxyribonucleic acid

803 CE: collision energy

804 CHI chalcone isomerase

805 Chl *a*: chlorophyll *a*

806 Chl *b*: chlorophyll *b*

807 ChlD: magnesium-chelatase subunit D

808 CHLG: chlorophyll synthase

809 ChlH: magnesium-chelatase subunit H

810 ChlI: magnesium-chelatase subunit I

811 CHLM: magnesium protoporphyrin IX methyltransferase

812 CHS: chalcone synthase

813 CIE: International Commission on Illumination

814 CLH: chlorophyllase

815 CUR: curtain gas

816 CV: coefficient of variation

817 ddH<sub>2</sub>O: double distilled water

818 DEGs: differential expressed genes

819 DEMs: differential expressed metabolites

820 DFR: dihydroflavonol 4-reductase

821 DNA: deoxyribonucleic Acid

822 DP: declustering potential

823 DV Pchl *a*: 3,8-divinyl protochlorophyllide *a*

824 DVR: divinyl reductase

825 ECH:  $\epsilon$ -carotene hydroxylase

826 ESI: electrospray ionization

827 F3'H: flavanone 3'-hydroxylase

828 F3'5'H: flavanone 3',5'-hydroxylase

829 F3H: flavanone 3-hydroxylase

830 FDR: false discovery rate

831 gDNA: genomic deoxyribonucleic acid

832 GluTR: glutamyl-tRNA reductase

833 GO: Gene Ontology

834 GGPP: geranylgeranyl pyrophosphate

835 GSA: glutamate 1-aldehyde aminotransferase

836 HCAR: 7-hydroxymethyl chlorophyll *a* reductase

837 HCl-ethanol: hydrogen chloride ethanol

838 HMMER: Hidden Markov Model for Ergodic Regions

839 IS: ion spray voltage

840 KEGG: Kyoto Encyclopedia of Genes and Genomes

841 LCYB: lycopene  $\beta$ -cyclase

842 LCYE: lycopene  $\epsilon$ -cyclase

843 Mg-Ch: magnesium chelatase

844 MgPPIX: Mg-protoporphyrin IX

845 MRM: multiple reaction monitoring

846 MS: mass spectrometry

847 NOL: NYC1-like

848 NR: Non-Redundant Protein Sequence Database

849 NYC: Non-Yellow Coloring 1

850 OD: optical density

851 PAL: phenylalanine ammonia-lyase

852 PCA: principal components analysis

853 POR: protochlorophyllide oxidoreductase

854 PPG: polypropylene glycol

855 PSY: phytoene synthase

856 qRT-PCR: real-time quantitative polymerase chain reaction

857 RNA: ribonucleic acid

858 SD: standard deviation

859 SGR: Stay-Green protein

860 SVR: support vector regression

861 UDP: uridine diphosphate

862 UFGT: UDP-glucose flavonoid 3-O-glucosyltransferase

863 UPLC: ultra performance liquid chromatography

864 VIP: variable importance in projection

865 VDE: violaxanthin de-epoxidase

866 ZEP: zeaxanthin epoxidase

## 867 **Authors' contributions**

868 Xiuling Li, experiment design, experiment, data analysis, article writing and funding  
869 assistance; Yuankai Yi, experiment, data analysis, and article writing; Donghui Peng  
870 and Zhongjian Liu, supervision and revision of the experiment design and manuscript;  
871 Minqin Ye, manuscript revision; Jizheng Fan and Changyan Huang, experiment and  
872 resources management; Xiaoshuang Tang, Guangqing Dang, and Xinyu Yang, data  
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## 880 **Supplementary files**

881       Supplementary file 1: Figure S1–4 and Table S1.

882       Supplementary file 2: Table S2.

883       Supplementary file 3: Relative content of flavonoid metabolites of *P.* ‘GXAAS  
884 Prince’.

885       Supplementary file 4: Absolute content of carotenoid metabolites of *P.* ‘GXAAS  
886 Prince’.

887       Supplementary file 5: Expression matrix with annotation of all unigenes of *P.*

888 ‘GXAAS Prince’.

## 889 **Data availability**

890 The raw RNA-seq data have been submitted to NCBI SRA under the project number:  
891 PRJNA1303329.

## 892 **Declarations**

### 893 **Ethics approval and consent to participate**

894 Not applicable

### 895 **Consent for publication**

896 Not applicable

### 897 **Competing interests**

898 The authors declare no conflict of interest.

899

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