Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Transcriptome and flavonoid metabolome analysis reveals the molecular basis of floral color divergence between the hybrid and their parental species in Paphiopedilum

Quanshu Luo
Guizhou Institute of Biology
Junming Chen
Guilin University of Aerospace Technology
Cheng Wan
Guizhou Institute of Biology
Leijia Li
Guizhou Institute of Biology
Sheng Chen
Guizhou Institute of Biology
Jihong Wang
Guizhou Institute of Biology
Li Zhou
Minzu Normal University of Xingyi
Keyun Deng
Qianxinan Lvyuan Animal AND Plant Science and Technology Development Co. LTD
Ying Wang
luoquanshu@icloud.com

Guizhou Institute of Biology

Research Article

Keywords: Paphiopedilum, floral color, Transcriptome, flavonoid metabolome

Posted Date: July 17th, 2025

DOI: https://doi.org/10.21203/rs.3.rs-6878590/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Abstract

Background The artificial hybridization of *Paphiopedilum* began in 1869. With the breakthrough in aseptic sowing techniques, the number of hybrids surged. Over 30,000 hybrids have been registered by the Royal Horticultural Society (RHS). Hybrids derived from *Paphiopedilum delenatii* typically retain its prominent characteristics: large, symmetrical petals and a magenta-purple labellum. In this study, the hybrid of *P. delenatii* and *P. primulinum* exhibited an overall creamy-white coloration. This study focuses on the divergence in floral coloration between the hybrid and their parental lines. Through comparative transcriptome analysis and flavonoid metabolic profiling across three distinct *Paphiopedilum*, we aim to elucidate the molecular mechanisms underlying how hybrids deviate from the red and yellow-green phenotypes of their parents to develop a cream-colored pigmentation.

Results The labellum and dorsal sepal morphology closely resembled *P. primulinum*, while the petal traits combined the wavy margins of *P. primulinum* and smooth surface of *P. delenatii*. Flavonoid metabolome analysis of the floral tissues, a total of 29 flavanones, 93 flavones and flavonols, 13 anthocyanins, 23 isoflavonoids, six flavonoids, and four chalcones and dihydrochalcones were detected. Remarkably, anthocyanin content is significantly higher in *P. delenatii* compared to *P. primulinum* and the hybrid. Transcriptome analysis highlighted significant up-regulation of genes in the flavonoid biosynthesis pathway and phenylpropanoid biosynthesis pathway when comparing hybrids to *P. primulinum*. Conversely, early-stage biosynthetic genes were down-regulated in hybrid relative to *P. delenatii*, likely contributing to metabolite content differences.

Conclusion This study employed multi-omics analysis to compare the floral tissues of *P. delenatii* and *P. primulinum*, along with their hybrid progeny. It identified differences in flavonoid metabolite content among them. By analyzing differentially expressed genes (DEGs) within the KEGG flavonoid biosynthesis pathway, the molecular mechanism underlying the divergent floral pigmentation pattern of the hybrid compared to both parental species was explored. Analysis of transcriptomic data, through comparison with the non-redundant (NR) protein database, revealed a closer phylogenetic relationship between *Paphiopedilum* and the genus *Dendrobium*, whereas morphologically *Paphiopedilum* is classified under *Cypripedium*-related taxa. These findings provide novel insights and molecular evidence for understanding *Paphiopedilum* floral coloration and phylogenetic relationships.

Introduction

Orchidaceae are the most diverse family of angiosperms, comprising 28000 species spread across 736 genera [1,2]. The *Paphiopedilum* genus (commonly known as " Lady's Slipper Orchid ") comprises 96-100 species globally and is primarily distributed in Southeast Asia, the Himalayan lowlands, and Southwest China [3]. Renowned for its slipper-shaped floral morphology and vibrant color variations, this genus holds significant horticultural value and is a prized ornamental plant in global markets [4]. China is the country with the richest diversity of *Paphiopedilum* species in the world. There are 27 native species indigenous to China, accounting for one-third of the globally recorded wild *Paphiopedilum* species, and

most are distributed in Yunnan or Guangxi Province [3]. All *Paphiopedilum* species are included in Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). The natural distribution range of *Paphiopedilum delenatii* Guill. (*Orchidaceae*) spans northern Guangxi Province and southeastern Yunnan Province in China, as well as southern Vietnam [5]. Its floral morphology exhibits a diameter of 6-8 cm, with the dorsal sepal and synsepal presenting a pure white coloration, while the labellum displays a distinct pink pigmentation. This species displays notable adaptability to cultivation under controlled horticultural conditions [3]. On the other hand, *Paphiopedilum primulinum* M. W. Wood & P. Taylor is native to Sumatra and Java, Indonesia [6]. The flower has a diameter of 6.5-7 cm, with the dorsal sepal and synsepal displaying greenish-yellow coloration, while the petals and labellum are yellow[7]. Because of their uniqueness in flower color and morphology, they are widely sought after by orchid lovers. Therefore, the hybrid varieties of *P. delenatii* or *P. primulinum* as their parents are also welcomed by *Paphiopedilum* lovers, and have won a large number of prizes in the orchid exhibition [3].

Flowering plants typically rely on multi-sensory media, such as color, scent, and morphology, to attract pollinators, thereby ensuring successful reproduction [8]. Although some plants increase their attractiveness to pollinators through a combination of olfactory and visual signals [9], visual signals are still decisive for attracting pollinators [10]. The color of flowers is determined by secondary metabolites, including flavonoids, carotenoids, and betalains, which collectively regulate pigmentation based on their chemical structures and cellular environment [11]. Numerous studies on floral pigmentation have shown that flowers displaying red, purple, or blue colors exhibit high anthocyanin content, whereas pale or white flowers primarily contain flavonols or chalcones [12, 13]. Generally, within the same species, darker flowers colors are typically associated with higher flavonoid content, particularly anthocyanins [14], but the effect of total flavonoid content on flower color did not show obvious correlation [15].

The flavonoid biosynthesis pathway constitutes a key branch of the phenylpropanoid pathway extended via chalcone synthase (CHS), through which a multitude of flavonoid secondary metabolites are generated. These metabolites are broadly categorized into six classes: flavones, flavonols, flavanones, flavanols, anthocyanins, and isoflavones [16]. The flavonoid biosynthesis pathway is governed by a series of critical enzymes and genes, including phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL) [17]. The enzymatic pathway for flavonoid biosynthesis as follows: 4CL generates flavanones, such as naringenin, through the catalytic actions of chalcone synthase (CHS) and chalcone isomerase (CHI). When catalyzed by dihydroflavonol 4-reductase (DFR) and anthocyanidin synthase (ANS), flavanone can be converted into anthocyanins; under the catalysis of flavonol synthase (FLS) along with flavonoid 3'-hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H), it generates flavonols; additionally, isoflavone synthase (IFS) and flavone synthase (FNS) mediate the production of isoflavones and flavones respectively[17]. These enzymes are regulated by their corresponding genes [18]. With the development of science and technology, transcriptomics has been gradually applied to botanical research. Through RNA sequencing technology, the gene expression pattern of plants can be comprehensively analyzed, and the regulatory mechanism of genes can be explained [19], and the complex gene expression network can be revealed [20]. In research of orchid plants, using metabolomics-transcriptomics combined analysis, reveal *Dendrobium nobile* flavonoid

metabolite content and metabolic pathway different genes on flower color diversity impact, and obtain regulatory gene network [21]. The three *Paphiopedilum* plants used in this study exhibited yellow-green, purplish-red, and cream-white flower colors, respectively. Through integrated analysis of flavonoid metabolomics and transcriptomics, we performed a comparative analysis of the differences in flavonoid metabolism and gene expression between the hybrid and its parental lines. This approach revealed the molecular mechanisms underlying the segregation phenomenon of flower coloration between the hybrid plant and its parents.

Results

Characteristics of flavonoid metabolizing substances in *Paphiopedilum L.*

The floral structure of the native species *P. delenatii* exhibit two broad, smooth, and glabrous white petals. The dorsal sepal is nearly white, while both the synsepal and the labellum display a uniform purple coloration (Fig. 1a). *P. primulinum* features petals with undulate margins and ciliate edges. The labellum is yellow-green, while the dorsal sepal and synsepal are green (Fig. 1b). The hybrid combines the broad petal morphology inherited from *P. delenatii* and the undulate petal margins from *P. primulinum*. The synsepal is yellow-green with sparse reddish pigmentation, while the dorsal sepal, petals, and labellum are uniformly cream-colored (Fig. 1c). Results of our determination of flavonoid metabolic content in these three *Paphiopedilum* plants a total of 29 flavanones were detected, among which Hesperetin 5-O-glucoside, Eriocitrin, Poncirin, Naringenin and Butin were much higher in *P. delenatii* than in the other two. The contents of Naringenin and Butin in the cream-colored hybrid were higher than *P. primulinum* (Fig. 2A, Table S1). 93 flavones and flavonols were detected. There was no significant difference in the content of most compounds between *P. primulinum* and hybrid, while some metabolites, such as Kaempferol-3-O-rutinoside, Chrysoeriol 7-O-rutinoside, were found to be higher in *P. delenatii* than in the others (Fig. 2B). Among the 13 anthocyanins detected, the content of Cyanidin in *P. delenatii* was consistently higher than that in *P. primulinum* and the hybrid. In contrast, the concentration of Procyanidin A2 in the hybrid significantly exceeded those in its parental species (Fig. 2C). Additionally, 23 isoflavonoids, 6 flavonoids, and 4 chalcones and dihydrochalcones were identified. Among 168 flavonoid metabolites, the hybrid exhibited 45 metabolites with significant differential abundance compared to *P. delenatii,* of which 36 were down-regulated. In contrast, only 15 flavonoid metabolites showed significant differential abundance relative to *P. primulinum,* with 12 being up-regulated (Table 1).

Table 1 The number of flavonoid metabolites in *Paphiopedilum*

Compared Samples	Num. of Total Ident.	Num. of Total Sig.	Num. of Sig. Up	Num. of Sig. Down
Hybrid vs Pd	168	45	9	36
Hybrid vs Pp	168	15	12	3
Pd vs Pp	168	47	40	7

Num. of Total Ident.: The number of total identified metabolites.

Num. of Total Sig.: The number of totals significantly differential metabolites.

Num. of Sig. Up: The number of totals significantly upregulated metabolites.

Num. of Sig. Down: The number of totals significantly downregulated metabolites

Analysis of transcriptome dataset

The transcriptome data were compared with the annotation results of seven databases. 45.6 % of unigenes could be compared to NR database, only 8.28 % could be compared to KOG database. And more than 26% in GO, NT, PFAM and SwissProt databases, they were 26.82 %, 27.35 %, 26.82 % and 28.12 %, respectively (Fig. 3A, Table S2). When compared with the NR database maintained by NCBI, 20.3 % of the unigenes showed significant sequence alignment with *Dendrobium catenatum*, followed by *Dendrobium nobile* (14.7 %) and *Dendrobium chrysotoxum* (12.7 %) (Fig. 3B, Table S3).

Transcriptomic data from three color morphs of *Paphiopedilum* floral tissues were subjected to pairwise sample comparisons to quantify differentially expressed genes (DEGs). Our analysis revealed 16,621 DEGs between *P. delenatii* (paternal parent) and *P. primulinum* (maternal parent), with comparable numbers of up-regulated (8,842) and down-regulated (7,779) genes in the yellowish-green *P. primulinum* relative to *P. delenatii* (Fig. 4A). The hybrid progeny exhibited 3,338 DEGs compared to *P. delenatii* and 4,623 DEGs relative to the *P. primulinum* (Fig. 4A). Obviously, the majority of DEGs in hybrid comparisons displayed up-regulation regardless of the parental line. Intersectional analysis identified 194 shared DEGs across all three *Paphiopedilum* plants (Fig. 4B).

Analysis of differential expressed genes related flavonoid biosynthesis.

KEGG (http://www.kegg.jp/ or http://www.genome.jp/kegg/) is an integrated database resource for biological interpretation of genome sequences and other high-throughput data. Molecular functions of genes and proteins are associated with ortholog groups and stored in the KEGG Orthology (KO) database [22]. Comparative analysis against the KO database revealed distinct unigene associations with flavonoid biosynthesis pathways in the hybrid progeny relative to *P. delenatii* (paternal parent) and *P. primulinum* (maternal parent). Specifically, 47 unigenes were annotated to the general flavonoid biosynthesis pathway (ko00941), 3 unigenes to anthocyanin biosynthesis (ko00942), and 14 unigenes to flavone/flavonols biosynthesis (ko00944). Notably, no unigenes correlated with isoflavonoid biosynthesis (ko00943) were identified in either hybrid-parent comparison (Fig. 5).

Discussion

Artificial hybridization breeding in the genus *Paphiopedilum* commenced in the 1860s [3]. Driven by market demand, the proliferation of artificially hybridized varieties accelerated significantly over time. This trend is quantitatively reflected in *Sander's List of Orchid Hybrids*, which documents a dramatic

increase in registered orchid hybrids from 750 species in 1946 to over 130 000 by 2019 [23, 24]. Among numerous *Paphiopedilum* hybrids derived from *P. delenatii* as a parental species, the majority retain the characteristic large and symmetrical petals inherited from this species. A subset of these hybrids exhibits distinct floral features including magenta-purple labella or pure white petals, as exemplified by *Paphiopedilum* Magic Lantern 'Ralph' and *Paphiopedilum* Joyce Hasegawa 'Ruey-Hwa' [25]. The hybrid plant material utilized in this study was developed through artificial hybridization breeding techniques between *P. primulinum* and *P. delenatii*. The resulting hybrid exhibits a creamy-white coloration across its entire floral structure, with a slightly yellowish tint compared to the petals of *P. delenatii*. Morphological analysis revealed that the labellum and dorsal sepal closely resembled those of *P. primulinum* in morphology. Prominently, the petal morphology demonstrated an intermediate inheritance pattern: the undulate margins characteristic of *P. primulinum* were preserved, combined with the large, glabrous surface texture typical of *P. delenatii* (Fig. 1). This chimeric expression pattern suggests incomplete dominance in petal trait inheritance during interspecific hybridization. Analysis of flavonoid detection in floral tissues revealed that *P. delenatii* exhibited a higher abundance of flavonoid metabolites compared to *P. primulinum*. Similarly, the hybrid demonstrated flavonoid metabolite levels higher than those of *P. primulinum* but lower than those of *P. delenatii* when compared to their parental species (Fig. S2). In numerous studies on flower color, the content of flavonoids, especially anthocyanins, in dark flowers was higher than that in light flowers among the same species [26]. In this study, the anthocyanin content in *P. delenatii*, characterized by magenta-purple labella, was found to be significantly higher than that of the yellowish-green *P. primulinum* and hybrid cultivar, except for Petunidin 3-O-glucoside. Notably, Petunidin 3-O-glucoside, a compound which substantial accumulation induces deep red to purple pigmentation in plant tissues, was also detected at elevated levels in the Zikui tea plant (*Camellia sinensis cv.* Zikui). Petunidin 3-O-glucoside, alongside cyanidin glycosides, is considered to co-regulate the expression of purple pigmentation traits in plant [27]. The Petunidin 3-O-glucoside in *P. primulinum* was significantly higher than that in the other two species, but did not show purple color, which may be due to the low content of cyanidin 3-O-glucoside. Beyond the direct chromatic effects of anthocyanins, other flavonoid metabolites, such as flavonoids and isoflavonoids, demonstrate substantially elevated levels in dark-pigmented floral tissues compared to lighter-colored counterparts [28], suggesting their synergistic or regulatory roles in pigmentation intensity [29]. In the transcriptome analysis, we also found three unigenes related to anthocyanin synthesis, 14 unigenes related to flavone and flavonol biosynthesis, and 47 unigenes related to flavonoid biosynthesis. Although it is not clear what role these genes play in the formation of the color of the *Paphiopedilum.*

In this study, we performed transcriptome sequencing on the flowers of three *Paphiopedilum* plants, and it can be intuitively seen from the number of DEGs that most of the differentially expressed genes were up-regulated in the hybrid compared with its parents. In KEGG enrichment analysis, pairwise comparisons were conducted to examine the top 20 most significantly enriched pathways of differentially expressed genes among them. The DEGs enriched in flavonoid synthesis pathway and phenylpropanoid biosynthesis showed particularly significant enrichment (Fig. S3). The phenylpropanoid biosynthesis pathway and the flavonoid biosynthesis pathway share upstream pathways in

phenylpropanoid metabolism but utilize distinct enzymes. In the flavonoid biosynthesis pathway, the phenylpropanoid biosynthesis pathway provides essential precursor substances [30]. In comparison with *P. primulinum*, the differentially expressed genes involved in the phenylpropanoid biosynthesis pathway and flavonoid biosynthesis pathway were all highly significant in hybrids. To visually explore the expression differences among the three *Paphiopedilum* plant within the flavonoid biosynthesis network, we first examined the pathway map of KEGG pathway ko00941 (Flavonoid Biosynthesis) (Fig. S4). Compared with *P. primulinum*, genes at most nodes in the flavonoid biosynthetic pathway of the hybrid were up-regulated (Fig. S4A). In contrast, when compared with *P. delenatii*, although genes at some nodes in the later stages of biosynthesis were up-regulated, the majority of genes at nodes in the early stages of biosynthesis were down-regulated. For example, multiple genes in pathways such as EC: 2.3.1.74 (CHS) and EC: 1.14.1491 (trans-cinnamate 4-monooxygenase) exhibited this pattern (Fig. S4B). The up-regulation and down-regulation of these genes might have influenced the differences in the final flavonoid metabolite content.

With the application of multi-omics in botanical research, some scholars have conducted in-depth studies on the genomics and transcriptomics of *Orchidaceae* plants [31]. Researchers analyzed transcriptomic data of *P. armeniacum*, *P. concolor*, *P. hirsutissimum* and *P. malabellumoense*, revealing three whole-genome duplication (WGD) events during the evolutionary history of *Paphiopedilum,* the resultsuggested that *Paphiopedilum* belongs to the subfamily *Cypripedioideae*, which diverged from its sister clades within the subfamily approximately 29.9 Mya (14.6–39.1 Mya) [32]. On the other hand, in the *Orchidaceae* family, the earliest diverged subfamily is *Apostasioideae*, followed by *Vanilloideae*, *Cypripedioideae*, *Orchidoideae*, and finally *Epidendroideae* [1].

In this study, when comparing the transcriptome data of three *Paphiopedilum* species with the NR database, the results showed that 47.7% of sequences were aligned to the genus *Dendrobium* within the subfamily *Epidendroideae*, while 7.8% and 7.6% matched sequences from the subfamilies *Vanilloideae* and *Apostasioideae*, respectively. Noticeably, no significant matches were detected to other sister clades within the subfamily *Cypripedioideae*, which may be attributed to the insufficient genomic data coverage of *Cypripedioideae* plants in the NR database (Fig. 3A). On the other hand, when we aligned *Paphiopedilum* transcriptomic data to six other databases, the coverage rates were all below 30% (Fig. 3B). This phenomenon may primarily be due to *Paphiopedilum* being a non-model organism, coupled with a lack of molecular genetic research reports and the absence of its gene functional annotations in databases like KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) [33]. Additionally, it is also possible that due to the uniqueness of *Paphiopedilum*, a large number of novel genes or species-specific genes exist in its transcriptome, which cannot be annotated through homology-based mapping to functional databases [34, 35]. Molecular analysis of *Paphiopedilum* is an essential approach to comprehensively understand this species, and it also provides a theoretical foundation for the conservation of *Paphiopedilum* populations and the breeding of new cultivars.

Conclusion and future directions

This study analyzed flavonoid metabolites in the floral organs of diverse *Paphiopedilum* species and their hybrid progeny, providing a critical foundation for understanding the molecular mechanisms underlying floral coloration in *Paphiopedilum*. Integrated with transcriptomics analysis, it offers novel insights into the regulatory mechanisms controlling the biosynthesis of color-related compounds in *Paphiopedilum* plants. The hybrid exhibited cream-colored floral colour, distinct from the purple and yellow-green phenotypes of the parental lines. In this process, anthocyanin content plays a pivotal role in purple pigmentation. Upregulation or downregulation of genes at specific nodes within the flavonoid biosynthesis pathway resulted in altered flavonoid accumulation, ultimately leading to the loss of coloration in the hybrids.

Future research should further investigate the differentially expressed genes within the flavonoid biosynthesis pathway, integrating sequences and expression of related candidate genes to identify key regulatory genes control floral pigmentation in *Paphiopedilum*. Furthermore, metabolomic analysis revealed that only a minor subset of flavonoid metabolites showed significant content differences between the hybrid and *P. primulinum*. This result may be attributed to the yellow-green pigmentation of *P. primulinum* correlating with non-flavonoid metabolites rather than flavonoids. Consequently, we will extend our investigation to compare the contents of carotenoids, betalains, and chlorophyll in the floral organ of these three *Paphiopedilum* plants. *Paphiopedilum* cultivars are highly sought after in the floriculture market, particularly for their vibrant coloration. Breeding novel varieties with unique ornamental traits remains a primary objective for horticulturists. This study will continue to explore the regulatory genes associated with floral color and the dynamics of pigment-related metabolites, thereby providing a theoretical foundation for molecular breeding strategies in *Paphiopedilum*.

Method and Materials

Plant Materials

The plant materials were cultivated in the tissue culture room and greenhouse located in Xingyi City, Guizhou Province (104°54´E, 25°09´N). The tissue culture room maintained a constant temperature of 25°C, while the greenhouse temperature fluctuated between 10–30°C. The Paphiopedilum varieties included *P. delenatii, P. primulinum*, and their hybrid (Fig. 1). *P. delenatii* plant and *P. primulinum* plant were initially sown in the tissue culture room in December 2017 and transplanted to the Xingyi greenhouse eight months later. For hybrid development, crosses were performed in April 2020 using *P. delenatii* as the paternal parent and *P. primulinum* as the maternal parent. The hybrid seeds were sown in tissue culture bottles in December 2020 and transplanted to the Xingyi greenhouse eight months post-germination. Sampling of floral tissues was conducted according to the respective flowering periods: *P. delenatii* plant and *P. primulinum* plant were sampled in April 2024, while the hybrid was sampled in May 2024.

Biochemical analysis

Flavonoid-targeted metabolite components were sampled from petals and labella and identified and quantified using a liquid chromatography-mass spectrometry (LC-MS) system.

De novo transcriptome assembly and annotation

RNA integrity was assessed using the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Low-quality reads were filtered from raw sequencing data to obtain clean reads. De novo transcriptome assembly was performed using Trinity software [36], generating unigenes with N50 and N90 values of 1205 and 393, respectively (Table S4). Transcriptome completeness was evaluated using BUSCO (Benchmarking Universal Single-Copy Orthologs), with the Trinity assembly achieving a BUSCO completeness score of 70.1% (Fig. S5). Gene function was annotated based on the following databases, such as NCBI non-redundant protein sequences (Nr), NCBI nucleotide sequences (Nt), Protein family (Pfam), Clusters of orthologous groups for eukaryotic complete genomes (KOG), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO). Coding sequences (CDS) were predicted by aligning unigenes against the NCBI non-redundant protein database (Nr) and Swiss-Prot database. For unigenes without hits in these databases, open reading frames (ORFs) were predicted using TransDecoder (v3.0.1), yielding putative CDS sequences.

Analysis of differentially expressed genes

RSEM and bowtie was used for comparing and normalizing the mapping read to calculating the gene expression levels by the fragments per kilobase of transcript per million mapped reads (FPKM). Differential expression of the two genes was analyzed using DESeq2, and the criteria for identification of differentially expressed genes were based on log2(FoldChange) |≥1 and the false discovery rate (FDR)≤0.05. GO and KEGG pathway enrichment analysis were performed from significant DEG using clusterProfiler R software. PPI analysis of differentially expressed genes was based on STRING database which contains known and predicted protein-protein interactions, via diamond (version 0.9.13).

Declarations

Acknowledgements

Not applicable

Author’s contributions

Q. L. and Y. W. figured the original idea; J. C. and S. C. analyzed the data; C. W., L. L. and J. W. conducted experiments; L. Z. and K. D bred and cultivated the plant materials, including hybrid, *P. delenatii* and *P. primulinum*. Q. L. wrote the paper with input from other authors.

Funding

The financial assistance for this research was provided by the grants from the Guizhou Academy of Sciences project "Gene Screening and Identification for Flower Color Formation in Hybrid Paphiopedilum" (No. QKYRZ202401), directed by Q. L. Technical support for plant material development was obtained from the discipline construction project "Conservation and Development of Mountain Characteristic Plant Resources" (No. XKJS202312) at Minzu Normal University of Xingyi, led by L. Z.

Data availability

The raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under the BioProject accession number PRJNA1266858, which can be found at the following https://dataview.ncbi.nlm.nih.gov/object/.

Ethics approval and consent to participate

All samples collected fully adhere to national and local legal requirements. The *Paphiopedilum* plant materials used in this study (including but not limited to seeds, seedlings, and tissue cultures) were derived exclusively from an aseptic artificial cultivation system, with no collection from wild resources whatsoever. This study is a genetic and physiological exploration of ornamental flowers, with plants as the research subjects. It does not involve any human participants, medical interventions, health outcome or patient data. According to the International Committee of Medical Journal Editors (ICMJE) definition of clinical trials and WHO registration guidelines, clinical trials require human subjects undergoing health-related interventions. Therefore, this study is exempt from clinical trial registration.

Consent for publication

Not applicable.

Conflict of interest statement

The authors declare no conflicts of interest.

References

1. Givnish TJ, Spalink D, Ames M, Lyon SP, Hunter SJ, Zuluaga A, et al. Orchid phylogenomics and multiple drivers of their extraordinary diversification. Proc Biol Sci. 2015;doi: 10.1098/rspb.2015.1553.
2. Li X, Fan J, Luo S, Yin L, Liao H, Cui X, et al. Comparative transcriptome analysis identified important genes and regulatory pathways for flower color variation in *Paphiopedilum hirsutissimum*. BMC Plant Biol. 2021;21:495.
3. Liu ZJ, Chen XQ, Chen LJ, Lei SP. The genus Paphiopedilum in China. Beijing: Science Press 2009. P. 1-12,48-54,210-232.

4. Yao N, Zheng B, Wang T, Cao X. Isolation of *Tulasnella spp.* from cultivated Paphiopedilum orchids and screening of germination-enhancing fungi. J Fungi (Basel) 2023;9:597.
5. Vu HT, Tran N, Nguyen TD, Vu QL, Bui MH, Le MT, Le L. Complete chloroplast genome of Paphiopedilum delenatii and phylogenetic relationships among Orchidaceae. Plants (Basel) 2020;9:61.
6. Safitri S, Yalapuspita DC, Handini E, Aprilianti P, Isnaini Y, Semiarti E. Improvement of growth rate in in vitro culture of Paphiopedilum primulinum and Paphiopedilum glaucophyllum using banana enrichment media. Trop Life Sci Res. 2024;35:109–120.
7. Koopowitz H. An updated, annotated checklist of the genus *Paphiopedilum*. Orchid 2012;76:178–215.
8. Willmer P. Pollination and Floral Ecology. Princeton: Princeton University Press 2011.
9. Galizia CG, Kunze J, Gumbert A, Borg-Karlson AK, Sachse S, Markl C, Menzel R, et al. Relationship of visual and olfactory signal parameters in a food-deceptive flower mimicry system. Behav Ecol. 2005;16:159–168.
10. Jersáková J, Jürgens A, Šmilauer P, Johnson, S.D. The evolution of floral mimicry: Identifying traits that visually attract pollinators. Funct Ecol. 2012;26:1381–1389.
11. Grotewold E. The genetics and biochemistry of floral pigments. Annu Rev Plant Biol. 2006;57:761–780.
12. Tanaka Y, Sasaki N, Ohmiya A. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. Plant J. 2008;54:733–749.
13. Zhao D, Tao J. Recent advances on the development and regulation of flower color in ornamental plants. Front Plant Sci. 2015;6:261.
14. Zhu M, Zheng X, Shu Q, Li H, Zhong P, Zhang H. Relationship between the composition of flavonoids and flower colors variation in tropical water lily (*Nymphaea*). PLoS One 2012; doi: 10.1371/journal.pone.0034335.
15. Taylor LP, Grotewold E. Flavonoids as developmental regulators. Curr Opin Plant Biol. 2005;8:317–323.
16. Tian J, Zhang CB, Sun YX. Advances in genetic engineering, chemical synthesis of flavonoids and their structural modification. Food Ferment Ind. 2021;47:252–258.
17. Song XW, Wei JB, Di SK, Pang YZ. Recent advances in the regulation mechanism of transcription factors and metabolic engineering of anthocyanins. Chin Bull Bot. 2019;54:133–156.
18. Anusha MR, Mohammad A. Integrating omics approaches for climate-resilient crops: A comprehensive review. J Adv Biol Biotechnol. 2024;27:351–363.
19. Raza A, Salehi H, Bashir S, Tabassum J, Jamla M, Charagh S, et al. Transcriptomics, proteomics, and metabolomics interventions prompt crop improvement against metal(loid) toxicity. Plant Cell Rep 2024;43:80.

20. Qiu Y, Cai C, Mo X, Zhao X, Wu L, Liu F, et al. Transcriptome and metabolome analysis reveals the effect of flavonoids on flower color variation in *Dendrobium nobile.* Front Plant Sci 2023;doi: 10.3389/fpls.2023.1220507.
21. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29:644–652.
22. Kanehisa M, Sato Y, Kawashima M, Furumichi M, Tanabe M. KEGG as a reference resource for gene and protein annotation. Nucleic Acids Res 2016; doi: 10.1093/nar/gkv1070
23. Sanders DF, Wreford MW. Sander's Complete List of Orchid Hybrids. London: David Sander's Orchids Limited 1947.
24. Royal Horticultural Society. Quarterly supplement to the International Register and Checklist of Orchid Hybrids (Sander's List): January–March 2022 registrations. Oxford: RHS 2022.
25. Frowine SA. Fragrant Orchids: A Guide to Selecting, Growing, and Enjoying. Portland: Timber Press 2006. p. 120–136.
26. Zhao Y, Qi X, Liu Z, Zheng W, Guan J, Liu Z, et al. Transcriptome and metabolome profiling to explore the causes of purple leaves formation in non-heading Chinese cabbage (*Brassica rapa L. ssp.* chinensis Makino var. multliceps Hort). Foods 2022;11:1787.
27. Cai J, Lv L, Zeng X, Zhang F, Chen Y, Tian W, et al. Integrative analysis of metabolomics and transcriptomics reveals molecular mechanisms of anthocyanin metabolism in the Zikui tea plant (*Camellia sinensis cv.* Zikui). Int J Mol Sci. 2022;23:4780.
28. Mekkiou R, Touahar H, Dijoux-Franca MG, Mariotte AM, Benayache S, Benayache F. A new isoflavone from Genista saharae (Fabaceae). Biochem Syst Ecol. 2005;33:635–638.
29. Ren C, Wang J, Xian B, Tang X, Liu X, Hu X, et al. Transcriptome analysis of flavonoid biosynthesis in safflower flowers grown under different light intensities. Peer J. 2020; doi: 10.7717/peerj.8671.
30. Winkel-Shirley B. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 2001;126:485–492.
31. Cembrowska-Lech D, Krzemińska A, Miller T, Nowakowska A, Adamski C, Radaczyńska M, et al. An integrated multi-omics and artificial intelligence framework for advance plant phenotyping in horticulture. Biology (Basel) 2023;12:1298.
32. Wang M, Wang T, Xia Z, Li TZ, Jin XH, Yan YH, et al. Advances in the study of flower color variation in orchids. Chin Bull Bot 2021;56:699–714.
33. Pruitt KD, Tatusova T, Maglott DR. NCBI Reference Sequences (RefSeq): a curated non-redundant sequence database of genomes, transcripts and proteins. Nucleic Acids Res. 2007;35:61–62.
34. Baker W, van den Broek A, Camon E, Camon E, Hingamp P, Sterk P, Stoesser G, et al. The EMBL nucleotide sequence database. Nucleic Acids Res 2000;28:19–23.
35. Zhang Y, Zhang GQ, Zhang D, Liu XD, Xu XY, Sun WH, et al. Chromosome-scale assembly of the *Dendrobium chrysotoxum* genome enhances the understanding of orchid evolution. Hortic Res. 2021;8:183.

36. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29:644–

Figures

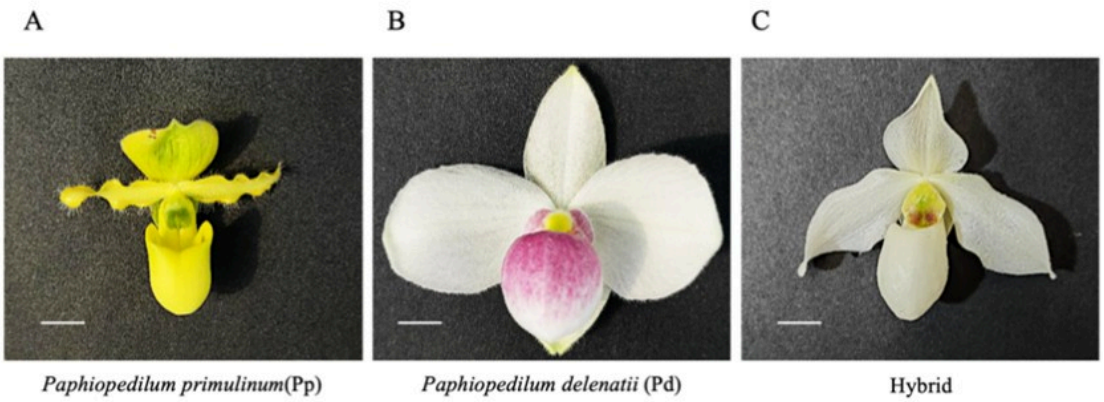


Figure 1

Phenotypic comparison of three *Paphiopedilum L. Paphiopedilum primulinum* (A), *Paphiopedilum delenatii* (B) and their hybrid progeny (C), Scale bars = 1cm.

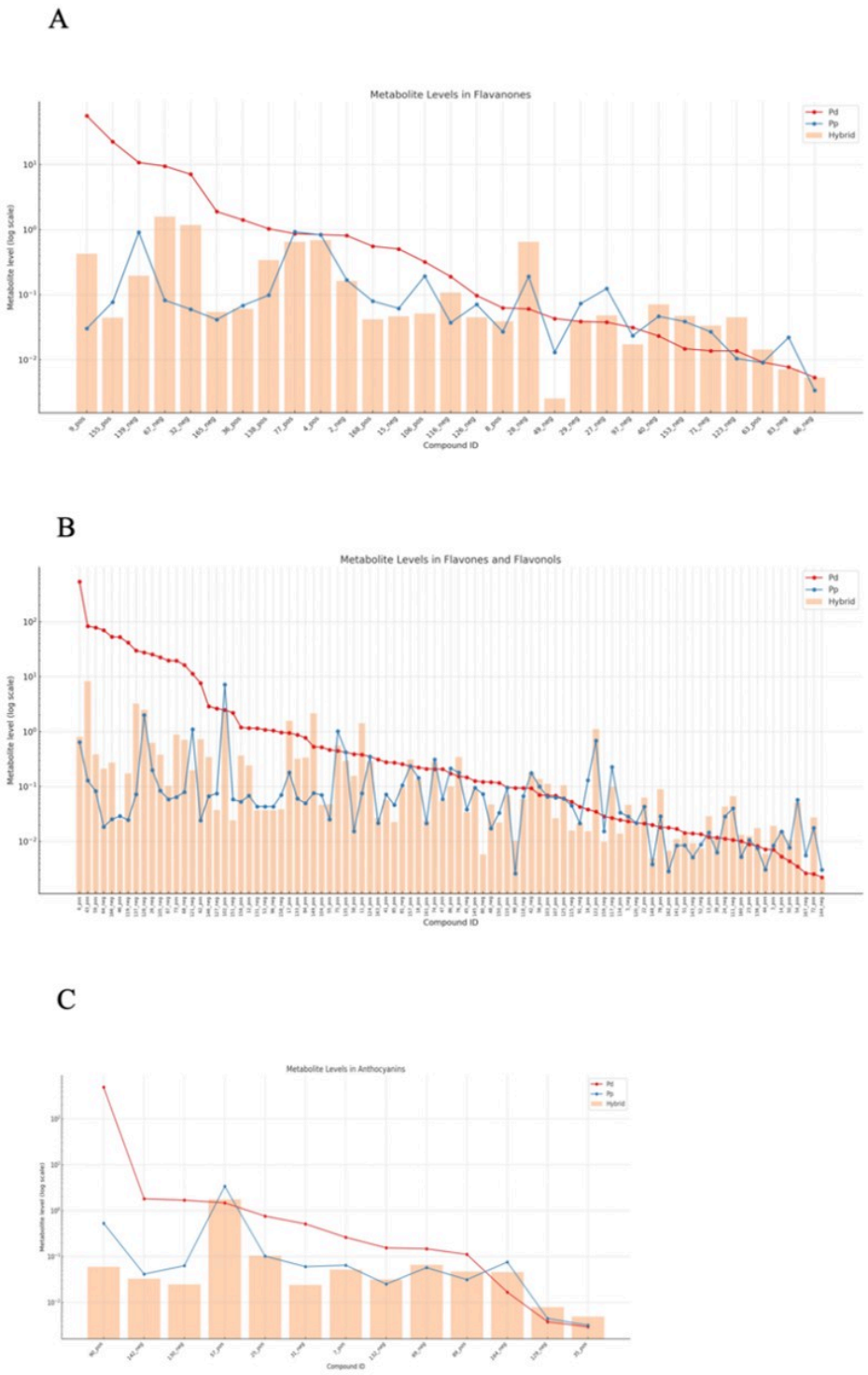


Figure 2

Relative quantitative values of flavonoid metabolites in three flowers of *Paphiopedilum*. The figure shows only the relative quantification values of three flavonoid subclasses：flavanones (A), flavones and flavonols (B) and anthocyanins (C). Pp: *P. primulinum,* Pd: *P. delenatii,*Hybrid: The hybrid progeny of *P. delenatii* and *P. primulinum*.

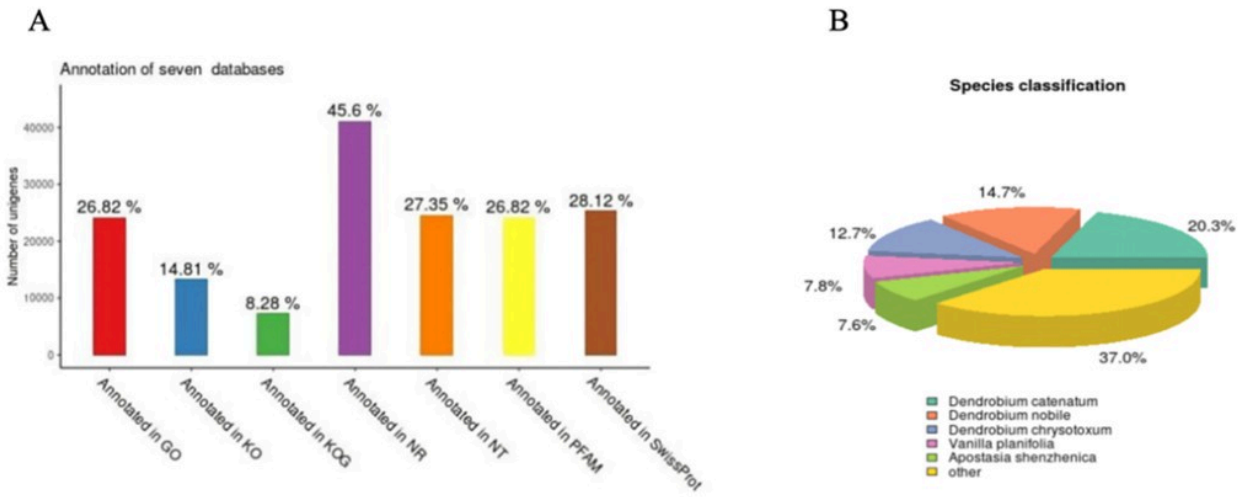


Figure 3

Comparison of transcriptome data with databases. A: Percentage of unigene numbers annotated in seven databases, GO: Gene Ontology, KO: Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology, KOG: Clusters of orthologous groups for eukaryotic complete genomes, Nr: NCBI non-redundant protein sequences, Nt: NCBI nucleotide sequences, Pfam: Protein family, Swiss-Prot: Swiss-Prot Protein Knowledgebase; B: Species distribution diagram of matched sequences in the Nr database.

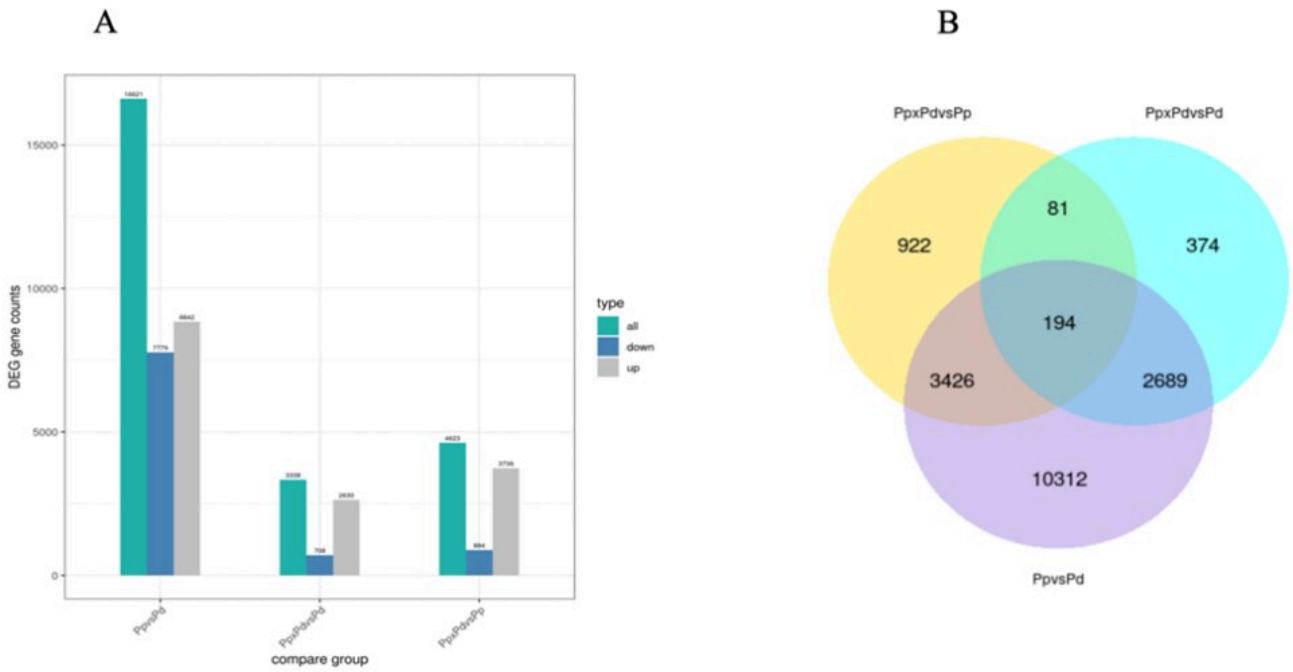


Figure 4

Statistical Quantification of Differentially Expressed Genes. A Statistical Bar Chart of Differentially Expressed Genes (DEGs). Compare denotes the names of comparative groups; All indicates the total number of DEGs identified in each comparison; Up and Down represent the counts of upregulated and downregulated DEGs, respectively. Threshold specifies the analytical software (e.g., DESeq2/edgeR); B: Venn Diagram of DEG Overlaps, Venn diagram visualizing overlaps of DEGs among multiple comparative groups.

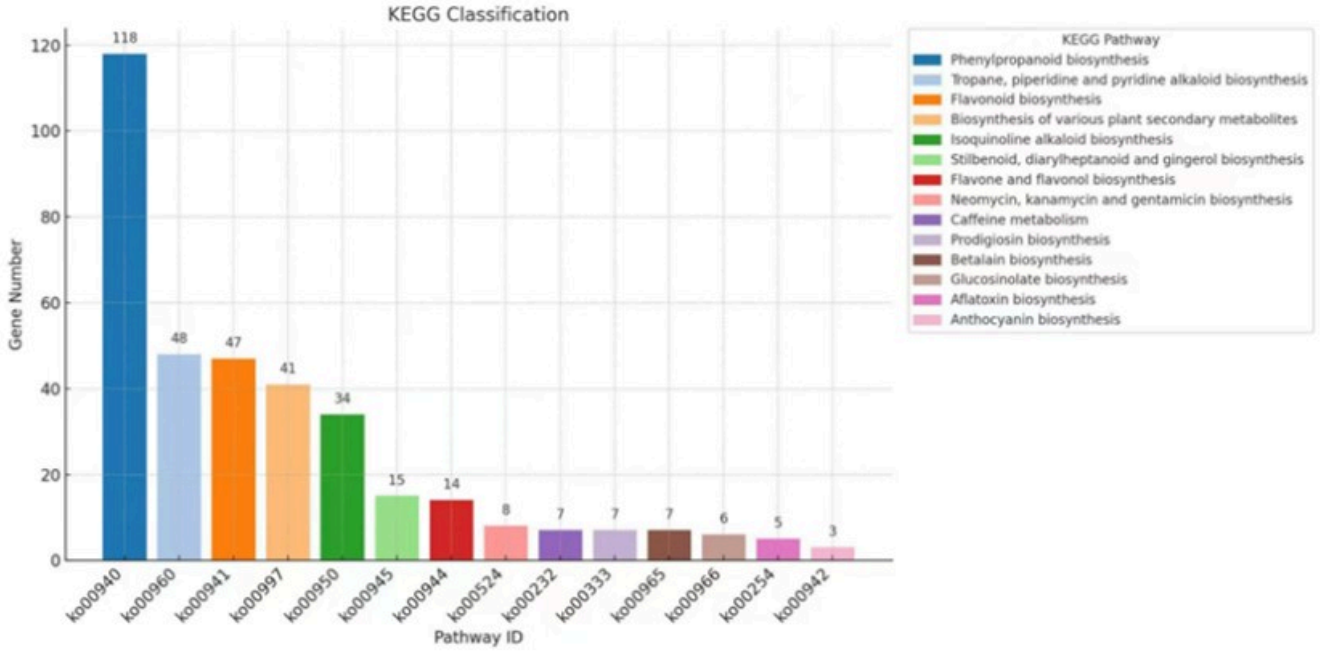


Figure 5

KEGG metabolic pathway classification statistical diagram. The x-axis represents KEGG Orthology (KO) metabolic pathway names, while the y-axis indicates the number of genes annotated to each pathway. Box annotations specify the KEGG pathway names corresponding to their respective KO identifiers (e.g., ko00940).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- abbreviationlist.xlsx
- Supplementaryfigures.pdf
- TableS1.csv
- TableS2.csv
- TableS3.csv
- TableS4.csv