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Transcriptomic and targeted metabolomic analyses provide insights into the flavonoids biosynthesis in the flowers of *Lonicera macranthoides*

Ling Ling Lv^{1**}, Li Yun Li¹, Long Qian Xiao^{1†} and Jian Hui Pi¹

Abstract

Background Flavonoids are one of the bioactive ingredients of *Lonicera macranthoides* (*L. macranthoides*), however, their biosynthesis in the flower is still unclear. In this study, combined transcriptomic and targeted metabolomic analyses were performed to clarify the flavonoids biosynthesis during flowering of *L. macranthoides*.

Results In the three sample groups, GB_vs_WB, GB_vs_WF and GB_vs_GF, there were 25, 22 and 18 differentially expressed genes (DEGs) in flavonoids biosynthetic pathway respectively. A total of 339 flavonoids were detected and quantified at four developmental stages of flower in *L. macranthoides*. In the three sample groups, 113, 155 and 163 differentially accumulated flavonoids (DAFs) were detected respectively. Among the DAFs, most apigenin derivatives in flavones and most kaempferol derivatives in flavonols were up-regulated. Correlation analysis between DEGs and DAFs showed that the down-regulated expressions of the *CHS*, *DFR*, *C4H*, *F3H*, *CCoAOMT_32* and the up-regulated expressions of the two *HCTs* resulted in down-regulated levels of dihydroquercetin, epigallocatechin and up-regulated level of kaempferol-3-O-(6"-O-acetyl)-glucoside, cosmosiin and apigenin-4'-O-glucoside. The down-regulated expressions of *F3H* and *FLS* decreased the contents of 7 metabolites, including naringenin chalcone, proanthocyanidin B2, B3, B4, C1, limocitrin-3,7-di-O-glucoside and limocitrin-3-O-sophoroside.

Conclusion The findings are helpful for genetic improvement of varieties in *L. macranthoides*.

Keywords *Lonicera macranthoides*, Transcriptome, Targeted metabolome, Flavonoid biosynthesis, Flavone, Flavonol, Anthocyanin, Flower

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Background

Lonicera macranthoides Hand. Mazz (*L. macranthoides*), the most widely cultivated species of Lonicerae flos grown in south China, is one of the traditional Chinese medicinal plants. Lonicerae flos was separated from honeysuckle (*Lonicera japonica*) grown in north China in the Chinese Pharmacopoeia in 2005. The main medicinal ingredients of honeysuckle are luteoloside and chlorogenic acid, and the market price of its fresh flowers is about 360 yuan per kilogram [1]. The main medicinal ingredients of *L. macranthoides* are phenolic acids, flavonoids and carotenoids, especially chlorogenic acid, and the market price of its fresh flower buds or early flowers is about 130 yuan per kilogram [1]. Low prices due to differences in medicinal ingredients hinder the sustainable development of the *L. macranthoides* industry.

Flavonoids, including flavonols, flavones, flavanols, iso-flavones, flavanones and anthocyanins, are a major class of secondary metabolites existing in all higher plants [2]. They are not only very important pigments that contribute to a range of flower colors from yellow to purple [3], but also important bioactive ingredients. They have antibacterial, anti-inflammatory, antioxidant, anticancer; antiviral and antimicrobial and other functions [4–7], and can be used for the treatment of diseases such as throat arthralgia, erysipelas, heat toxic blood dysentery, wind heat cold, warm heat disease, etc. [8–9]. They also played important roles in the treatment of the severe acute respiratory syndrome (2003) and COVID-19 (2019) outbreak in China. Twenty-one flavonoids have been isolated from *L. macranthoides*, including kaempferol-3-O- β -D-glucoside, quercetin-3-O- β -D-glucoside and luteolin-7-O- β -D-glucoside, etc [10–13]. The biosynthetic pathways of flavonoids in apple [14], *Salvia miltiorrhiza* [15], *Arabidopsis thaliana* and maize [16] have been well studied.

Compared with honeysuckle, the research of *L. macranthoides* in all aspects lags behind seriously. At present, the researches on *L. macranthoides* mainly focus on the determination of medicinal ingredients, RNA sequencing, cloning and verification of functional genes [17–19]. These studies are fragmentary and lack systematicness. In recent years, the regulation mechanism of functional ingredients have been elucidated through the combined analysis of transcriptome and metabolome in honeysuckle [20], cucumber [21], dendrobium [22] and Longan [23]. Pan et al. [24] reported the regulatory mechanism of chlorogenic acid (CGA) biosynthesis in *L. macranthoides* and the effect of corolla dehiscence on the quality of medicinal function. In this work, combined transcriptomic and metabolomic analyses were performed to clarify the flavonoids biosynthesis in the flowers of *L. macranthoides*. The findings are helpful for breeding new varieties of *L. macranthoides* with

high content of medicinal ingredients and increasing the economic income of growers.

Results

Transcriptome analysis

To evaluate the gene expression profile in *L. macranthoides* flowers at GB, WB, WF and GF, 12 cDNA libraries were constructed and sequenced. The raw data has been deposited in the China National GeneBank DataBase (CNGBdb, <https://db.cngb.org/>) with accession code CNP0003782. A total of 158.96 Gb clean data was obtained and the GC content of each sample was more than 44.3%. The percentages of Phred-like quality score at the Q30 level (an error probability of 1%) ranged from 90.94 to 93.72%.

There were 11,692, 8,058, and 8,656 differentially expressed genes (DEGs) in the three sample groups (GB_vs_WB, GB_vs_WF and GB_vs_GF) respectively (Fig. 1). Among the three sample groups, the most DEGs were found in GB_vs_WB, with 5,208 DEGs up-regulated and 6,484 DEGs down-regulated. Venn diagram analysis showed that 5,095 DEGs were common to all three sample groups (Fig. 1).

Gene ontology (GO) classification analysis assigned 57,129, 29,156 and 36,957 unigenes to the three major categories (Biological Process, Cell Component and Molecular Function), respectively (Supplementary Fig. S1). In the 'Biological Process' category, the subcategory with the most unigenes was 'metabolic process' (GO:0008152, 14,175 unigenes), followed by 'cellular process' (GO:0009987, 13,316 unigenes) and 'biological regulation' (GO:0065007, 3,948 unigenes). In the 'Molecular Function' category, the most enriched subcategory was 'binding' (GO:0005488, 18,510 unigenes). In addition, 'nucleic acid binding transcription factor activity' (GO:0001071) included 678 unigenes and 'molecular function regulator' (GO:0098772) included 485 unigenes.

All DEGs were BLAST against the KEGG Ortholog database [25] and the top 20 KEGG categories in the three sample groups were presented respectively in Fig. 2A–C. In GB_vs_WB, GB_vs_WF and GB_vs_GF, there were 25, 22 and 18 DEGs in flavonoids biosynthesis (ko00941) respectively. Among the DEGs in flavonoids biosynthetic pathway, *PAL*, *C4H*, two *CHSs*, *CHI*, *F3H*, *F3'H*, two *DFRs*, *CCoAOMT_32*, *ANS* and *ANR* were all down-regulated, while *FLS_73*, *C4H_90*, *CCoAOMT_73* and three *HCTs* were up-regulated in the three sample groups.

Validation of RNA-seq data by qPCR analysis

To validate the RNA-Seq data, 9 DEGs (two transcription factors and 7 genes in flavonoids biosynthetic pathway) were selected and their expression levels at GB, WB, WF and GF were analyzed by qPCR. As shown in Fig. 3, the

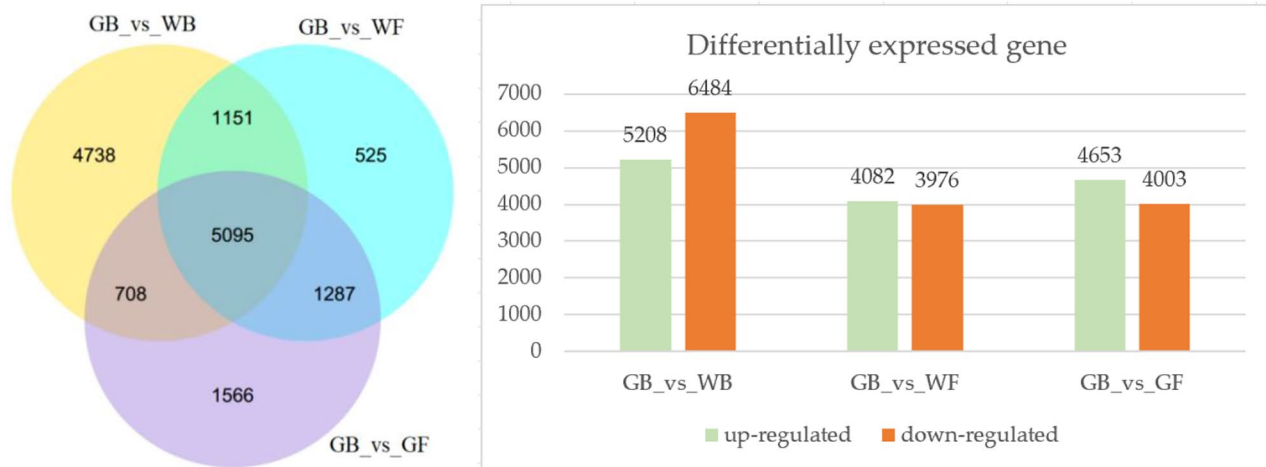


Fig. 1 Venn Diagram and the up- and down-regulated differentially expressed genes in the three sample groups. GB, green flower bud; WB, white flower bud; WF, white flower; GF, golden flower

expressions of 8 DEGs were higher in RNA-seq than in qPCR. Though the expression levels of these DEGs were different, the trends in gene expression of qPCR were highly consistent with those of RNA-seq, which suggested that RNA-seq data was credible and could be used for subsequent experiments.

Flavonoids assay

A total of 339 flavonoids were detected and quantified at four developmental stages of flower in *L. macranthoides*, including 135 flavones, 105 flavonols, 16 flavanols, 4 anthocyanins, 7 proanthocyanidins, 21 chalcones and 51 other flavonoids (Fig. 4). Among the three sample groups, the most differentially accumulated flavonoids (DAFs) were found in GB_vs_GF, with 45 DAFs up-regulated and 118 DAFs down-regulated (Fig. 5A-B). Venn diagram analysis showed that 67 DAFs were common to all three sample groups (Fig. 5A). Based on the accumulation patterns of flavonoids, the clustering heat map was shown in Fig. 6. The biological replicates were all grouped together, indicating a high reliability of the metabolome data.

5,7,3,4'-tetrahydroxy flavone (Luteolin) and its derivative of luteolin-7-O-(6"-malonyl)-glucoside (LOG) were detected in the three sample groups. The former was all down-regulated, while the latter was all up-regulated (Fig. 7). Four and five other luteolin derivatives were detected in GB_vs_WF and GB_vs_GF respectively, all of them were down-regulated. Cyanidin-3,5-O-diglucoside and pelargonidin-3,5-O-diglucoside were detected in GB_vs_WB and GB_vs_WF, and they were all up-regulated. Cyanidin-3-O-glucoside was only detected in GB_vs_WB and also up-regulated (Fig. 7). In the three sample groups, a total of 61 differentially accumulated flavonols were detected. The up-regulated flavonols were mainly kaempferol derivatives, while the down-regulated

flavonols were mainly limonin derivatives. Four differentially accumulated dihydroflavonols were detected, among which dihydroquercetin was down-regulated in all three sample groups. Seven differentially accumulated procyanidins, A1, B1, B2, B3, B4, C1, C2, were detected and procyanidin A1 was only found in GB_vs_GF. The other 6 procyanidins were detected in the three sample groups and their contents were all down-regulated. In addition, the DAFs of naringenin chalcone, naringenin, eriodictyol, catechin and epigallocatechin were all down-regulated in the three sample groups (Fig. 7).

Correlation analysis between transcripts and flavonoids

Thirteen DEGs and 17 DAFs in the flavonoids biosynthetic pathways (Fig. 7) were obtained to organize an interaction network (Fig. 8) using Cytoscape 2.0 software. *CHS*, *DFR*, *C4H*, *F3H* and *CCoAOMT_32* were positively correlated with dihydroquercetin and epigallocatechin and negatively correlated with kaempferol-3-O-(6"-O-acetyl)-glucoside (K3OG), apigenin-7-O-glucoside (Cosmosiin) and apigenin-4'-O-glucoside. The regulatory effects of *HCT_60*, *HCT_32* and the above five genes were just opposite (Fig. 8). The down-regulated expressions of *F3H* and *FLS* decreased the contents of 7 metabolites, including naringenin chalcone, proanthocyanidin B2, B3, B4, C1, limocitrin-3,7-di-O-glucoside and limocitrin-3-O-sophoroside (Figs. 7 and 8). In addition, *F3H* was positively correlated with epigallocatechin and catechin. *HCT_60* and *HCT_32* were negatively correlated with luteolin, while *CHS*, *DFR*, *CCoAOMT_32* and *CHI* were positively correlated with it. The flavonoids associated with *CCoAOMT_32* and *CCoAOMT_73*, respectively, were completely different. There were similarities and differences among the flavonoids associated with the three *HCTs* respectively (Fig. 8).

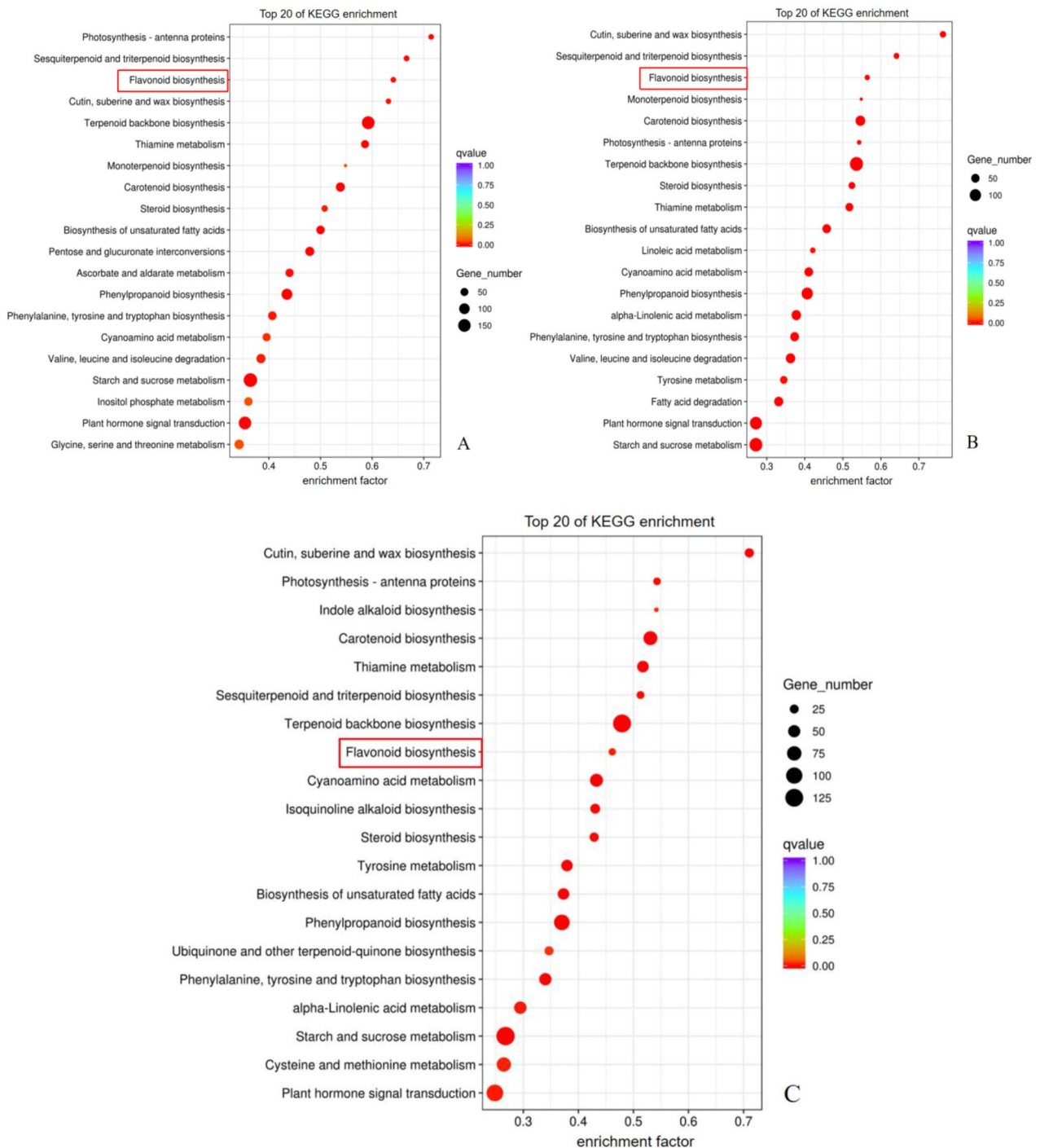


Fig. 2 KEGG pathway classification of differentially expressed genes in the three sample groups of *L. macranthoides*. (A) In GB_vs_WB. (B) In GB_vs_WF. (C) In GB_vs_GF.

Discussion

Flavone is a major class of flavonoids and 135 flavones were detected in this work, 89 of which were DAFs. The contents of LOG, cosmosiin and apigenin-4'-O-glucoside increased in the three sample groups (Fig. 7), and their contents at GF were 5.29, 7.81 and

7.72 times of those at GB respectively. *HCT_60* and *HCT_32* were positively correlated with the accumulations of LOG, apigenin-4'-O-glucoside and cosmosiin, while *C4H* and *CCoAOMT_32* were negatively regulated them (Fig. 8). In addition, apigenin-7-O-(2''-glucosyl)arabinoside, apigenin-6-C-(2''-xylosyl)-glucoside,

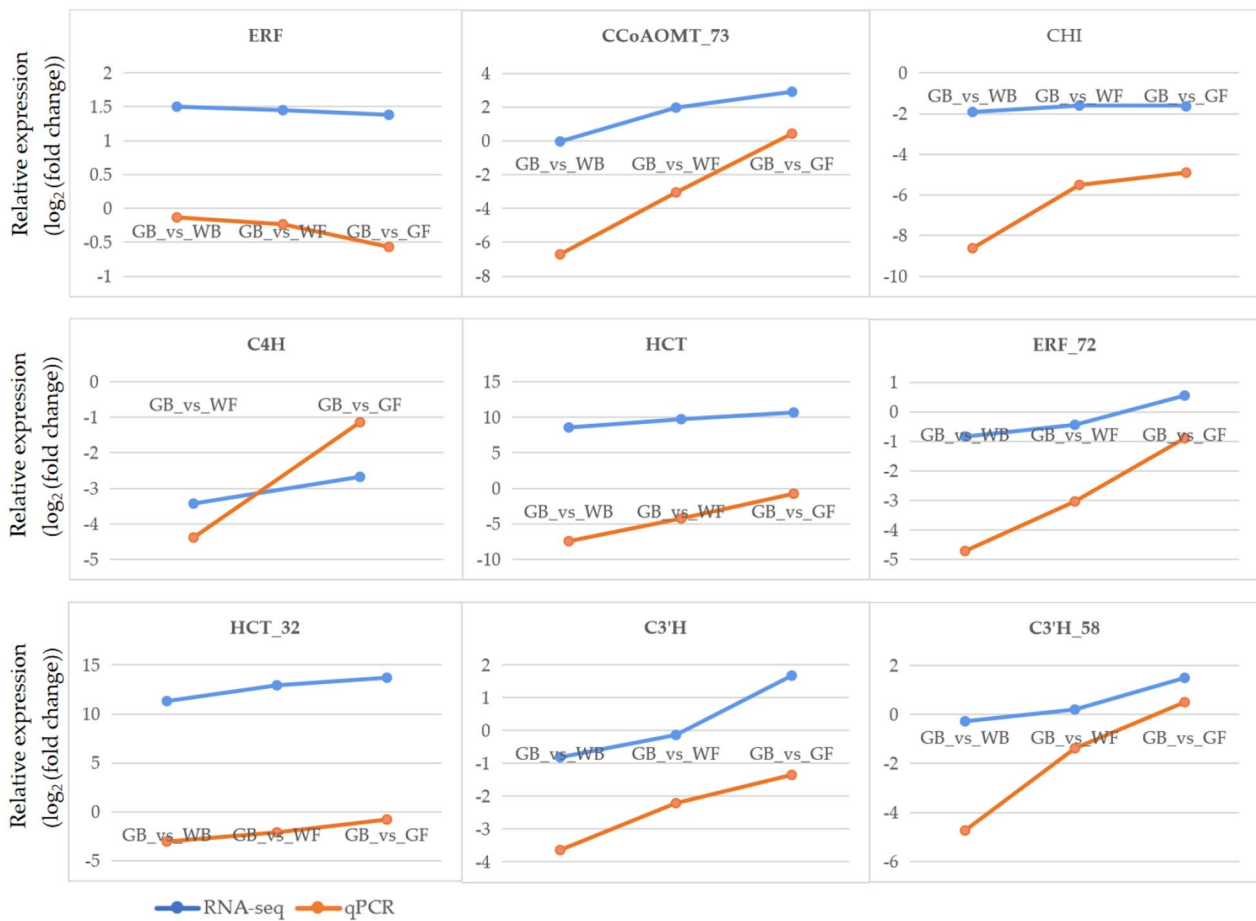


Fig. 3 qPCR validation of RNA-seq data using 9 related genes in *L. macranthoides*. *18S rRNA* expression values were used as the internal reference. Values are the means \pm SD of three biological replicates. GB, green flower bud; WB, white flower bud; WF, white flower; GF, golden flower

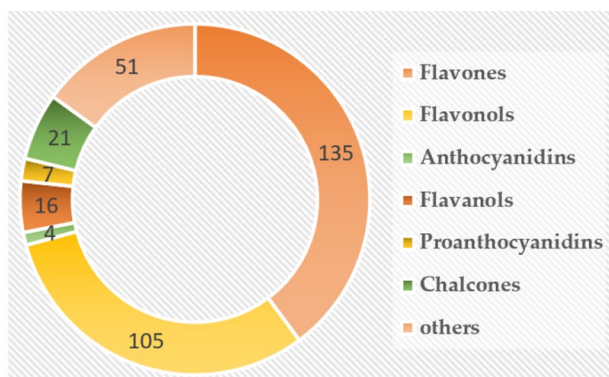


Fig. 4 The total flavonoids detected in the samples

apigenin-8-C-(2''-xylosyl)-glucoside and apigenin-7-O-(6''-acetyl)-glucoside were also up-regulated in the three sample groups. Tricin-7-O-(2''-malonyl)rhamnoside (TMR) had the largest fold change of 56.13 in differentially accumulated flavone in GB_vs_GF. These suggested that the flavones of LOG, apigenin derivatives and TMR

were one of the main medicinal ingredients in the late flowers of *L. macranthoides*. Yang et al. [26] reported that flavones had antibacterial, antioxidant and anticancer effects. Apigenin had been demonstrated to have medicinal functions in the treatment of cancer [27], epilepsy [28] and hyperlipemia [29].

Flavonols have the functions of anti-allergy, anti-inflammation, anticancer and preventing diabetes [30–31]. FLS is a key enzyme in the synthesis of flavonols and different *FLS* genes may play the same or different roles [32]. In this study, *FLS* positively correlated with proanthocyanidins C1, B2, B3, B4, naringin chalcone and limonin derivatives, while *FLS_32* negatively regulated kaempferol-3-O-(6''-acetyl)glucosyl-(1→3)-galactoside (K3OGG). The other three *FLS*s showed no correlation with flavonoids accumulation (Fig. 8). Among the six *FLS*s in *Arabidopsis Thaliana*, *AtFLS1* plays an important role in flavonoid synthesis [33]. The up-regulated flavonols were mainly kaempferol derivatives, such as K3OGG, K3OG, kaempferol-3-O-galactoside-4'-O-glucoside and kaempferol-3-O-(6''-malonyl)galactoside,

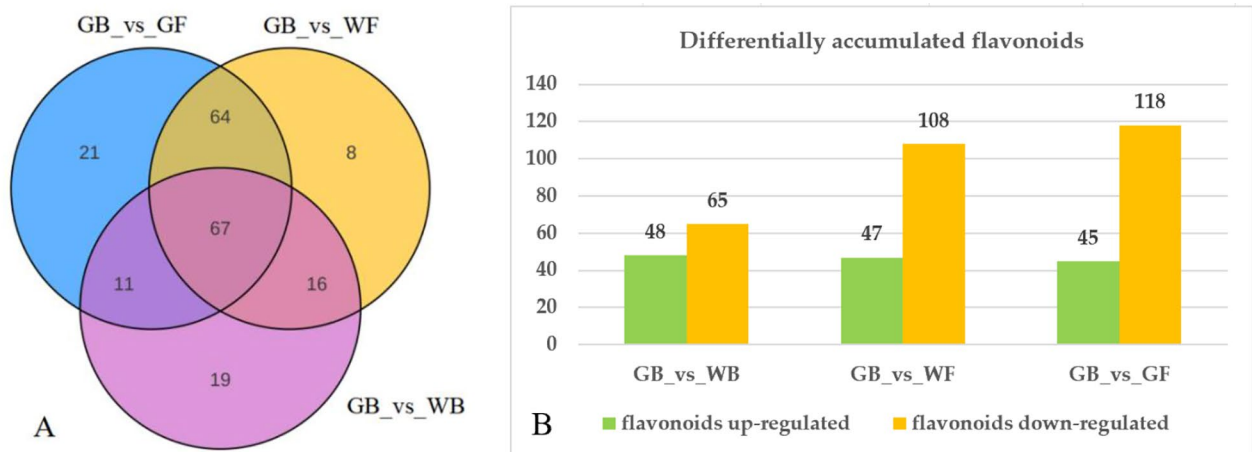


Fig. 5 The differentially accumulated flavonoids in the three sample groups. **(A)** Venn diagram depicting the shared and specific flavonoids. **(B)** The up- and down-regulated flavonoids. GB, green flower bud; WB, white flower bud; WF, white flower; GF, golden flower

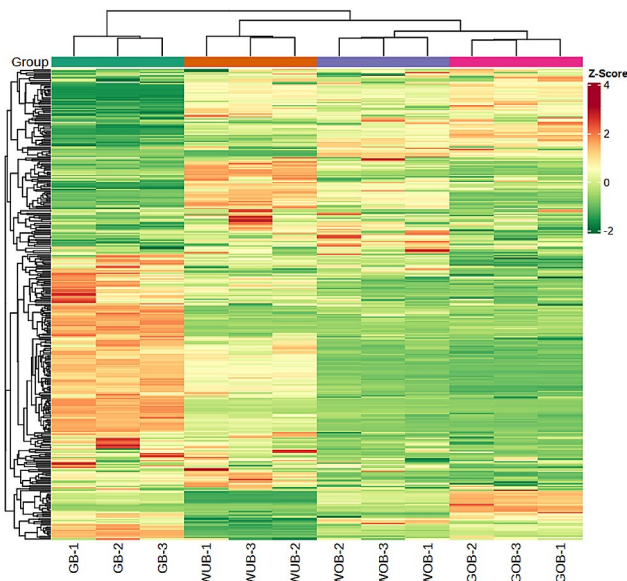


Fig. 6 Clustering heat map of the flavonoids detected in the total samples. Each sample is visualized in a single column, and each metabolite is represented in a single row. Red indicates high abundance of metabolites, while green indicates low abundance. GB, green flower bud; WB, white flower bud; WF, white flower; GF, golden flower

which suggested that *FLS*s catalyzed dihydrokaempferol to form kaempferol, rather than dihydroquercetin to quercetin or dihydromyricetin to myricetin. The substrates of *FLS* are dihydroquercetin, dihydrokaempferol and dihydromyricetin, and the binding of *FLS* to the substrates is specific [34]. The content of *K3OGG* decreased slightly in *GB_vs_WB*, then increased sharply, and had the largest fold change of 52.60 in differentially accumulated flavonols in *GB_vs_GF*.

Anthocyanins have anti-inflammatory and antioxidant effects [4–5], and they are synthesized in the flavonoid pathway (Fig. 7). In this work, two differentially

accumulated cyanidin derivatives, cyanidin-3,5-O-diglucoside and cyanidin 3-O-glucoside, and differentially accumulated pelargonidin-3,5-O-diglucoside were detected in *GB_vs_WB* and *GB_vs_WF*. Moreover, the contents of cyanidin derivatives were considerably higher than that of pelargonidin derivative. Yi et al. [23] reported the similar result in longan pericarp. The contents of the three anthocyanins reached the highest at *WB* and then decreased gradually (Fig. 7). Xue et al. [20] reported that anthocyanins were detected at *S1* and *S2* of early flowering and were not present at *S3–S6*. *DFR* has selective specificity for dihydrokaempferol, dihydroquercetin and dihydromyricetin [35]. In this work, it seems that *DFR*s catalyzed dihydroquercetin to produce cyanidin, rather than dihydromyricetin to delphinidin. Similar result was reported in *Ficus carica* L [36]. The expression of *CHS* was significantly correlated with anthocyanin accumulation [37]. Surprisingly, in this study, the expressions of key genes in anthocyanin biosynthetic pathway, such as *CHS*, *DFR*, *ANS*, etc. were all down-regulated, while the contents of the above three anthocyanins were up-regulated in *GB_vs_WB* and *GB_vs_WF*. Is there another anthocyanin biosynthetic pathway in the flowers of *L. macranthoides*?

HCT belongs to plant acyltransferase family and can catalyze a variety of substrates, including shikimic acid, 4-hydroxyphenyllactic acid, quinic acid, 4-hydroxyphenylethylamine and gentianic acid, to form ester or amide compounds [38]. *HCT* plays an important role in the biosynthesis of lignin and CGA [39–40]. In this work, *p-coumaroyl-CoA* was catalyzed by *HCT*, *C3'H* and *CHS* to synthesize eriodictyol, thus participating in flavonoid biosynthesis (Fig. 7). Although the expressions of three *HCT*s were significantly up-regulated, the content of eriodictyol was down-regulated (Fig. 7). It might be because

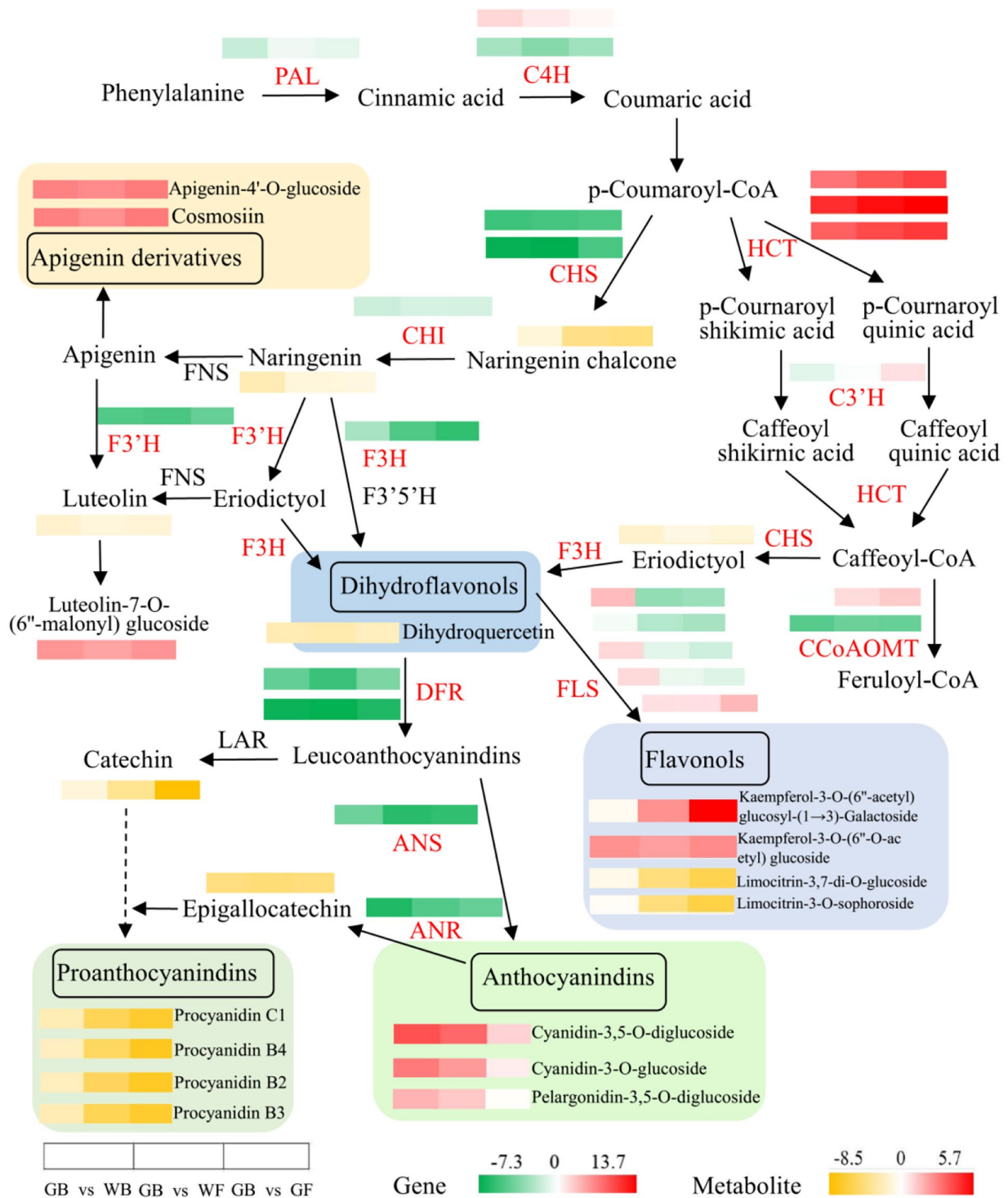


Fig. 7 The pathway associated with flavonoid biosynthesis in *L. macranthoides* and heat maps of differentially expressed genes (DEGs) and differentially accumulated flavonoids in three sample groups. The red font represents DEG. ANS, anthocyanidin synthase; ANR, anthocyanidin reductase; CCoAOMT, caffeoyl-CoA O-methyl-transferase; CHI, chalcone isomerase; CHS, chalcone synthase; C4H, cinnamate-4-hydroxylase; C3'H, p-coumarate 3'-hydroxylases; DFR, dihydroflavonol reductase; F3'5'H, flavonoid 3'/5'-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; HCT, hydroxycinnamoyl CoA shikimatelquinatate hydroxycinnamoyltransferase; LAR, leucocyanidin reductase; PAL, phenylalanine ammonialyase; 4CL, 4-coumaric acid coenzyme a ligase

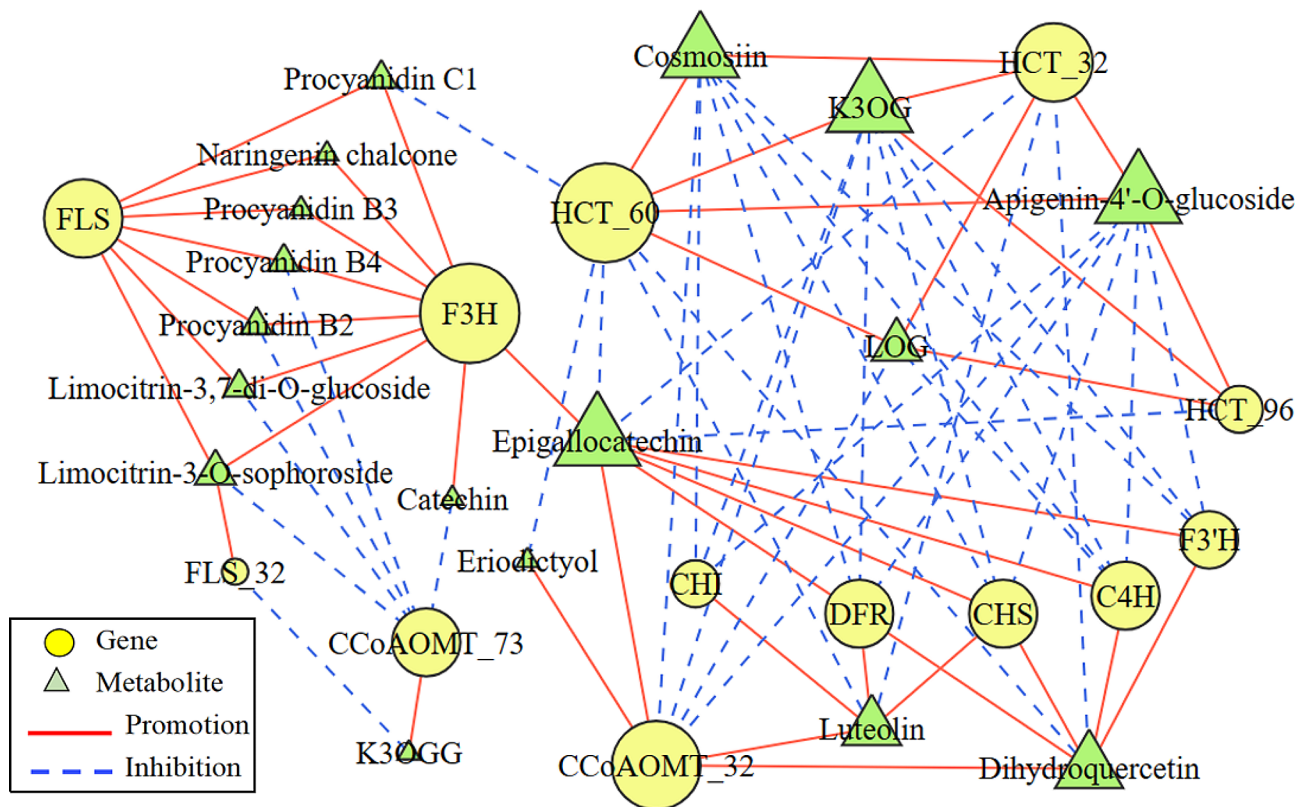


Fig. 8 Connection network between genes and flavonoids. K3OG, Kaempferol-3-O-(6"-O-acetyl)- glucoside; LOG, luteolin-7-O-(6"-malonyl)-glucoside; K3OGG, Kaempferol-3-O-(6"-acetyl)glucosyl- (1→3)-galactoside. The size of the triangle and the circle represents the number of genes or metabolites associated with them, respectively

most of the synthesized caffeoyl-CoA rapidly entered the biosynthesis of downstream metabolites, resulting in a decrease in the amount of eriodictyol.

In this work, transcriptomic and targeted metabolomic analyses provided insights into the flavonoids biosynthesis in the flowers of *L. macranthoides*. However, the specific regulatory mechanisms of flavonoids, including the interesting finding in the study, need a further research.

In short, the synthesis of flavonoids in the flowers of *L. macranthoides* is a dynamic balance of various regulatory networks. The same metabolic process may be regulated by multiple genes and a gene may be involved in the regulation of multiple metabolic processes. The ultimate medicinal value of *L. macranthoides* is determined by the whole gene regulation network, and is ultimately reflected by the types and contents of metabolites.

Conclusions

Flavonoids are one of the bioactive ingredients in the flowers of *L. macranthoides*. In this study, correlation analysis between DEGs and DAFs showed that the down-regulated expressions of the *CHS*, *DFR*, *C4H*, *F3'H*, *CCoAOMT_32* and the up-regulated expressions of the two *HCTs* resulted in up-regulated level of K3OG, cosmosiin and apigenin-4'-O-glucoside. The down-regulated

expressions of *F3H* and *FLS* decreased the contents of 7 metabolites, including naringenin chalcone, proanthocyanidin B2, B3, B4, C1, limocitrin-3,7-di-O-glucoside and limocitrin-3-O-sophoroside. The results showed that the early flower buds mainly contained limonin derivatives, procyanidins, catechin, luteolin and cyanidin derivatives, while the late flowers mainly contained kaempferol derivatives, apigenin derivatives and TMR. These indicated that flavonoids exerting medicinal functions were different in flowers at different developmental stages. The findings are helpful for genetic improvement of varieties in *L. macranthoides*.

Materials and methods

Plant materials

'XiangLei', a local main cultivar of *L. macranthoides* in Hunan province, China, identified by Chen et al. [41], was used as material. The fresh flower buds or opening flowers were sampled respectively at the four developmental stages: green flower bud (GB, green, rod-shaped, 2–3 cm long), white flower bud (WB, white, rod-shaped, 4–5 cm long), white flower (WF, white, fully open flower) and golden flower (GF, golden yellow, fully open flower) (Fig. 9). The samples were immediately frozen in liquid nitrogen and stored at -80°C until further use in



Fig. 9 The four developmental stages of flower in *L. macranthoides*. From left to right, green flower bud (GB), white flower bud (WB), white flower (WF) and golden flower (GF).

metabolite, RNA sequencing and qPCR analysis. Three biological replicates were performed for each sample.

Complementary DNA library construction and RNA sequencing

Total RNA was extracted from flower buds or flowers using a plant RNA extraction kit (HUAYUE YANG, Inc. Beijing, China). The quantity and quality of the RNA samples were verified using Nucleic acid/protein analyzer (Eppendorf, Germany). Twelve libraries were constructed and sequenced using Illumina HiSeq. 4000 platform by GenePioneer Biotech Corporation (Nanjing, China).

Quantitative real-time PCR (qPCR) analysis

To compare the expression data between RNA-seq and qPCR, the relative expression level was transformed to \log_2 (fold change). Screening of DEGs and the qPCR analysis were done as that reported by Lv et al. [2]. The *18 S rRNA* was used as an internal control for normalization of gene expression [42]. The gene-specific primers are listed in supplementary Table S1.

Flavonoids extraction and analysis

The sample preparation, extraction, identification, quantification and analysis of flavonoids were performed at Metware Biotechnology Co., Ltd. (www.metware.cn, Wuhan, China) following its standard procedures and previously fully described by Pan et al. [24] and Xia et al. [43] respectively. The sample solutions were analyzed by an ultra performance liquid chromatography system (UPLC, SHIMADZU Nexera X2) and a tandem mass spectrometry (MS/MS) system (Applied Biosystems 6500 QTRAP) [24]. Variable importance in projection (VIP) ≥ 1 combined with $|\log_2$ (fold change)| ≥ 1 and the False Discovery Rate ≤ 0.05 was used to screen DAFs.

Correlation analysis between metabolome and transcriptome

Correlation analysis was performed using quantitative values of transcripts and metabolites in all samples by calculating Pearson's correlation coefficients (PCC). The coefficients were calculated from \log_2 (fold change) of each transcript and metabolite with the Excel 2023. Correlations with $|PCC| > 0.8$ and p value < 0.05 were selected.

Statistical analysis

All determinations were conducted in triplicate, and results are presented as mean \pm standard deviation.

Abbreviations

DAF	Differentially accumulated flavonoid
DEG	Differentially expressed gene
GO	Gene ontology
LOG	Luteolin-7-O-(6"-malonyl)-glucoside
K3OG	Kaempferol-3-O-(6"-O-acetyl)-glucoside
K3OGG	Kaempferol-3-O-(6"-acetyl) glucosyl-(1 \rightarrow 3)-galactoside
GB	Green flower bud
WB	White flower bud
WF	White flower
GF	Golden flower
VIP	Variable importance in projection
PCC	Pearson's correlation coefficients

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12896-024-00846-5>.

Supplementary Material 1

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Not applicable.

Author contributions

Ling Ling Lv and Long Qian Xiao conceived and designed the research. Ling Ling Lv, Long Qian Xiao acquired the data. Ling Ling Lv and Li Yun Li analyzed

the data. Ling Ling Lv and Jian Hui Pi drafted and revised the manuscript. All authors reviewed the manuscript.

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Data availability

The datasets supporting the conclusions of this article has been deposited in the China National GeneBank DataBase (CNGbDb, <https://db.cngb.org/>) with accession code CNP0003782 or is included within the additional files.

Declarations

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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