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Integrated transcriptome and targeted metabolome analyses provide insights into flavonoid biosynthesis in kiwifruit (*Actinidia chinensis*)

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ABSTRACT: So far, a variety of metabolite components of kiwifruit have been elucidated. However, the identification and analysis of flavonoids in different tissue of kiwifruit are rarely carried out. In this study, we performed transcriptome and metabolome analyses of roots (Gkf_R), stems (Gkf_T), leaves (Gkf_L) and fruits (Gkf_F) to provide insights into the differential accumulation and regulation mechanisms of flavonoids in kiwifruit. Results showed that total of 301 flavonoids were identified, and in four tissues with the different accumulation trends, and a large proportion of flavonoids with high accumulation in Gkf_L and Gkf_R. A total of 84 genes have been identified involved in the flavonoid biosynthesis pathway, and the expression level of five *LAR*, two *DFR*, and one *HCT* were significantly correlated with the accumulation of 16 flavonoids and co-localized in the flavonoid biosynthesis pathway. In addition, a total of 2,362 transcription factors were identified, mainly included MYB, bHLH, ERF, bZIP and WRKY, among which the expression level of *bHLH74*, *RAP2.3L/4L/10L*, *MYB1R1* and *WRKY33* were significantly correlated with 25, 56, 43 and 24 kinds of flavonoids. Our research will enrich the metabolomic data, provide useful information for the directed genetic improvement and application in the pharmaceutical industry of kiwifruit.

Keywords Kiwifruit, Flavonoids, Biosynthesis, Transcriptome, Metabolome

Introduction

Kiwifruit is native to China and has recorded since the Spring and Autumn Period, and rich in vitamins, dietary fiber, lipids, amino acids, flavonoids, minerals, and other

nutrient components that are considered beneficial to human health^[1-4]. A total of 54 species and 21 varieties in the genus of *Actinidia* have been recognized worldwide, in which 52 species are native to China. According to the *Compendium of Materia Medica* and *Herbal Supplements*, the roots, stems, leaves, and fruits of kiwifruit can be used as medicine. Modern medical research also shows that kiwifruit contains a variety of physiological active ingredients, with anti-tumor, anti-mutation, anti-viral, anti-inflammatory, anti-lipid peroxidation, improving immunity, and other pharmacological effects^[5-8]. For example, kiwifruit juice can effectively block the synthesis of carcinogens nitrosamine, N-nitrosamide, N-nitromorpholine, and N-nitrosoproline^[9-10]. The root extracts of kiwifruit has a good effect on scavenging free radicals, inhibiting lipid peroxidation, maintaining the normal structure of plasma membrane, and avoiding cell damage^[11]. Through metabolomics analysis study found that the most important bio-active metabolites in kiwifruit were flavonoids^[12-14]. Since the development of kiwifruit globalization in the 1960s, so far the world's kiwifruit cultivation area of more than five million mu, production of more than four million tons. But all of them are for the purpose of producing kiwifruit fresh fruit, and the production and research work for the medicinal development are rarely carried out.

Flavonoids are by far the largest group of polyphenols, with an estimated number of over 8000 flavonoid metabolites. They can be divided into the following categories: flavones, biflavones, flavanones, flavanonols, chalcones, isoflavones, flavonols, flavanols, flavonoid carbonoside, anthocyanidins, and proanthocyanidins. Among them, a variety of flavonoids have anti-oxidant, anti-inflammatory, anti-tumor, liver protection, lipid-lowering, cholesterol-lowering, reducing blood lipid, protect the nervous system, prevent cardiovascular diseases and other effects^[15-21]. As the main secondary metabolites of plants, flavonoids also play a crucial role in the growth and development of plants. Such as, affect the synthesis of cell wall^[22], participates in root growth^[23], pollen development^[24], color formation^[25], and improves plant resistance^[26-30].

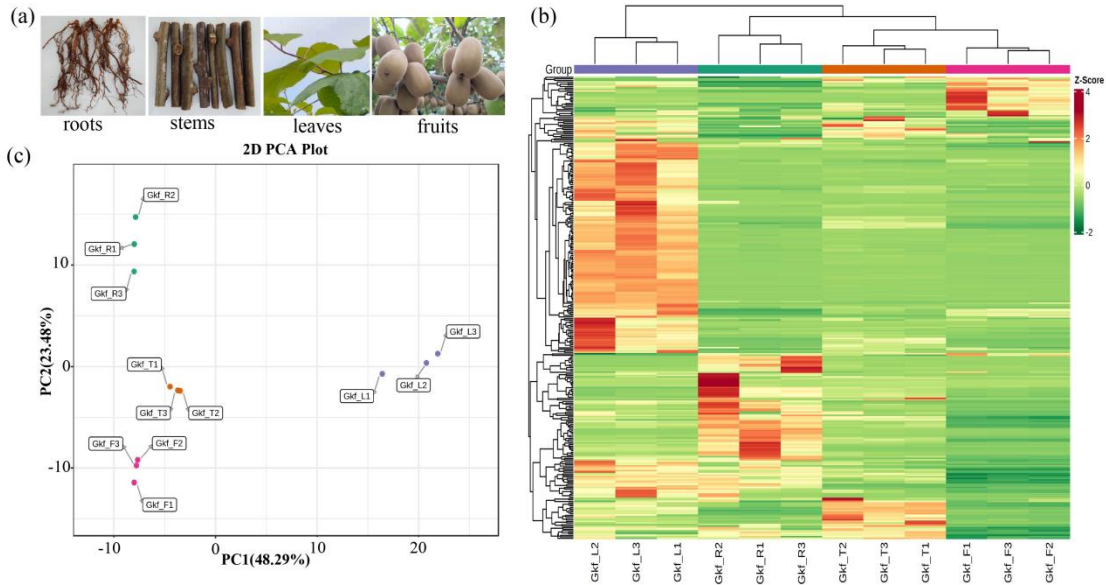
The pathway involved in flavonoid metabolites include flavonoid biosynthesis pathway (map00941), anthocyanin biosynthesis pathway (map00942), isoflavone biosynthesis pathway (map00943), and flavone and flavonol biosynthesis pathway (map00944). Among them, map00941 synthesized upstream substances of the other three pathways, included apigenin and kaempferol in the map00944 pathway, liquiritigenin and naringenin in map00943 pathway, and pelargonidin, cyanidin and delphinidin in map00942 pathway, respectively. The biosynthesis of flavonoids starts with phenylalanine, and catalyzed by phenylpropane-related enzymes phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL) to form coumaroyl-CoA. Subsequently, coumaroyl-CoA is catalyzed by the series enzymes chalcone synthase (CHS), chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid-3'-hydroxylase (F3'H), and flavonoid-3',5'-hydroxylase (F3'5'H), leucoanthocyanidin reductase (LAR), dihydroflavonol 4-reductase (DFR). UDP-glucose flavonol 3-O-glucosyltransferase (UFGT), anthocyanidin synthase (ANS) and anthocyanidin reductase (ANR) to form anthocyanins and the corresponding series products chalcone, flavanone, dihydroflavonol, flavonols, leucocyanidin, anthocyanidins, etc^[31-34]. In addition to the above key enzyme genes, a variety of key regulatory factors, such as bHLH, MYB, WRKY, bZIP, ERF have been reported to involved in flavonoid biosynthesis ^[35-38].

With the development of metabolomics, more and more endogenous small molecule metabolites can be identified and quantified. So far, a variety of metabolite components of kiwifruit have been elucidated. However, the identification and analysis of flavonoid compounds in different tissue of kiwifruit are rarely carried out. In order to elucidate the composition and content differences of flavonoids in different tissue of kiwifruit, and the potential regulatory mechanism of biosynthetic pathway. The transcriptome and metabolome analyses were used to investigate the gene expression and flavonoids detection in this study. Our research will enrich the metabolomic data, provide useful information for the directed genetic improvement, and application in the pharmaceutical industry of kiwifruit.

87 **Results**

88 **Metabolomic profiling**

89 The UPLC-ESI-MS/MS technology platform was used for qualitative and relative
90 content analysis of flavonoids in *Actinidia chinensis* cv Jinyan. A total of 301
91 flavonoids were identified, mainly include flavonols and flavones (Table 1,
92 Supporting Information File 1). Among them, 218, 274, 286, and 231 flavonoids were
93 identified in Gkf_R, Gkf_T, Gkf_L and Gkf_F, respectively. A total of 184 flavonoids
94 were detected in all four tissues (Supporting Information File 1). In addition, the
95 1,7-di-o-galloyl-d-sedoheptulose (cmsn001563) specifically accumulated in Gkf_R,
96 biochanin A-7-o-glucoside (pme3400) and acacetin-7-o-galactoside (pmp000573)
97 specifically accumulated in Gkf_T, delphinidin-3-o-sophoroside-5-o-glucoside
98 (lmqp001553), baicalein (mws0041), isosinensetin (pmp001076) specifically
99 accumulated in Gkf_F, and 14 kinds of flavonoids specifically accumulated in Gkf_L
100 (Supporting Information File 2).



101
102 **Fig. 1.** The sampling photos of roots, stems, leaves and fruits tissues (a), Principal
103 component analysis (PCA) (b), and hierarchical clustering heatmap analysis of
104 metabolites identified (c) in *Actinidia chinensis* cv Jinyan.

105
106 The PCA was used to elucidate the overall flavonoids differences between
107 groups as well as the variability between samples within groups. The results showed

that PC1 and PC2 could explain 48.29% and 23.48% of the total variance of the samples, indicating that there were obvious flavonoids differences in different tissue (Fig. 1c). The heatmap of hierarchical clustering analysis was used to classify the accumulation and variation model of metabolites in Gkf_R, Gkf_T, Gkf_L, and Gkf_F. The results suggested that the flavonoids in four tissue with the different accumulation trends, and a large proportion of flavonoids with high accumulation in Gkf_L (Fig. 1b). Therefore, we performed further analysis of flavonoids that differentially accumulated in different tissues.

Table 1 Categories list of the 301 flavonoids identified in *Actinidia chinensis* cv Jinyan

Category	Number	Category	Number
Anthocyanidins	16	Flavanonols	13
Biflavones	2	Flavonols	93
Chalcones	16	Flavonoid carbonoside	15
Dihydroisoflavones	1	Isoflavones	12
Flavanols	20	Proanthocyanidins	9
Flavanones	21	Tannin	22
Flavones	61		

Differentially accumulated flavonoids analysis

The univariate and multivariate statistical analysis were used to screen DAFs for each comparison group. The OPLS-DA and 200-response sorting test results showed that the model was stable and reliable (Fig. 2, Fig. S1), and the VIP analysis in the OPLS-DA model could be used to screen DAFs. A total of 279 DAFs were screened from the six comparison groups under the set threshold, and the DAFs in each comparison group are presented in Table 2. The comparison group of Gkf_L_vs_Gkf_F had the largest number of DAFs, with more than 200 flavonoids being more abundant in Gkf_L than in Gkf_F, and only 17 flavonoids being more abundant in Gkf_F. Followed by Gkf_R_vs_Gkf_L comparison group, 157 flavonoids up-regulated in Gkf_L, and 47 flavonoids down-regulated in Gkf_R. The smallest

difference in flavonoids content was found between Gkf_R and Gkf_T tissues, only 162 DAFs were identified in Gkf_R_vs_Gkf_T comparison group. In addition, a total of 29 differentially accumulated flavonoids were present in all six comparison groups (Supporting Information File 3), meaning that the contents of these 29 flavonoids were significantly different in all four tissues.

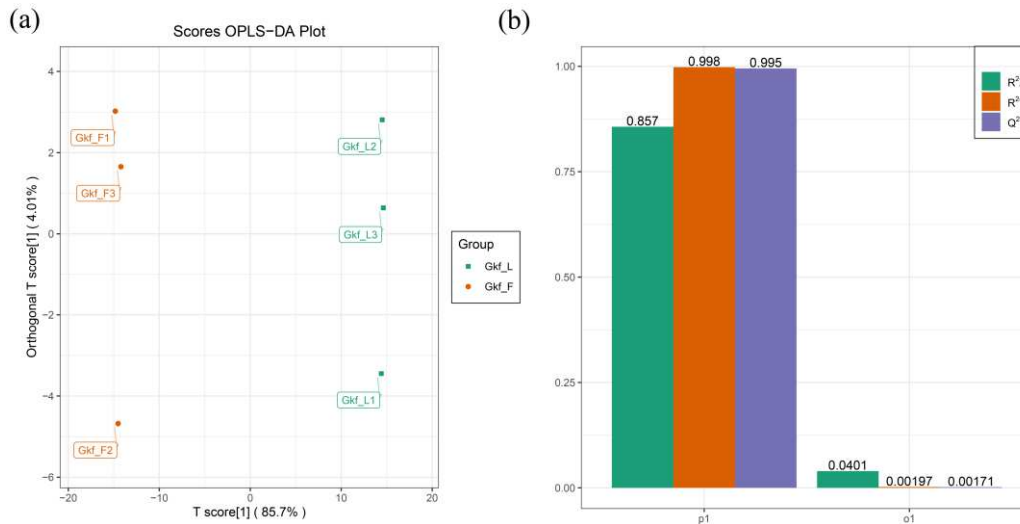


Fig. 2. Analysis of differentially accumulated flavonoids (DAFs) identified in the Gkf_L_vs_Gkf_F comparison group. Orthogonal partial least squares-discriminant analysis (OPLS-DA) (a) and 200-response sorting tests (b). Q^2 is an important parameter for evaluating the OPLS-DA model, and the R^2X and R^2Y represent the percentage of OPLS-DA model that can explain X and Y matrix information, respectively.

K-means analysis showed that the 279 DAFs could be divided into six accumulation patterns (Fig. 3). Compared with Gkf_R, Gkf_T, and Gkf_F, a total of 121 flavonoids were highly accumulated in Gkf_L, mainly include 65 flavonols, 21 flavones, eight flavanonols, seven flavanones, and six anthocyanidin. Compared with Gkf_T, Gkf_L, and Gkf_F, a total of 63 flavonoids were highly accumulated in Gkf_R, mainly include 14 flavones, 10 flavonols, seven flavanols, six flavanones, six tannin, and five proanthocyanidins (Supporting Information File 4). In addition, a total of 22 and 30 flavonoids were significantly high accumulation in Gkf_F and Gkf_T, respectively.

Table 2 Summary of the differentially expressed genes (DEGs) and differentially accumulated flavonoids (DAFs) among six library pairs

Comparison groups	Total	Down_meta	Up_meta	Total	Down_gene	Up_gene
Gkf_L_vs_Gkf_F	222	205	17	9550	4001	5549
Gkf_R_vs_Gkf_F	169	108	61	11163	5207	5956
Gkf_R_vs_Gkf_L	204	47	157	10568	6250	4318
Gkf_R_vs_Gkf_T	162	54	108	6351	4196	2155
Gkf_T_vs_Gkf_F	198	175	23	9307	2031	5276
Gkf_T_vs_Gkf_L	185	31	154	5371	2487	2884

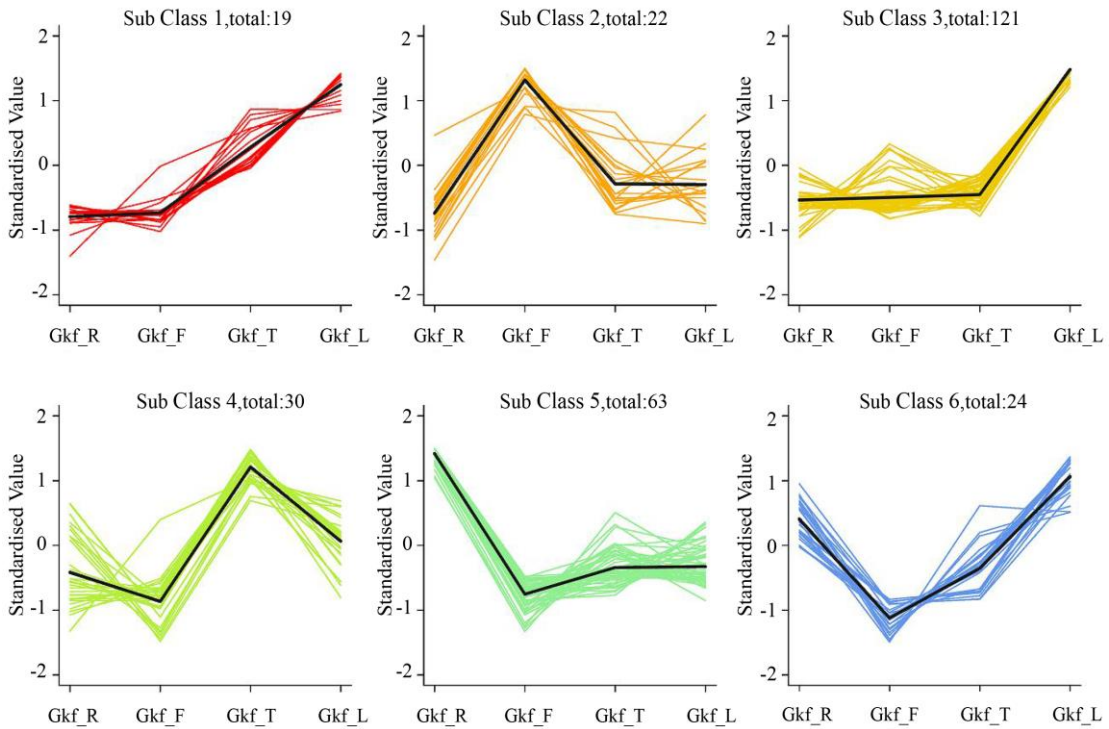


Fig. 3. K-means analysis of 279 differentially accumulated flavonoids (DAFs)

The hierarchical clustering heatmap analysis showed that in Gkf_L_vs_Gkf_F comparison group, 38 DAFs with KEGG pathway functional annotation information were all highly accumulated in Gkf_L than Gkf_F (Fig. 4). In Gkf_R_vs_Gkf_F comparison group, a total of 29 DAFs obtained KEGG pathway functional annotation, among them, eight DAFs were highly accumulated in Gkf_F, and 21 DAFs were

highly accumulated in Gkf_R (Fig. S2). In Gkf_R_vs_Gkf_T comparison group, 25 DAFs obtained KEGG pathway functional annotation, and six DAFs up-regulated in Gkf_R, 19 DAFs up-regulated in Gkf_T (Fig. S3). In Gkf_T_vs_Gkf_F comparison group, 39 DAFs obtained KEGG pathway functional annotation, and three DAFs up-regulated in Gkf_T, 36 DAFs up-regulated in Gkf_F (Fig. S4). In Gkf_T_vs_Gkf_L and Gkf_R_vs_Gkf_L comparison group, most DAFs with KEGG pathway functional annotations were highly accumulated in Gkf_L (Fig. S5, S6). These results indicate that in *Actinidia chinensis* cv Jinyan, the most abundant flavonoid components and contents are found in Gkf_L, followed by Gkf_R, Gkf_F, and Gkf_T.

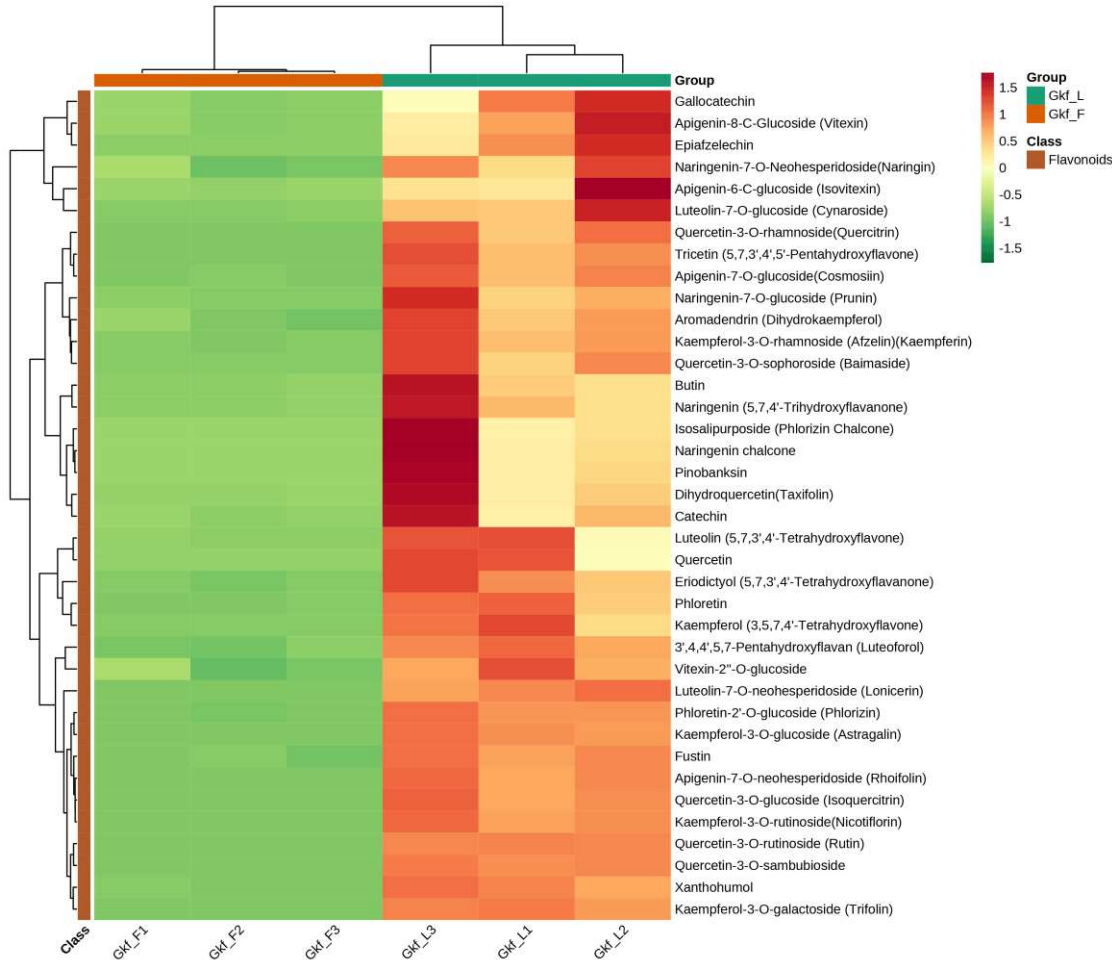


Fig. 4. The hierarchical clustering heatmap analysis of 38 differentially accumulated flavonoids (DAFs) obtained KEGG pathway functional annotation.

Transcriptomic profiling

A total of 12 cDNA libraries for Gkf_R, Gkf_T, Gkf_L, and Gkf_F of *Actinidia chinensis* cv Jinyan were constructed and sequenced to obtain gene expression signatures at the transcription level. The average Q30 and GC content of the 12 libraries were 94.52% and 45.03%, respectively. A total of 28,783,394 clean reads were obtained and assembled into 50,439 unigenes, and 45,478 (90.16%) unigenes were annotated (Supporting Information File 5). Among them, 45,284 (99.57%), 45,120 (99.21%), 21,267 (46.76%), 27,639 (60.77%), and 35,980 (79.12%) unigenes were annotated in the Nr, UniProt, KEGG, KOG, and GO databases, respectively. Among them, a total of 84 genes have been identified involved in the flavonoids biosynthesis pathway, mainly include *LAR*, *DFR*, *ANS*, *CHI*, *HCT*, *FLS*, *CHS*, *F3H* and *CYP81E/75B1/73A* (Supporting Information File 6). In addition, a total of 2,362 transcription factors genes were identified, including 231 *MYB*, 188 *bHLH*, 133 *ERF*, 107 *bZIP* and 112 *WRKY* (Supporting Information File 7).

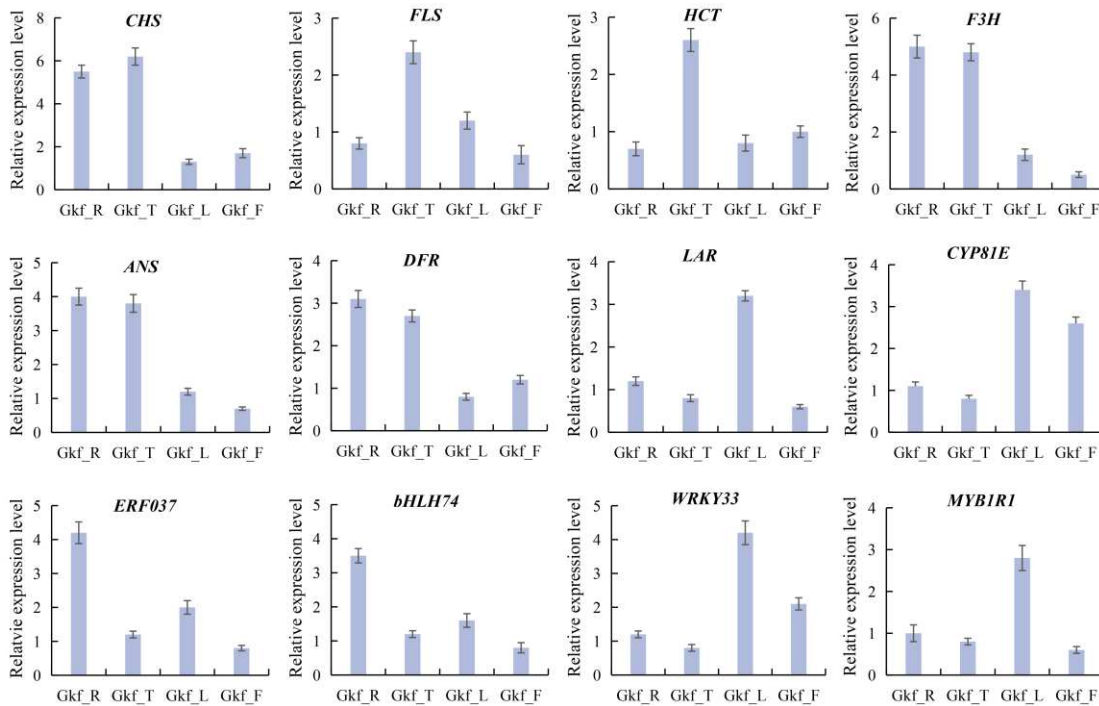


Fig. 5. The relative expression level in roots, stems, leaves and fruits of *Actinidia chinensis* cv Jinyan of 12 genes obtained by quantitative real-time PCR (qRT-PCR) analysis. The error bars represent the SD from three replicates.

Differentially expressed genes analysis

Differential expression analysis showed that a total of 20,542 DEGs were identified in six comparison groups, with the threshold values. A large proportion of DEGs were obtained from Gkf_R_vs_Gkf_L and Gkf_R_vs_Gkf_F comparison groups, and only 6,351 DEGs and 5,371 DEGs were identified in Gkf_R_vs_Gkf_T and Gkf_T_vs_Gkf_L comparison groups, respectively (Table 2). The flavonoid biosynthesis pathway genes *FLS*, *HCT* and *CYP73A* were highly expressed in Gkf_T, the *F3H*, *ANS*, *DFR* and *CHS* were highly expressed in Gkf_R and Gkf_T, the *LAR* were highly expressed in Gkf_L, and the *CYP81E* were highly expressed in Gkf_F and Gkf_L (Supporting Information File 6). In addition, a total of 97 transcription factors previously reported to be involved in the regulation of flavonoid biosynthesis were significantly differentially expressed in four tissues of *Actinidia chinensis* cv Jinyan. Among them, 11 *MYB*, six *bHLH*, two *ERF* and one *WRKY* genes were significantly highly expressed in Gkf_F, and 12 *WRKY*, 10 *ERF*, nine *MYB* and one *bHLH* genes were significantly highly expressed in Gkf_L, and 21 *ERF*, eight *MYB*, seven *bZIP*, six *bHLH* and three *WRKY* genes were significantly highly expressed in Gkf_R (Supporting Information File 8). To further verify the reliability of candidate gene expression levels and transcriptome data, 12 genes involved in flavonoid biosynthesis were randomly selected for qRT-PCR analysis. The results showed that the expression characteristics of 12 genes in four tissues of *Actinidia chinensis* cv Jinyan were consistent with the results of transcriptome sequencing (Fig. 5).

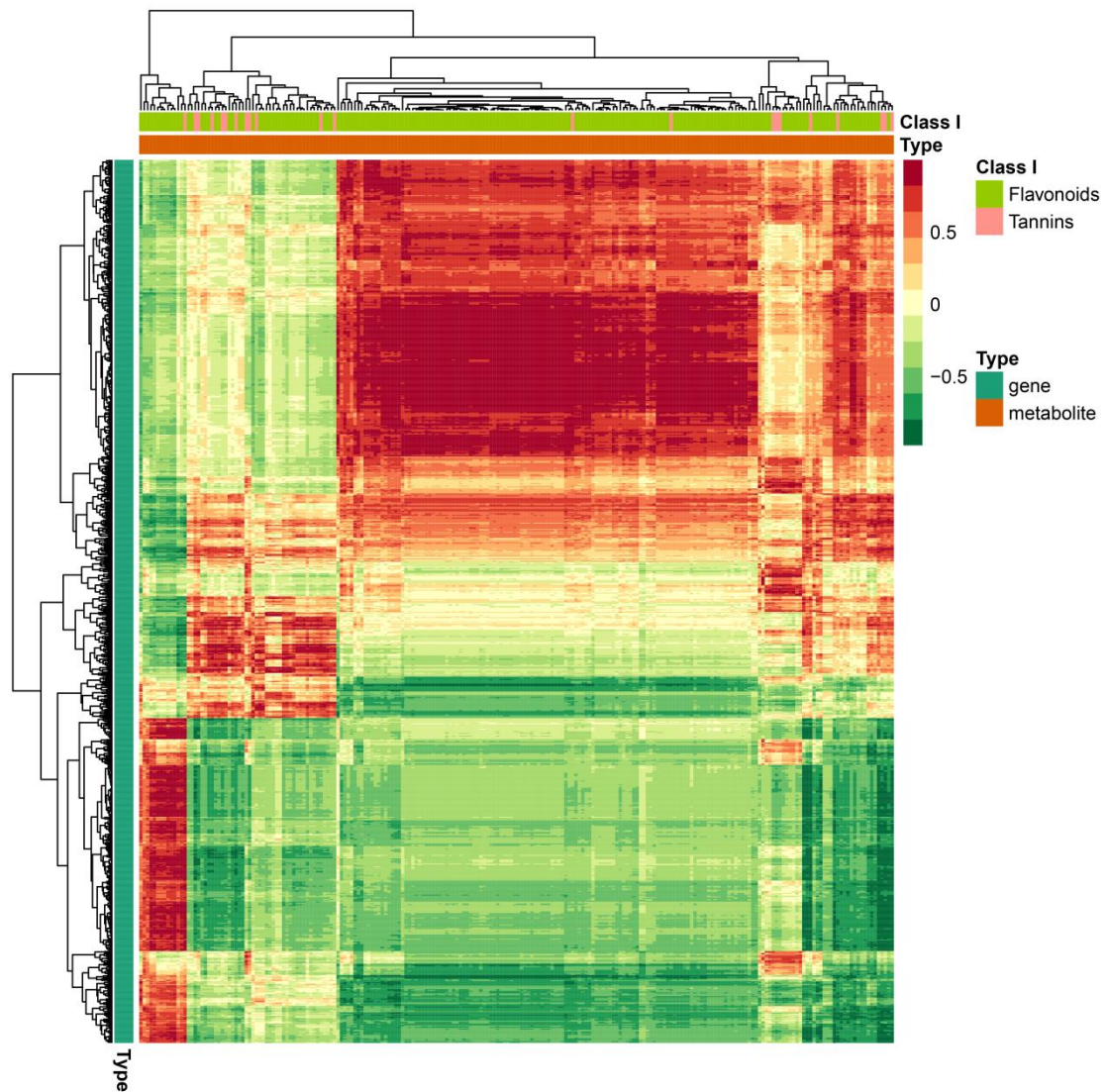


Fig. 6. The hierarchical clustering heatmap analysis of correlation between differentially expressed genes (DEGs) and differentially accumulated flavonoids (DAFs), each row represents a DEG, each column represents a DAF, red indicates a positive correlation between DEGs and DAFs, green represents a negative correlation.

To further clarify the intrinsic mechanisms underlying the differential accumulation of flavonoids in different tissues of kiwifruit. We performed an integrated analysis of transcriptome and metabolome data. The results showed that a total of 18,510 DEGs were significantly correlated with 279 DAFs (Fig. 6, Supporting Information File 9), and the number of positively correlated DEGs was significantly higher than that of negatively correlated DEGs (Fig. 7a, Fig. S7). The CCA analysis also demonstrated a high correlation between transcriptome and metabolome data (Fig.

7b, Fig. S8). However, KGML analysis showed that the vast majority of DEGs were located in glyoxylate and dicarboxylate metabolism pathway (Ko00630), purine metabolism pathway (Ko00230), Glycolysis/ gluconeogenesis biosynthesis pathway (Ko00010), glycine, serine and threonine metabolism pathway (Ko00260), pentose phosphate pathway (Ko0030) (Fig. 7c, Fig. S9). and only nine DEGs (five *LAR*, two *DFR*, two *HCT*) and 16 DAFs were co-located in the flavonoid biosynthesis pathway (Ko00941). Among them, the expression of *HCT* genes was significantly positively correlated with the accumulation level of epiafzelechin (mws1422), galocatechin (mws0049), and kaempferol (mws1068), the expression of *DFR* genes was significantly negatively correlated with the accumulation level of fustin (mws1000), and the expression of *LAR* genes was significantly positively correlated with the accumulation level of the other 15 DAFs except for mws1000 (Supporting Information File 10, Fig. 7d).

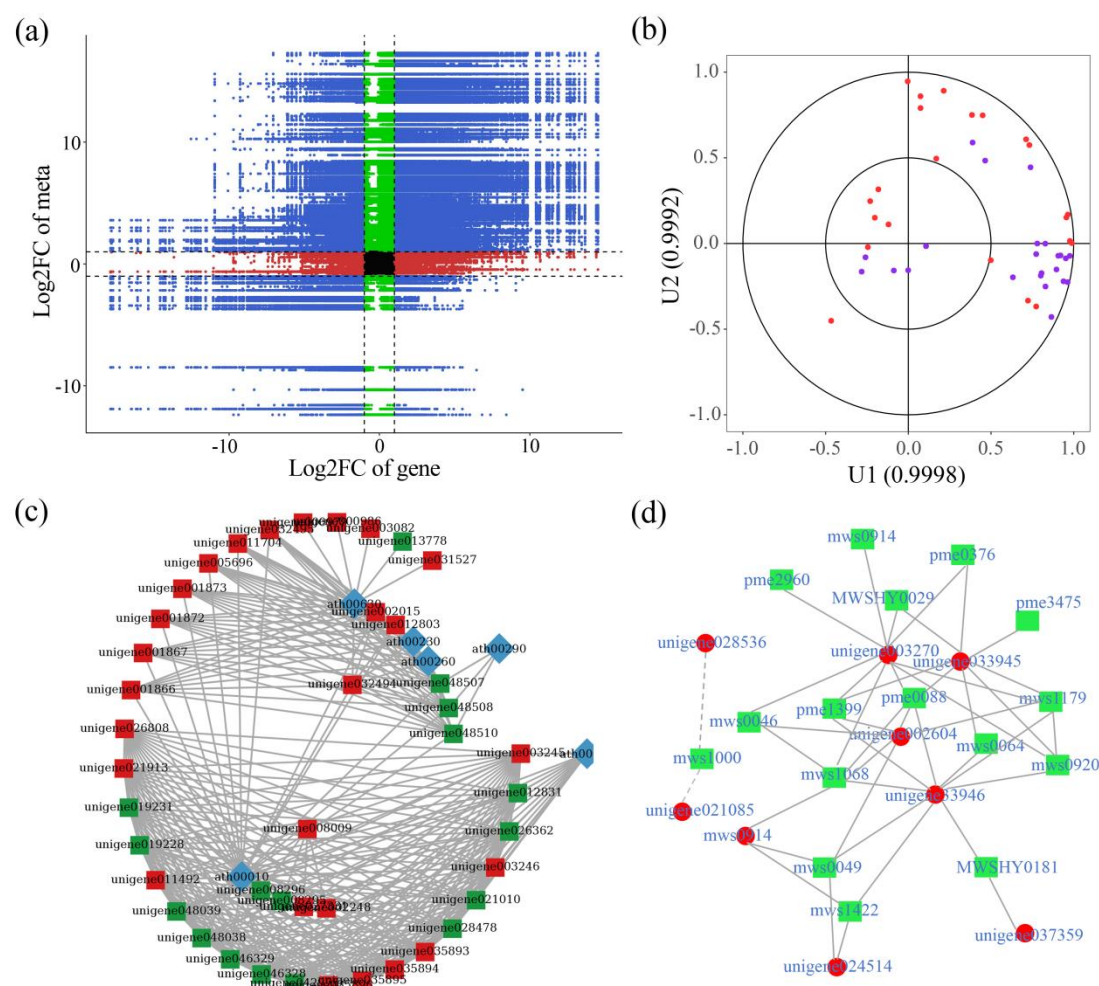


Fig. 7. Integrated analysis of transcriptome and metabolome data. (a) The ninequadrants analysis of differentially expressed genes (DEGs) and differentially accumulated flavonoids (DAFs) in Gkf_L_vs_Gkf_F comparison group. (b) The canonical correlation analysis of DEGs and DAFs in Gkf_L_vs_Gkf_F, purple and red dots represent DEGs and DAFs, respectively, and within the same region, further away from the origin indicates stronger correlation. (c) The KGML analysis of DEGs and DAFs in Gkf_L_vs_Gkf_F, squares represent DEGs, rhombus represent DAFs, red indicates the DEGs or DAFs up-regulation, and green indicates down-regulation. (d) The correlation network analysis, red circles represent DEGs, squares represent DAFs, solid lines indicates significant positive correlations, and dashed lines represent significant negative correlations.

In addition, the specific high expression of *bHLH74* gene in fruits were significantly correlated with the accumulation levels of 25 kinds of flavonoids, the specific high expression of ERF transcription factor genes *RAP2.3L/4L/10L* in roots were significantly correlated with the accumulation levels of 56 kinds of flavonoids, the specific high expression of *MYB1R1* gene in leaves were significantly correlated with the accumulation levels of 43 kinds of flavonoids, the specific high expression of *WRKY33* gene in leaves and stems were significantly correlated with the accumulation levels of 24 flavonoids (Supporting Information File 11). Further correlation analysis showed that the expression levels of *bHLH74* and *DRF*, *MYB1R1* and *HCT*, *WRKY33* and *LAR* were significantly positively correlated, respectively.

Discussion

Flavonoids not only play an important role in the growth, development, flowering, fruiting, antibacterial and disease prevention of plants, but also have important medicinal value^[39]. *Actinidia* species is rich in bioactive flavonoids, but most of the related studies have focused on *Actinidia arguta* ^[14,40]. For the flavonoids composition, tissue distribution characteristics, and potential mechanism regulation of flavonoid biosynthesis in *Actinidia chinensis* remains unclear. In this study, we conducted a

metabolomic analysis on the Gkf_R, Gkf_T, Gkf_L and Gkf_F of *Actinidia chinensis* cv Jinyan. The results showed that a total of 301 flavonoids were identified, the number of flavonoids identified was much higher than that reported in *Actinidia arguta* (118 flavonoids) [41]. This may be due to the fact that our test samples simultaneously contained roots, stems, leaves and fruits. Another reason may be due to the differences in kiwifruit species. The results of differential analysis showed that the composition and content of flavonoids were also significantly different in different tissues of *Actinidia chinensis* cv Jinyan. 279 of the 301 flavonoid compounds differentially accumulated in two or more tissues. A total of 121 and 63 flavonoid compounds were significantly high accumulation in Gkf_L and Gkf_R, respectively. This may be related to the fact that roots and leaves are the most important and direct plant tissues in response to external environmental changes. It also shows that the root and leaves of *Actinidia chinensis* cv Jinyan has potential value of pharmaceutical development and utilization.

Flavonoid biosynthesis in plants are mainly regulated by two types of genes [42-43]. The first is structural genes encoding enzymes that can catalyze the biosynthesis of different flavonoids. The second is regulatory genes that regulate transcription of structural genes [43-44]. In this study, we performed transcriptome sequencing analysis to determine 84 genes encoding enzymes known to be involved in flavonoid biosynthesis: *LAR*, *DFR*, *ANS*, *CHI*, *FLS*, *CHS*, *F3H*, *CYP81E*, *CYP75B1* and *CYP73A*. However, *C4H*, *4CL*, *ANR*, *UFGT* and *UGT* were not present in the flavonoids biosynthesis pathways of the involved *Actinidia chinensis* cv Jinyan, probably due to interspecies differences [39]. In many plants, naringin, naringenin, saonol, kaempferol, myricetin, dihydromyricetin, dihydroquercetin, pelargonidin are catalyzed by positively regulating the expression of *CHS*, *CHI*, *F3H*, *DFR* and *FLS* [6, 45-50]. This is basically consistent with the results of the present study.

Previous studies have shown that MYB, bHLH, WD40 and their complexes (MBW) are the major regulatory genes regulating flavonoid biosynthesis [51-53]. In the present study, the largest number of transcription factors identified was MYB followed by bHLH. Notably, no WD40 TFs were identified in the *Actinidia chinensis*

cv Jinyan, suggesting that MYB and bHLH are the main TF families regulating flavonoid biosynthesis in *Actinidia chinensis* cv Jinyan^[39]. This is consistent with a previous report by^[54], MYB and bHLH interact and thus exert functions on structural genes (*AcMYBF110-AcbHLH-AcWDR*). It has also been reported in apricot fruits that *MYB5*, *bHLH* and *bHLH* were positively correlated with *ANS7* and *CYP75B11*, key structural genes for flavonoid biosynthesis^[39]. In this study, it was also found that the expression of transcription factor genes *bHLH74*, *MYB1R1*, *WRKY33*, *RAP2.3L/4L/10L* were significantly correlated with the accumulation of many flavonoids and the expressi of flavonoid biosynthesis pathway genes *DFR*, *LAR* and *HCT*. We speculate that these candidate transcription factors are involved in regulating flavonoid biosynthesis. In conclusion, in this study, the flavonoid components present in *Actinidia chinensis* cv Jinyan were identified by metabolomic analysis, and clarified the distribution characteristics of each flavonoid component in Gkf_R, Gkf_T, Gkf_L and fruit. The flavonoids related genes were identified by transcriptomic analysis, and elucidated the expression characteristics in Gkf_R, Gkf_T, Gkf_L and Gkf_F of *Actinidia chinensis* cv Jinyan. Our results provide new insights into the development and utilization and the regulation of flavonoid of *Actinidia chinensis* cv Jinyan.

Materials and methods

Plant materials

Actinidia chinensis cv Jinyan tissues samples were obtained from Fengxin County Doctor Kiwifruit Base (E114°45, N28°34'), Yichun City, Jiangxi Province, China. A total of 15 Five-year-old kiwifruit plants were selected, and lateral roots (Gkf_R) at a depth of 15 to 20 cm underground, fully lignified stems (Gkf_T), and fully mature leaves (Gkf_L) and fruits (Gkf_F) were collected at the physiological maturity stage (the end of September) in 2022 (Fig. 1a), identical tissue parts from each of five kiwifruit plants were mixed together for a total of three biological replicates, and then immediately frozen in liquid nitrogen for metabolites and total RNA extraction, respectively.

Total RNA extraction and transcriptome sequencing analysis

Total RNA from each sample was extracted using a Plant RNA Purification Reagent (Invitrogen) according to the manufacturer's instructions. The cDNA libraries were carried out according to the protocol reported by Li et al^[55]. Libraries were sequenced using the Illumina Novaseq 6000 platform at the Science Corporation of Gene (Guangzhou, China). The RNA sequence data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the study accession number PRJNA1071734 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1071734>). After filtering out low-quality sequencing data, the obtained clean reads were aligned and assembled using the HISAT2 software^[56] and Trinity method^[57], respectively. Based on sequence similarity, the assembled genes were against with Non-redundant (Nr), Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-Prot, Eukaryotic Orthologous Groups (KOG), and Gene Ontology (GO) public databases with an E-value $< 10^{-5}$ to obtain functional annotation information.

Differential expression and quantitative real-time PCR analyses

The expression levels of the genes were normalized and calculated as the value of fragments per kilobase of transcripts per million mapped fragments (FPKM). Differentially expressed genes (DEGs) that satisfied the parameters with $|\log_2FC| \geq 1$ and adjusted p-value ≤ 0.05 screening was carried out using the R package DESeq. A total of 12 candidate genes were selected to investigate the expression profiles by Quantitative Real-Time PCR (qRT-PCR) analyses. Reverse transcription was performed using a PrimeScript TMRT reagent kit with a gDNA Eraser (Code No: RR047A, TaKara, Dalian, China) following the instructions of the manufacturer. The primers sequence details for the 12 candidate gene and internal control gene *AcActin* were shown in Table S1. The qRT-PCR analyses were performed using the LightCycler 480 System (Roche, Basel, Switzerland) and a TB Green Premix Ex Taq II kit (Code No: RR820A, TaKara). Three biological replicates of each tissue of kiwifruit were assessed, and each qRT-PCR reaction included three technical

replicates. The relative expression levels of the candidate genes were calculated using the $2^{-\Delta\Delta C_t}$ method^[58].

Metabolite extraction and widely targeted metabolome analysis

The samples were freeze-dried and crushed using a mixer mill (MM400, Retch) with zirconia beads for 1.5 min at 30 Hz. The powder (100 mg) was weighed and submerged in 1.2 ml of 70% aqueous methanol, vortex 30 s every 30 min for 6 times in total, place the sample in a refrigerator at 4°C overnight. Following centrifugation at 12,000 rpm for 10 min, the supernatant was absorbed and filtrated (0.22-μm pore size) for ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analyses by Wuhan Metware Biotechnology Co., Ltd., China.

The sample extracts were analyzed based on the UPLC-ESI-MS/MS (electrospray ionization, ESI) system. The analytical conditions were conducted as previously described, UPLC column, Agilent SB-C18 (1.8μm, 2.1 × 100 mm). The mobile phase consisted of solvent A, which was pure water with 0.1% formic acid, and solvent B was acetonitrile with 0.1% formic acid. The gradient program was 95% A and 5% B at 0 min, a linear gradient to 5% A and 95% B within 9 min, and 5% A and 95% B for 1 min, and 95% A and 5% B adjusted within 0.1 min and maintained for 2.9 min. The flow rate was 0.35 mL/min with a column temperature of 40°C, and an injection volume of 4 μl. The MS/MS conditions used an electrospray ionization temperature of 550°C, ion spray voltages of 5,500 V (positive ion mode) and -4,500 V (negative ion mode), ion source gas I (50 psi), gas II (60 psi), and curtain gas (25 psi), and the collision-activated dissociation was set to high. Each ion pair was scanned based on the optimized collision energy and declustering potential in the triple quadrupole (QQQ).

Qualitative and quantitative and differentially accumulated flavonoids analysis

Qualitative and quantitative analyses of the metabolites were performed using secondary spectral information based on the public metabolite database and the self-built MWBD database (Wuhan Metware Biotechnology Co., Ltd., China). The characteristic ions of each substance were screened out by the multiple reaction

monitoring of QQQ^[59], and the signal strengths of the characteristic ions were obtained in the detector. The Analyst 1.6.3 software were used to process the mass spectrometry data. The MultiaQuant software were used to calculate the relative content of the corresponding substances in the peak area of each chromatographic peak. The multivariate analysis of variable importance in projection (VIP) in the orthogonal partial least squares-discriminant analysis (OPLS-DA) model was used to initially screen differentially accumulated flavonoids (DAFs). The DAFs were identified based on a $VIP \geq 1$ and $|\log_2FC| \geq 1$. A permutation test with 200 permutations was performed to avoid overfitting.

Statistical analysis

The multivariate principal component analysis (PCA) and OPLS-DA were conducted using the base package and “MetaboAnalystR” in R. The K-means analysis and hierarchical cluster analysis were carried out by R package ComplexHeatmap. Pearson correlation coefficient (PCC) analysis of transcriptome and metabolome data were performed in the R. The network of genes and metabolites with $PCC > 0.8$ was visualized with the software Cytoscape^[60]. A canonical correlation analysis (CCA) was performed for genes and metabolites in the correlation network using the CCA package in the R language. The nine-quadrant graph was used to show the DEGs and DAFs in each group with a $PCC > 0.8$ among different groups using “ggplot2” and “getopt” in R. The KGML analysis was based on the KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>).

Data availability

The RNA sequence data were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under the study accession number PRJNA1071734 (<https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA1071734>). All data generated or analyzed in this work are included in this published article and its supporting information files. Any other data used and/or analyzed during the current study is available from the corresponding author on reasonable request.

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Author contributions

J.M: Conceptualization, Data curation, Formal analysis, Funding acquisition, Writing—original draf; Z.G: Data curation, Formal analysis, Investigation, Methodology; X.W: Conceptualization, Supervision, Validation, Writing—review & editing; D.Y, M.L, and L.C: Formal analysis, Data curation, Validation. All authors have reviewed and agreed to the published version of the manuscript.

Competing interests

The authors declare no competing interests.

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