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Multiomics analysis provides insights into flavonoids accumulation and biosynthesis in different planting years and locality of *Gongronemopsis tenacissima* (Dai-Bai-Jie)

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Abstract

The dried root of *Gongronemopsis tenacissima* (Roxb.) was the important Dai ethnic medicine, which is employed in folkloric medicine mainly for detoxification purposes. Due to the extensive utilization, the wild resources are becoming increasingly scarce. The plants have been domesticated in China. However, the accumulation patterns of the secondary metabolites and the main detoxifying component, flavonoids, as well as biosynthesis of flavonoids remain unclear. The differences in flavonoid accumulation and transcriptional regulatory mechanisms underlying the differential accumulation of flavonoid in Dai-Bai-Jie, cultivation for one, two, and three years in high altitude, as well as three years in low altitudes were investigated using transcriptome and widely targeted metabolome methods. A total of 1495 metabolites were identified by UPLC-MS/MS from Dai-Bai-Jie, and 943 differential accumulation metabolites were detected among four groups. All the flavonoids were grouped into six clusters by k-means cluster analysis. There is a regulatory relationship between genes such as PAL, CYP73A, 4CL, FLS and flavonoid components in Dai-Bai-Jie. However, significant differences in the Shannon, Chao1, or ACE indices of rhizosphere microorganisms across different plantation ages and localities were not detected. This study elucidates the regulatory mechanisms of flavonoids and the scientificity of harvesting years.

Keywords: *Gongronemopsis tenacissima*; metabolome; transcriptome; rhizosphere microbes; flavonoids

Introduction

Marsdenia tenacissima (Roxb.) Moon, a traditional medicine of the Dai ethnic group, holds significant value in the ethnomedical traditions of Southeast Asia. In the Dai language, it is referred to as "Ya Jie Xian Da," symbolizing its ability to purge the body of numerous toxins.

This medicinal herb has long been utilized in Dai-inhabited regions such as Xishuangbanna, Dehong, Ximeng, Menglian, Xinping, Yuanjiang, Mojiang, and Puer in China, as well as neighbouring countries like Laos and Myanmar (*Li et al., 1995*). The root of *M. tenacissima* is employed in folkloric medicine named Dai-Bai-Jie for detoxification purposes. It is known to counteract toxicities from various substances, such as food, animals, and even heat, water, and fire burns. Additionally, it is utilized to alleviate throat discomfort and swelling caused by excessive heat toxicity. With a rich historical background in traditional medicine, *M. tenacissima* (Dai-Bai-Jie) has found its way into contemporary hospital preparations at institutions like the Xishuangbanna Dai Hospital. These preparations include formulations such as Bai-jie Capsules, Ya-jie Gahan, and Banna Coolant. Modern pharmacological research has revealed that "Dai-Bai-Jie" exhibits inhibitory effects on cancer cells, protects against liver damage caused by certain drugs, demonstrates anti-HIV activity, possesses antioxidant properties, and exhibits antibacterial activities (*Gao et al.， 2014; Li et al.， 2021*) Currently, various bioactive compounds have been isolated from *M. tenacissima*, including organic acids, polyoxyprogesterone glycosides, volatile oils, and pyrrole alkaloids (*Liao et al., 2016; Pang et al.，2018; Song et al.，2018; Song et al., 2021*). These discoveries not only enhance our understanding of the medicinal properties of this herb but also pave the way for potential therapeutic applications in modern medicine.

For numerous years, Dai-Bai-Jie has been erroneously identified as the dried root of Dregea sinensis Hemsl., belonging to the genus Dregea of the Asclepiadaceae family (*Lin et al.， 2003*). However, in 2014, a pivotal study established that Dai-Bai-Jie is the dried root of *M. tenacissima*, a member of the Marsdenia genus (*Li et al.，2014; Li et al.，2023*). This identification was based on comprehensive molecular and morphological analysis, employing DNA fragments such as psbD-trnT, trnL-trnF, and ITS, in conjunction with observations of leaf morphology and floral traits. Importantly, it must be noted that the “tong-guan-teng” mentioned in the Chinese Pharmacopoeia, renowned for its broad-spectrum anticancer activities, corresponds to *M. cavaleriei* (*Chen et al.， 2022; Li et al., 2014*). Current scientific inquiries have revealed significant disparities in the chemical composition and therapeutic effects of these two species. Specifically, antidotal properties and gastrointestinal disease management are the primary therapeutic indications of Dai-Bai-Jie, whereas anticancer activity is the primary biological function attributed to *M. cavaleriei*. In 2022, *M. tenacissima* has been transferred to the genus Gongronemopsis, named *Gongronemopsis tenacissima* (Roxb.) (*Liede-Schumann* et al., 2022)

Flavonoids are secondary metabolites that are ubiquitously found in plants and possess diverse functions including antioxidant, anti-inflammatory, antitumor, antiviral, antibacterial, anti-vascular sclerosis, and anti-liver fibrosis activities (*Fang et al.， 2023; Wang et al.， 2020; Zhang et al.， 2023*). Recent studies suggested that its protective effect on intestinal mucosal barrier function may contribute to its detoxification mechanisms (*Yang et al.， 2020*). According to Dai medical theory, the occurrence of disease is closely associated with imbalances among the

four cosmic elements within the body, and these imbalances can be triggered by the presence of toxins (*Zhang et al.， 2023*). Such imbalances may stem from disturbances in antioxidant defences and imbalances between pro- and anti-inflammatory factors. Notably, recent studies have demonstrated a correlation between the levels of total flavonoids and total polyphenols in Dai-Bai-Jie with its antioxidant and anti-inflammatory activities (*Zhang et al. , 2023*). Therefore, Flavonoids maybe the most important active component for detoxification of *G. tenacissima*
Due to the extensive utilization of *G. tenacissima*, wild resources are becoming increasingly scarce. Fortunately, significant progress has been made in the artificial cultivation technology of *G. tenacissima*, resulting in small-scale cultivation in Xishuangbanna, Yunnan. Under natural conditions, the harvesting period is typically determined by empirical knowledge and generally occurs after at least two years of growth. Similarly, under cultivation conditions, the harvest period is usually 2-3 years, primarily considering the biomass of the roots.
Despite these advancements, the accumulation patterns of flavonoids in *G. tenacissima* under varying cultivation conditions remain unclear. To address this knowledge gap, this study investigated the flavonoids accumulation patterns and influence factors of *G. tenacissima* from the multi-omics perspective, which may lead to better understanding of *G. tenacissima* metabolism accumulative mechanism, as well as facilitate to elucidate scientifically optimal harvesting years for this medicinal plant.

Materials & Methods

2.1 Plant materials and sampling
The roots of one-year-old (CR1), two-year-old (CR2), and three-year-old (CR3) cultivated *G. tenacissima* (Dai-Bai-Jie) were collected from Menghun County, Xishuangbanna Dai Autonomous Prefecture, Yunnan, China (E 100.38°, N 21.82°; 1179 m) in November 2022 （Fig. 1）. Additionally, the roots of three-year-old Dai-Bai-Jie (CR4) cultivated in South Medicine Garden (E100.79°, N22.00°; 533.57m) also located in Xishuangbanna Dai Autonomous Prefecture, Yunnan Province, China, were gathered. Each plant was divided into two sections: one for transcriptome sequencing and the other for metabolome analysis, with three biological replicates per sample. Furthermore, the rhizosphere soil (CM1, CM2, CM3, CM4) corresponding to each plant (CR1, CR2, CR3, CR4) was collected and utilized for 16S rRNA and ITS analysis.
2.2 Metabolome analysis
After the freeze-dried samples were crushed (30 Hz, 1.5 minutes), the extraction solution (70% methanol water pre-cooled to -20℃) was added, and the mixture was vortexed for 30 seconds. Subsequently, the samples were vortexed six times (once every 30 minutes) and centrifuged at 12,000 rpm for 3 minutes. The supernatant was then filtered through a microporous filter membrane with a pore size of 0.22 μm and stored in an injection vial for UPLC-MS/MS analysis. Ultra High Performance Liquid Chromatography (ExionLC™ AD) was employed for sample collection and analysis, utilizing an Agilent SB-C18 column (1.8 μm, 2.1 mm × 100 mm). The mobile phase A consisted of 0.1% formic acid in water, while the mobile phase B was acetonitrile containing 0.1% formic acid. The column temperature was maintained at 40°C, and the automatic sampler temperature was set to 4°C. The flow rate was adjusted to 0.35 mL/min, and the injection volume was 2 μL. Applied Biosystems 6500 QTRAP was used for analysis. The typical ion source parameters were as follows: electrospray ionization (ESI) temperature of

500°C; ion spray voltage (IS) of 5500 V in positive ion mode and -4500 V in negative ion mode; ion source gas I (GSI), gas II (GSII), and curtain gas (CUR) were set to 50, 60, and 25 psi, respectively. The collision-induced dissociation parameters were set to high. SCIEX Analyst workstation software (version 1.6.3) was used for Multiple Reaction Monitoring (MRM) data collection and processing.

Using MS-Converter, MS raw data files were converted into TXT format for further analysis. An internal R program, along with a specialized database, was employed for peak detection and annotation. Prior to analysis, the raw data underwent preprocessing to filter out low-quality ion signals.

After obtaining the organized data, SIMCA (version 16.0.2) software was used for analysis PCA and OPLS-DA, which were used to explore the metabolic patterns and identify differential metabolites Metabolites (DAMs) with p-values < 0.05 and VIP (variable importance in projection) >1.

2.3 RNA-seq processing and data analysis

Total RNA was extracted and purified from the above samples. The extracted RNA was tested for purity, concentration, and integrity. After the samples were qualified, the mRNA was isolated and purified by Oligo (dt) for the construction of the cDNA library. Illumina Novaseq 6000 sequencing was performed after the library was qualified. Fastp software (*Chen et al.， 2018*) was used for quality control on the raw data.

After obtaining Clean Reads, Trinity assembly software is used to splice the Clean Reads to obtain reference sequences for subsequent analysis, trinity assembly software was used to stitch the clean reads to obtain reference sequences for subsequent analysis.

The RSeQC software (*Wang et al.， 2012*) was used to evaluate the quality of transcriptome data, and to analyze the sequencing data after passing the quality evaluation. FPKM (*Trapnell* et al.， 2010) was used to estimate gene expression level. The transcriptome assembly was assessed in terms of their completeness and the percentage of complete, fragmented, and missing fragments by using the BUSCO 5.3.2 (https://busco.ezlab.org, Simão et al. 2015). DESeq2 (*Iove et al.， 2014; Varet et al.， 2016*) was used for differential expression analysis between samples. The corrected p-value and FDR (False Discovery Rate) were used as the key indicators for the screening of differentially expressed genes (DEGs). Weighted Geneco-expression Network Analysis (WGCNA) was used to find the gene modules that are co-expressed and constructed the hierarchical clustering tree. The statistical power of this experimental design, calculated in RNASeqPower is 0.70.

The whole transcript data set can be found in the National Center for Biotechnology Information (NCBI) database (BioProject ID: PRJNA996325).

2.4 RT-qPCR validation

We selected five genes associated with flavonoids synthesis for RT-qPCR according to FMPK value. GAPDH was used as a reference gene and all genes used in this study are listed in Table 1. cDNA was synthesized using MonScript™ RTIII All-in-One Mix with ds DNase (Monad, China). According to the instructions of QuantiNova SYBR Green PCR Kit (QIAGEN, China), RT-qPCR was performed. The total volume of the system was 10 μL, including 5μL 2x SYBR Green PCR Master Mix, 0.7 μL upstream Primer with 0.7 μM, 0.7 μL down-stream primer with 0.7μM, 1μL cDNA with≤100ng/reaction, 2.55 μl RNase-free water, 0.05 μl QN ROX Reference Dye.

Microbial DNA extraction，16S rRNA and ITS gene sequencing

Genomic DNA was extracted using CTAB (Nobleryder, China). Dilute the DNA with sterile water to 1 ng/μL. 30 μL PCR amplification system was as follows: Phusion® High-Fidelity PCR Master and high fidelity polymerase Mix (New England Biolabs) 15μL, Primer 1 μL, DNA5-10 ng, ddH2O. 16S V4 Regional primer (GTGCCAGCMGCGCGGGGGTAA and 806R GGACTACHVGGGGTWTCTAAT) was used for identified bacterial diversity. ITS5-1737F 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS2-2043R 5'-GCTGCGTTCTTCATCGATGC-3' was used for identified fungal diversity. Reaction procedure was set at 98 C for 1 min, followed by 40 cycles at 98 ℃ for 10 s，0℃ for 38 s and 72 ℃ for 30 s, 72 ℃ extension for 5 minutes finally. PCR products was sequenced on the NovaSeq6000 platform (Maiwei Biotechnology Company).

Results

3.1 RNA-seq analysis and DEGs identification

We performed high-throughput transcriptome sequencing of CR1, CR2, CR3, and CR4 of Dai-Bai-Jie, with three biological replicates per sample. In total, we obtained 78.27 GB of clean data. The clean Data of all sample was not less than 6 Gb. The percentages of Q30 bases were all greater than 90%. After assembling and splicing, 85,346 unigenes were obtained. A BUSCO analysis was performed to evaluate the completeness, and we recovered 253 of the 255 conserved eukaryotic genes (99.2%) (Fig. 3A) .

Using the criteria of $|\log2Fold\ Change| \geq 1$ and $FDR < 0.05$, we screened for DEGs. The results revealed that 15,255, 8,170, 10,529, and 8,225 DEGs were identified in the comparisons of CR1 vs. CR2, CR1 vs. CR3, CR2 vs. CR3, and CR3 vs. CR4, respectively. Among them, there were 654 common DEGs shared of CR1, CR2, CR3, and CR4. Specifically, there were 6,043 unique DEGs in the comparison of CR1 vs. CR2, 1,243 unique DEGs in CR1 vs. CR3, 2,720 unique DEGs in CR2 vs. CR3, and 2,957 unique DEGs in CR3 vs. CR4 (Fig. 3C).

The DEGs in the four groups were analyzed by KEGG metabolic pathway. The results showed that the DEGs of CR1 vs. CR2, CR1 vs. CR3, CR2 vs. CR3, and CR3 vs. CR4 were annotated to 144, 140,143, and 140 KEGG metabolic and biosynthetic pathways, respectively. Notably, the "Metabolic pathways" category emerged as the most frequently annotated, encompassing 2492, 1428, 1669, and 1432 genes in each comparison, respectively. Closely following was the "biosynthesis of secondary metabolites" category, which annotated 1375, 800, 930, and 808 genes, respectively. The "Plant-pathogen interaction" pathway was annotated to 514, 271, 364, and 401 genes (Fig. 2).

WGCNA displayed that DEGs are divided into 27 co-expression modules of CR1, CR2, CR3, and CR4. Among them, the turquoise module has the highest number of genes with 11313, followed by the blue module with 5550 genes, and the least is the white module, which has 101 genes (Fig . 3B).

3.2 RT-qPCR validation

The RT-qPCR results for the five genes revealed that four of them (excluding cluster-60047.2) exhibited general consistency with the relative transcript abundance observed in the transcriptome analysis. This concordance validates the reliability of the RNA-seq data (Fig. 4).

3.3 Metabolomic profiling

A total of 1495 metabolites were identified by Ultra-Performance Liquid Chromatography coupled with Mass Spectrometry/Mass Spectrometry (UPLC-MS/MS) from Dai-Bai-Jie, including 378 amino acids and their derivatives（25.28%），265 phenolic acids (17.73%), 168 lipids (11.24%), 114 flavonoids (7.63%), 103 organic acids (6.89%), 92 alkaloids (6.15%), 80 nucleotides and their derivatives (5.35%), 55 lignans and coumarins (3.68%), and 42 terpenoids

(2.81%), 23 steroid（1.54%）and 75 metabolites belonging to other categories （11.71%）（Fig. 5A). Notably, the flavonoid category was further subdivided into 9 chalcones, 17 dihydroflavonoids, 8 dihydroflavonols, 36 flavonoids, 40 flavonols, and 4 flavanols.
Principal component analysis (PCA)was used to reveal the overall metabolite differences between the different groups. The results showed that both PC1 (38.39%) and PC2 (23.73%) explained 62.12% of the changes in the metabolic profile, indicating significant differences in four groups. The three samples within every group presented high aggregation and good repeatability (Fig. 5B).
A total of 943 Differential metabolites (DAMs) were detected using FC≥2 or ≤0.5 and VIP>1 as screening conditions, including 255 amino acids and their derivatives, 174 phenolic acids, 45 nucleotides and their derivatives, 79 flavonoids, 42 lignans and coumarins, 64 alkaloids, 30 terpenoids, 44 organic acids, 20 steroids and 83 lipids. Among them, there were one common DAMs shared of CR1, CR2, CR3, and CR4. Specifically, there were five unique DAMs in the comparison of CR1 vs. CR2, 273 unique DAMs in CR1 vs. CR3, 172 unique DAMs in CR2 vs. CR3, and 46 unique DAMs in CR3 vs. CR4 (Fig. 5D).
There were 627 DAMs in CR1 vs CR2, of which 183 were down-regulated and 444 were up-regulated. Compared with the CR1, the metabolite with a significant decrease in the CR2 was gofruside, and the metabolite with a significant increase was 4-O-(2"-O-acetyl-6"-P-coumaroyl-β-D-glucopyranosyl)-P-coumaric acid (Fig. 6A）. There was a total of 550 DAMs in CR1 vs CR3, of which 276 were up-regulated and 274 were down-regulated. Compared with the CR1, protocatechuic acid 4-O-(2"-O-Vanilloyl) glucoside was significantly reduced in the CR3, and eugenol was significantly increased (Fig. 6B). 449 DAMs were detected in CR2 vs CR3, of which 377 were down-regulated and 72 were up-regulated. The metabolite significantly reduced in CR3 was 6,7-dimethoxy-2-[2-(4'-hydroxy-3'-methoxyphenyl)ethyl]chromone compared to the CR2, and the significantly increased metabolite was sinapine（Fig. 6C）. A total of 259 DAMs were found in CR3 vs CR4, of which 117 were down-regulated and 142 were up-regulated. The metabolite that was significantly reduced in CR4 was rutin, and the metabolite that was significantly increased was 7-Hydroxycoumarin compared to CR3 (Fig. 6D). Cluster analysis was performed on the DAMs of the four groups. The differences between the four groups of samples were obvious, the phenolic acids were commonly higher in the CR2, and flavonoids were commonly higher in the CR1 and CR2 than other groups. The contents of amino acids and their derivatives were higher at CR3, while the contents of terpenes, nucleotides and their derivatives were higher CR4 (Fig. 5C).
To gain a deeper understanding of the accumulation patterns of metabolites of Dai-Bai-Jie across different plantation age and altitudes, we employed k-means cluster analysis to categorize all the metabolites. The analysis revealed that the metabolites clustered into six distinct groups (Fig. 6E). Notably, classes 1 and 6 exhibited the highest concentration of metabolites in CR2, with class 6 containing the largest number of metabolites among all six classes. Classes 2 and 4, on the other hand, demonstrated the highest abundance of metabolites in CR3. Class 3 was characterized by the highest amount of metabolites in CR4, while class 5 displayed the highest concentration of metabolites in CR1. This categorization provides valuable insights into the

specific patterns of metabolite accumulation within each growth year and altitude, enabling us to further investigate their potential biological significance.

3.4 Comparative metabolomic analysis aiming to flavonoids and flavonoid biosynthesis-related genes among the different plantation age and locality.

A total of 114 flavonoids were detected from Dai-Bai-Jie, including 34.21% flavonols, 31.58% flavonoids, 14.91% dihydroflavonoids, 7.02% dihydroflavonols, 7.89% chalcone , 3.50% flavanols, 0.88% flavonols, of which 79 flavonoids were differentially accumulated. Based on K-means analysis, nine flavonoids, including 3′,5-Dihydroxy-4′,6,7-trimethoxyflavanone, acacitin-7-O-galactide, robiniin-7-O-galactoside, phelamurin, huangbaioside, eriodictyol-7-O-glucoside, exhibited a relatively high accumulation in class 2 for CR2. 15 flavonoids including 3 ', 4', 7-trihydroxyflavone, cirsimaritin, hesperetin-7-O-glucoside, quercetin, exhibited a relatively high accumulation in class 6 for CR2. Six flavonoids including kaempferol-7-O-glucuronid, hesperetin-7-O-(6"-malonyl) glucoside，quercetin-3-O-(6"-O-galloyl) galactoside，myricetin-3-O-rhamnoside (Myricitrin)，diosmetin-7-O-glucuronide，syringetin-7-O-glucoside, exhibited a relatively high accumulation in class 2 for CR3 . Ten flavonoids including Rutin，hesperetin-5-O-glucoside，i sorhamnetin-3-O-rhamnoside，quercetin-3-O-robinobioside, exhibited a relatively high accumulation in class 4 for CR3. Five flavonoids including 3-Hydroxy-4',5,7-Trimethoxyflavanone，aromadendrin-7-O-glucoside，eriodictyol-8-C-glucoside，dihydromyricetin-3-O-glucoside，taxifolin-3'-O-glucoside, exhibited a relatively high accumulation in class 3 for CR4. 34 flavonoids including rhamnazin , quercetin-3,4'-dimethyl Ether, limocitrin-7-O-glucoside，kumatakenin, exhibited relatively high accumulation in class 5 for CR1.

To gain a deeper understanding of the molecular mechanisms underlying the differential accumulation of flavonoids across various planting year and planting environments, we conducted a comprehensive analysis of the expression patterns of genes involved in flavonoid metabolism. KEGG analysis revealed that the 15 flavonoids exhibiting differential accumulation were mapped to multiple biosynthetic pathways, including the flavonoid biosynthesis pathway (KO00941), flavonol biosynthesis pathway (KO00944), as well as the broader metabolic pathway (KO01100) and secondary metabolite biosynthesis pathway (KO01110)（Fig. 7A）. Correlation analysis was conducted between DAMs mapped to the KEGG pathway and the corresponding DEGs on the pathway, and the correlation > 0.8 or <-0.8 and the P-value <0.05 as the screening conditions. The analysis revealed complex regulatory relationship among phenylalanine ammonia-lyase (PAL Cluster-63886.0, Cluster-63886.1), 4-Coumarate: Coenzyme A Ligase (4CL，Cluster-58688.4, Cluster-62808.3), lavonol synthase (FLS, Cluster-46899.18, Cluster-46899.5, Cluster-50957.2, Cluster-57391.0, C12RT1(Cluster-45854.0）and metabolites of hyperin, lonicerin, vicenin-2, nicotiflorin, querceti, luteolin-7-O-(6"-malonyl) glucoside, Hesperetin-7-O-glucoside (Fig. 7B).

3. 5 Taxonomic features of the rhizosphere microbes of Dai-Bai-Jie

Plants recruit specific root-associated microbes, which allow plants to deliver photosynthates and root exudates to their root microbiome, thereby stimulating plant growth and productivity (Lareen et al.， 2016). Many research has indicated that the composition of microbial communities at roots, the so-called root microbiome, can have significant impacts both on plant development and their stress tolerance (Mendes et al.， 2011; Panke-Buisse et al.， 2015). The coverage index between the bacterial and fungal sample groups was above 0.965, indicating that the sequencing was representative and could truly and reasonably reflect the bacterial and

fungal diversity of the samples. The four groups of rhizosphere soil bacteria involved a total of 40 phyla, 71 classes, 154 orders, 300 families, and 695 genera, and fungi involved a total of 13 phyla, 61 classes, 168 orders, 406 families, and 875 genera. Crenarchaeota，Acidobacteriota，Chloroflexi，Firmicutes，Proteobacteria were the dominant bacteria in the rhizosphere soils, while Ascomycota，Basidiomycota，Mortierellomycota，Glomeromycota，Chytridiomycota，Rozellomycota were the dominant fungi.

The indices of the richness index (Alpha diversity, ACE, Chao 1) and Shannon diversity index of the microbial community and the number of OTUs in all the samples was studied. There was no significant difference of Shannon, Chao1 and ACE in rhizosphere microorganisms among the four groups (table 2).

A total of 1952 bacterial operational taxonomic units (OTUs) and 5230 fungi were detected in the rhizosphere microbiome. The co-possessed bacteria in the four rhizosphere soils are 2986 OTUs, 721 are unique to CM1, 406 are unique to CM2, 497 are unique to CM3, and 620 are unique to CM4 (Fig. 8A). The co-possessed fungi in the four rhizosphere soils are 5677 OTUs, 383 are unique to CM1, 223 are unique to CM2, 263 are unique to CM3, and 406 are unique to CM4 (Fig. 8B).

The community composition analysis showed that the community compositions were similar among all the twelve four rhizosphere soils at the phylum level. In addition to CM3.3, Acidobacteriota abundances of CM2 and CM3 were significantly higher than those of CM1 and CM4. The abundance of Proteobacteria in CM1 was higher than that in Acidobacteriota (Fig.8CD). However, the community compositions presented different to some extent among all the twelve rhizosphere soils at the genus level (Fig. 8EF).

Discussion

Growth duration is the paramount factor influencing the quality of medicinal plants. Until now, the harvesting period of Dai-Bai-Jie has primarily focused on biomass accumulation, with the accumulation of bioactive components remaining unknown. Despite the existence of numerous research reports exploring metabolites and anti-tumor properties of *G. tenacissima*, majority of these studies have not specifically targeted Dai-Bai-Jie due to inaccuracies in plant identification (*Li et al.， 2014; Li et al., 2023*). Up to now, little is known about the chemical composition and active ingredients of Dai-Bai-Jie (*Liao et al., 2016; Pang et al., 2018; Zhang et al., 2016; Li et al.，2017*). Consequently, there is a need for further scientific exploration to comprehensively understand the growth patterns and accumulation of bioactive components in Dai-Bai-Jie.

In this study, a comprehensive metabolic profiling of Dai-Bai-Jie was conducted using UPLC-MS/MS widely-targeted metabolomics analysis. A total of 1495 metabolites were successfully identified, demonstrating the rich metabolite content of Dai-Bai-Jie. These metabolites are likely to serve as the pharmacological material basis for the medicinal properties of Dai-Bai-Jie. 943 DAMs were detected from four group samples from distinct locations and three different plantation age, which suggests quality differences among them.

Flavonoids and total polyphenols were major contributors for detoxification of Dai-Bai-Jie (Zhang et al. ,2023). We detected a diverse array of secondary metabolites, including flavonoids, phenolic acids, alkaloids, and terpenoids. This finding demonstrates potentially contributing to its antioxidant and anti-inflammatory activities.

When comparing the accumulation of metabolites across different plantation age, it was observed that the total metabolite content in CR2 and CR3 was relatively abundant. Additionally, flavonoid levels were generally higher in CR1 and CR2. To achieve a balance between biomass,

economic benefits, and the biological activity of Dai-Bai-Jie, it is recommended that two-year harvesting serves as the optimal strategy.

Despite originating from the same plantation age, samples CR3 and CR4 exhibited in consistent metabolite accumulation trends, revealing a total of 259 DAMs. This variation can be attributed to diverse environmental factors, including altitude, temperature, and soil conditions. Although the number of DAMs identified was fewer compared to those observed between different years, it nonetheless underscores the significant impact of the environment on the accumulation of secondary metabolites in Dai-Bai-Jie. Furthermore, it suggests that cultivation at lower altitudes may result in a reduced abundance of secondary metabolites. The reason maybe that Dai-Bai-Jie is tropical plant, and the low temperature, as a stress, promoted the production of secondary metabolites in Dai-Bai-Jie.

Based on our widely targeted metabolome, flavonoids represent the secondary metabolites with the higher content in Dai-Bai-Jie. Notably, the flavonoid content is significantly higher in farmed one year and two years compared to those that farmed three years. Furthermore, the majority of differentiated flavonoid components exhibit a substantial accumulation in one-year and two-years plant.

Numerous flavonoids isolated from Dai-Bai-Jie have exhibited significant biological activities. Specifically, hesperetin-7-O-glucoside has been demonstrated to effectively modulate the gut microbiota composition and bile acid metabolism in murine models (*Wu et al.， 2022*). The antioxidative, antihypertensive, antidiabetic, anti-inflammatory and cardioprotective activities of rutin were reported, while rutin pretreatment before administration of ethanol can afford significant protection against mucosal hyperemia, necrosis, edema and mucosal or submucosal hemorrhage (*Akash et al.， 2024; Chua., 2013; Nicola et al.， 2024*). Quercetin is known to possess both mast cell stabilizing and gastrointestinal cytoprotective activity (*Anand David et al.， 2016; Catalina et al.， 2016*).

The flavonoid content in Dai-Bai-Jie varies significantly with its plantation age, which may be the result of DEGs patterns of genes involved in flavonoid biosynthesis. Until now, flavonoid biosynthetic pathway has been extensively studied, with the genes encoding enzymes involved in this pathway and their corresponding functions having been verified in many plants. Flavonoids, flavonols, and lignin are synthesized through various branching pathways originating from the phenylpropane biosynthetic pathway (*Froemel et al., 1985*). We screened nine DEGs related to flavonoid biosynthesis from Dai-Bai-Jie, PAL, 4CL, FLS, and C12RT1 included.

PAL catalyzes the first step in the phenylpropanoid pathway and plays an important role in the biosynthesis of phenylpropanoid and flavonoid compounds (*Levy et al.， 2018*). 4CL is the last enzyme in the general biosynthetic pathway of phenylpropane compounds, which catalyzes cinnamic acid and its hydroxyl or methoxy derivatives to generate corresponding coenzyme A esters (*Cao et al.， 2023; Lavhale et al.， 2018*). These intermediate products then enter the biosynthetic pathway of phenylpropane derivatives (*Tian et al.，2017*). FLS is a key enzyme specific to the flavonol pathway, which converts dihydroflavonol into the corresponding flavonol by introducing a double bond between C-2 and C-3 of the C-ring (*Forkmann et al., 1986; Shi et al., 2021*).

Correlation analysis conducted on flavonoid DAMs mapped to the KEGG pathway revealed that the expression patterns of genes PAL, 4CL, and FLS exhibited a concordant trend with the accumulation of nicotiflorin and lonicerin. Similarly, hesperetin-7-O-glucoside displayed a comparable trend with C12RT1. These DEGs may serve as key genes regulating the distinct accumulation patterns of flavonoid metabolites in Dai-Bai-Jie.

The RT-qPCR results showed that the expression trend of the key enzyme genes in the biosynthetic pathway of flavonoids in Dai-Bai-Jie was consistent with the results of transcriptome sequencing, indicating that the transcriptome data is reliable.
In general, plantation age has been found to elicit alterations in soil nutrient content and pH, subsequently driving changes in the composition and diversity of soil bacterial and fungal communities. For instance, *Na et al*.(2016) reported that fungal diversity decreased with the cultivation going on from 5 a to 10 a of Lycium bararum L. whereas bacterial diversity remained relatively unchanged. Conversely, *Li et al.* (2020) observed a significant increase in bacterial diversity and a decrease in fungal diversity in lily soil with increasing planting years. However, in our study on Dai-Bai-Jie, we did not detect any significant differences in the Shannon, Chao1, or ACE indices of rhizosphere microorganisms across different plantation ages and localities. This inconsistency suggests that the underlying mechanisms governing microbial community dynamics in Dai-Bai-Jie rhizospheres might differ from those observed in other plant species. The lack of significant changes in microbial diversity in our study merits further investigation, particularly from the perspectives of soil nutrients, pH, and moisture content.
In summary, this study comprehensively characterized the disparities in flavonoid metabolite profiles and abundances across varying cultivation environments and plantation age through integrated transcriptome and metabolome analyses. Key genes intricately associated with the differential accumulation of flavonoids were identified. The results laid a foundation for further regulation of the effective components and provided support for determining the scientific harvesting practices of Dai-Bai-Jie.

Conclusions

Dai-Bai-Jie is a traditional Dai nationality herb medicine for detoxification purposes. The accumulation patten of flavonoids and regulation patterns for remain undetermined. In this study, we collected roots and rhizosphere soils under three planting years (one years, two years, and three years) and three years with two different localities. We investigated the flavonoids accumulation patterns and influence factors of Dai-Bai-Jie from the multi-omics perspective. A total of 1495 metabolites were identified by UPLC-MS/MS from Dai-Bai-Jie, of which 943 DAMs were detected. 114 flavonoids were detected, of which 79 flavonoids were differentially accumulated. Maximum DAMs were appeared between one-year and two-year Dai-Bai-Jie. Complex regulatory relationship among phenylalanine ammonia-lyase (PAL Cluster-63886.0, Cluster-63886.1), 4-Coumarate: Coenzyme A Ligase (4CL，Cluster-58688.4, Cluster-62808.3), lavonol synthase (FLS，Cluster-46899.18, Cluster-46899.5, Cluster-50957.2, Cluster-57391.0, C12RT1(Cluster-45854.0）and metabolites of hyperin, lonicerin, vicenin-2, nicotiflorin, querceti, luteolin-7-O-(6''-malonyl) glucoside, Hesperetin-7-O-glucoside. Different plantation ages and localities did not cause the significant differences in the Shannon, Chao1, or ACE indices of rhizosphere microorganisms of Dai-Bai-Jie. The differences observed in flavonoid accumulation may be, to a certain extent, attributed to variations in the community compositions at the genus level.

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Figure 1

the sample used in this study

A, total plant of Dai-Bai-Jie. B, Root of cross-sections at different planting years. CR1: farmed for one year, CR2: farmed for two years, CR3, farmed for three years.

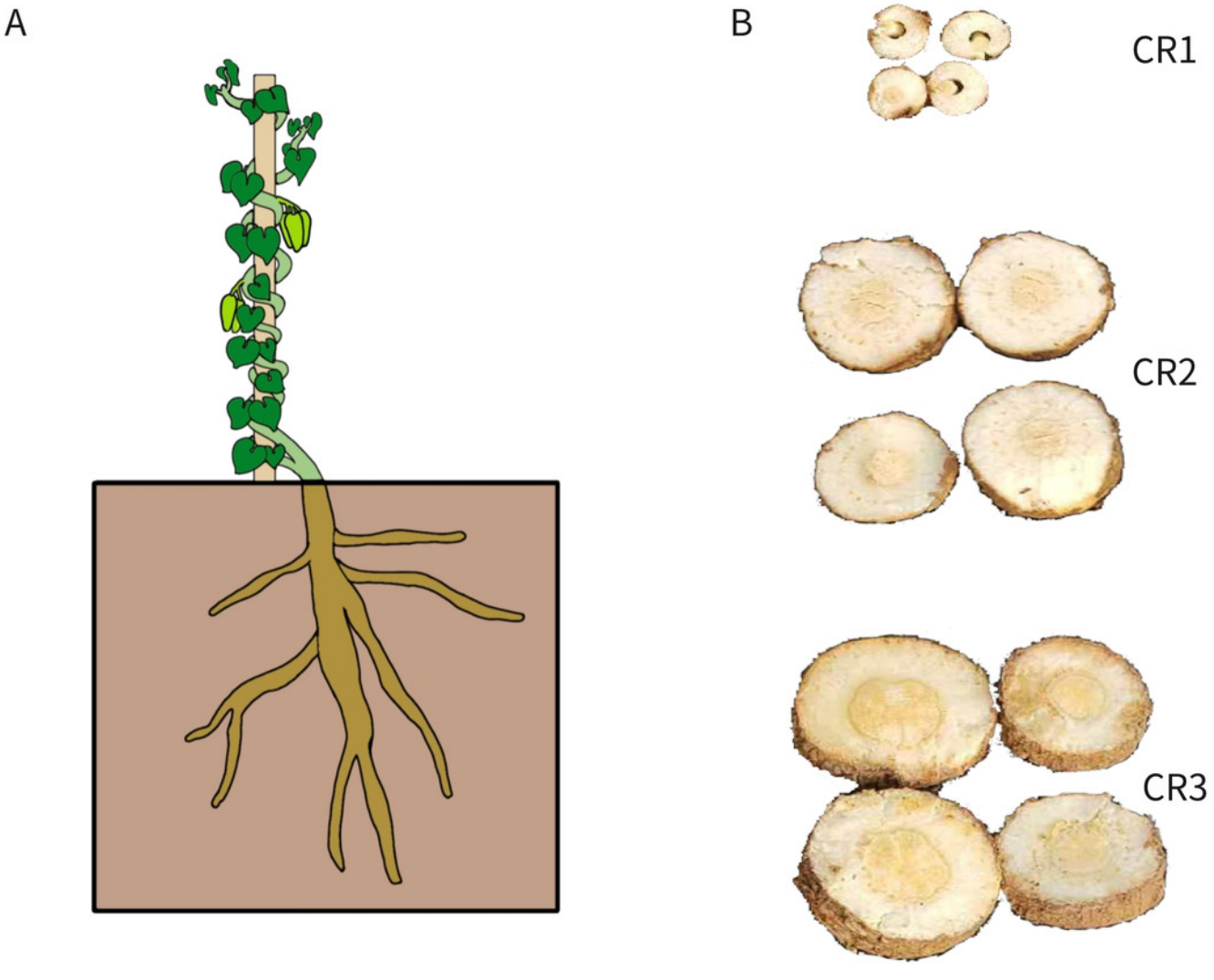

Figure 2

The DEGs in the four groups were analyzed by KEGG metabolic pathway

(A) CR1-CR2. (B) CR1-CR3. (C) CR2-CR3. (D)CR3-CR4.

Figure 3

Transcriptome analysis results

(A) BUSCO completeness assessments of the Dai-Bai-Jie transcriptome. (B) WGCNA clustering tree. (C) Venn Diagram representing the number of DEGs among four group sample.

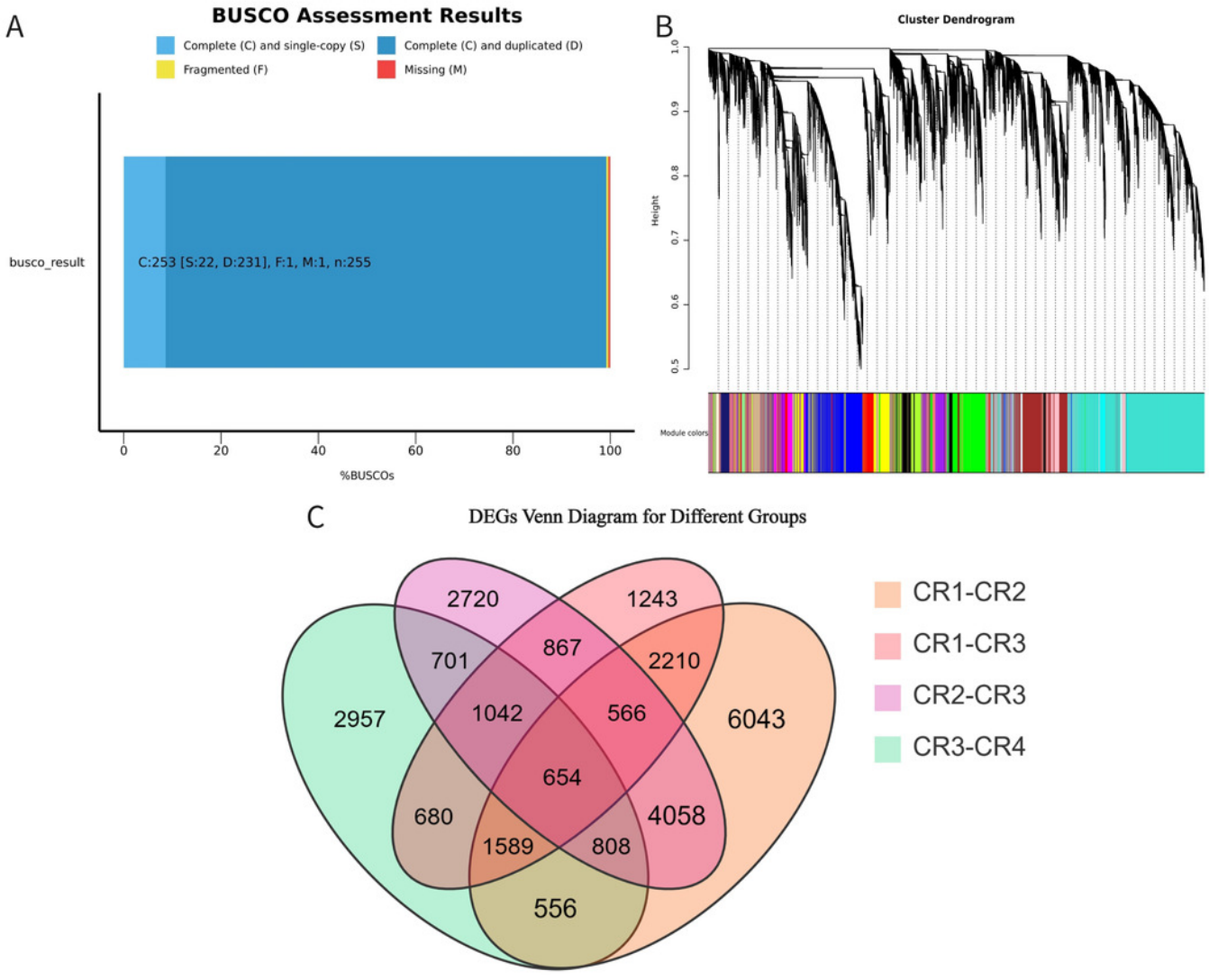

Figure 4

RNA-seq analysis of Dai-Bai-Jie and the qRT-PCR validation of five genes.

RNA-seq analysis of Dai-Bai-Jie and the qRT-PCR validation of five genes.

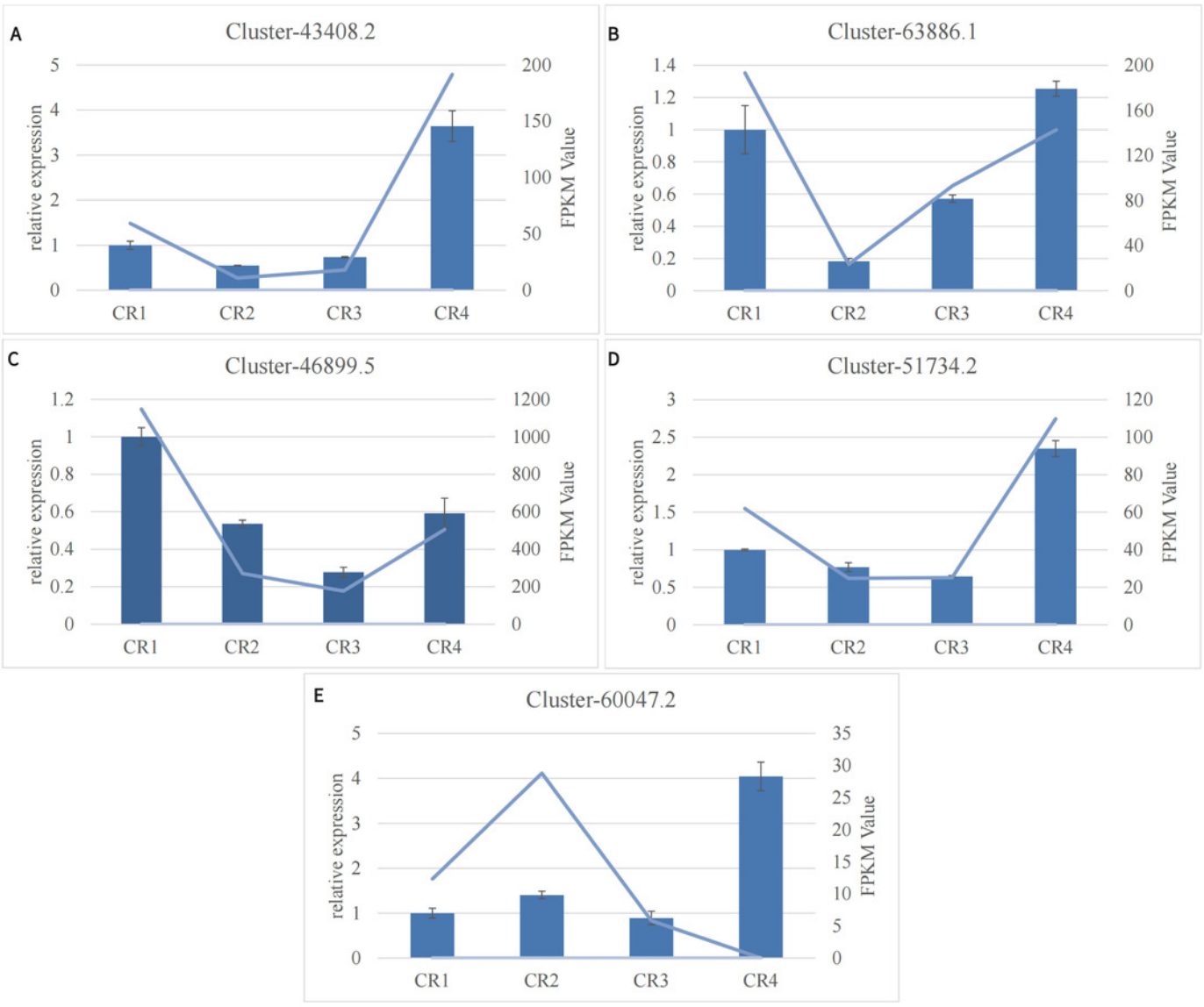

Figure 5

Metabolome analysis results

(A) Composition of metabolite in Dai-Bai-Jie. (B) PCA score plots for all samples. (C) Heat map of DAMs in four groups of samples. (D) Venn diagram of DAMs across groups.

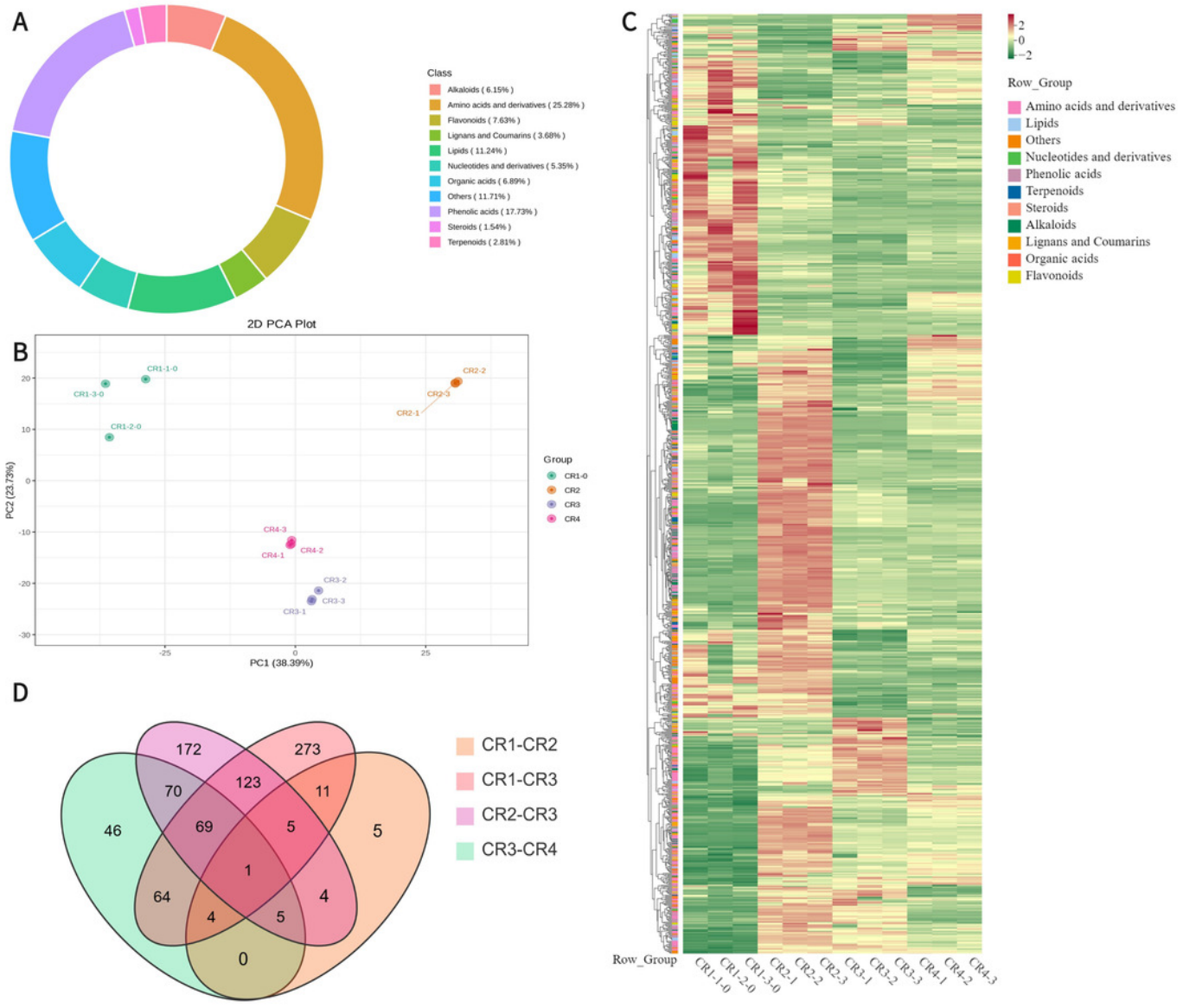

Figure 6

The volcano diagram and the k-means diagram of Metabolites.

(A) Volcano diagram of DAMs (CR1 vs. CR2). (B)Volcano diagram of DAMs (CR1vs. CR3). (C)Volcano diagram of DAMs□CR2 vs. CR3). (D)Volcano diagram of DAMs (CR3 vs.CR4). (E) The K-means analysis of all Metabolites. The black line in the figure represents the average pattern of all Metabolites in each class, and different colors represent different trend.

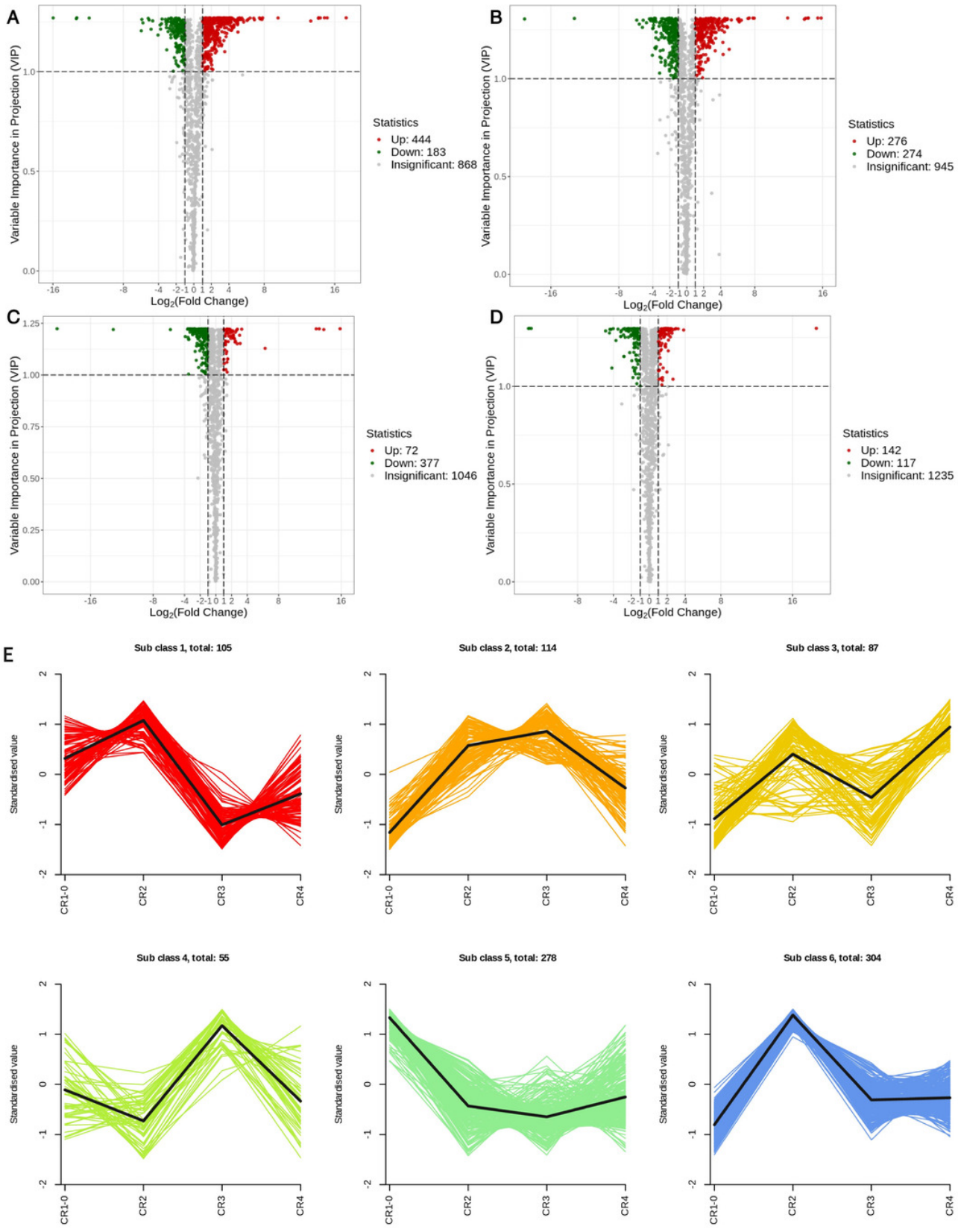
A
Variable Importance in Projection (VIP)
Log2(Fold Change)
Statistics
Up: 444
Down: 183
Insignificant: 868
B
Variable Importance in Projection (VIP)
Log2(Fold Change)
Statistics
Up: 276
Down: 274
Insignificant: 945
C
Variable Importance in Projection (VIP)
Log2(Fold Change)
Statistics
Up: 72
Down: 377
Insignificant: 1046
D
Variable Importance in Projection (VIP)
Log2(Fold Change)
Statistics
Up: 142
Down: 117
Insignificant: 1235
E
Sub class 1, total: 105
Sub class 2, total: 114
Sub class 3, total: 87
Sub class 4, total: 55
Sub class 5, total: 278
Sub class 6, total: 304
Standardised value
CR1-0
CR2
CR3
CR4

Figure 7

Flavonoid synthesis pathway and Network diagram

(A)Flavonoid synthesis pathway. (B) Network diagram of flavonoids and differential genes.

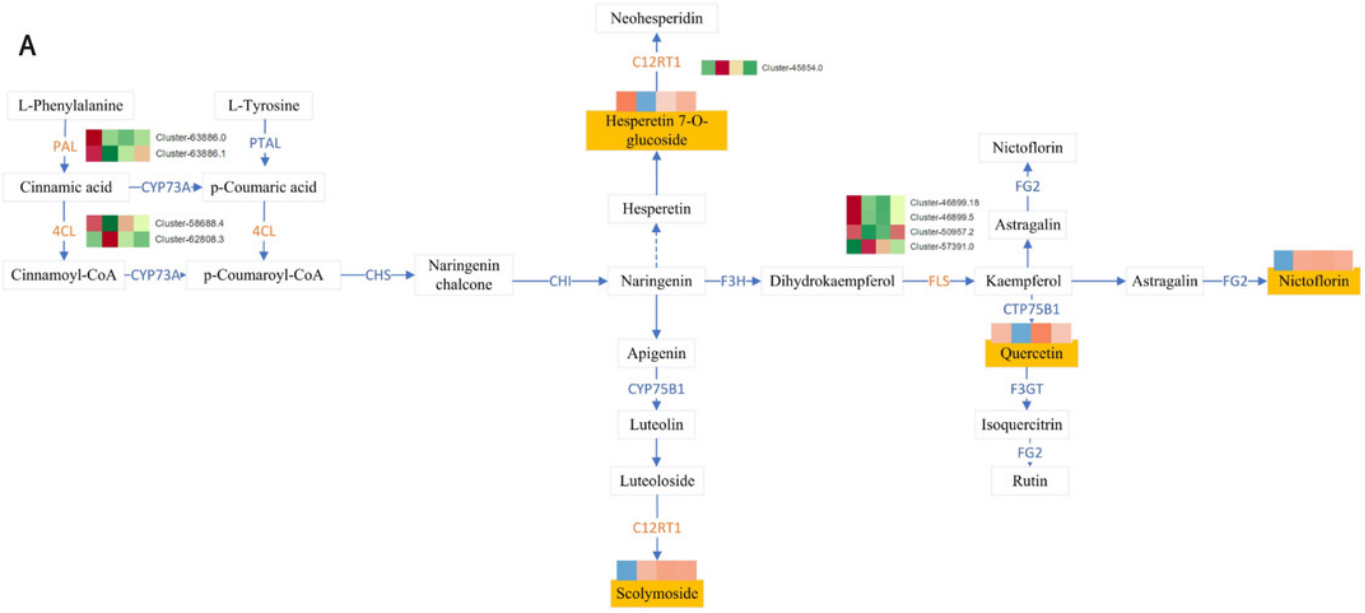

Figure 8

Venn diagram and the relative abundance of phylum and genus among CM1, CM2, CM3, and CM4 in rhizosphere soil of Dai-Bai-Jie.

(A) Venn diagram of bacterial. (B) Venn diagram of fungus. (C) relative abundance of bacterial phylum. (D)relative abundance of fungal phylum. (E) relative abundance of bacterial genus. (F)relative abundance of fungal genus.

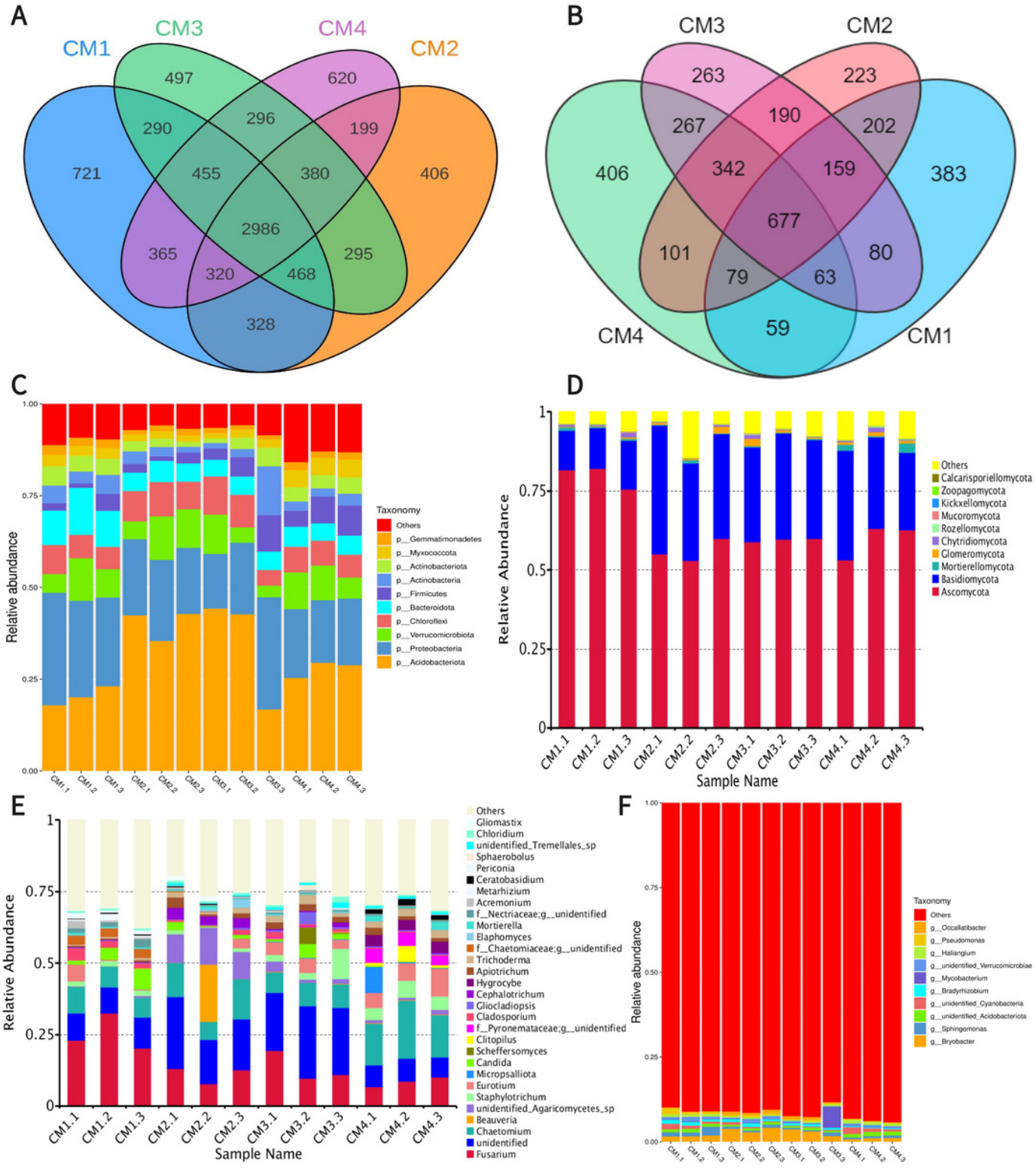
A
CM1
CM3
CM4
CM2
497
620
290
296
199
721
455
380
406
2986
365
320
468
295
328
B
CM3
CM2
263
223
267
190
202
406
342
159
383
677
101
80
79
63
CM4
59
CM1
C
Relative abundance
Taxonomy
Others
p__Gemmatimonadetes
p__Myxococcota
p__Actinobacteriota
p__Actinobacteria
p__Firmicutes
p__Bacteroidota
p__Chloroflexi
p__Verrucomicrobiota
p__Proteobacteria
p__Acidobacteriota
CM1.1
CM1.2
CM1.3
CM2.1
CM2.2
CM2.3
CM3.1
CM3.2
CM3.3
CM4.1
CM4.2
CM4.3
D
Relative Abundance
Sample Name
Others
Calcarisporiellomycota
Zoopagomycota
Kickxellomycota
Mucoromycota
Rozellomycota
Chytridiomycota
Glomeromycota
Mortierellomycota
Basidiomycota
Ascomycota
E
Relative Abundance
Sample Name
Others
Gliomastix
Chloridium
unidentified_Tremellales_sp
Sphaerobolus
Periconia
Ceratobasidium
Metarhizium
Acremonium
f__Nectriaceae;g__unidentified
Mortierella
Elaphomyces
f__Chaetomiaceae;g__unidentified
Trichoderma
Apiotrichum
Hygrocybe
Cephalotrichum
Gliocladiopsis
Cladosporium
f__Pyronemataceae;g__unidentified
Clitopilus
Scheffersomyces
Candida
Micropsalliota
Eurotium
Staphylotrichum
unidentified_Agaricomycetes_sp
Beauveria
Chaetomium
unidentified
Fusarium
F
Relative abundance
Taxonomy
Others
g__Occallatibacter
g__Pseudomonas
g__Haliangium
g__unidentified_Verrucomicrobiae
g__Mycobacterium
g__Bradyrhizobium
g__unidentified_Cyanobacteria
g__unidentified_Acidobacteriota
g__Sphingomonas
g__Bryobacter

Table 1(on next page)

primer of Five genes

1 Table 1 primer of Five genes
2

Gene	sequence(5'-3')	product size
Cluster-43408.2 FLS	F: TGATGAATGGGAAGCCCGAG R: TAGCGGTCCTGTTTTGGCTT	175bp
Cluster-46899.5 FLS	F: AGCCCTTGAAGAATTTGGTTGT R: ATCTCTTGTAAAGGCCGATCAAA	114bp
Cluster-51734.2 CYP73A	F:GGACCTGGCTAAGGAAGTGT R: TGTGAAGAAAGGCACCGTCA	166bp
Cluster-60047.2 4CL	F: GCATCCGTGGCGATCAAATC R: TGCCACTTGGAACCCTTTGT	179bp
Cluster-63886.1 PAL	F: CATGCCCTCCTCAACAACGA R: GGACCTGCACTCCTTGATCC	171bp
GAPDH	F: GGCATTGTCGAGGGTCTCAT R: CCGGTGCTGCTGGGAATAAT	131bp

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