

Variation in Columbin Content in Rhizome of different *Tinospora sagittata* Varieties

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Abstract

Columbin, a key secondary metabolite in *Tinospora sagittata*, exhibits diverse pharmacological properties, yet its content shows notable variation across different varieties. This study investigated the molecular and biochemical basis behind such variation, analyzing columbin content in 32 *T. sagittata* varieties ranging from 5.40 mg/g to 46.09 mg/g. High and low-columbin-content varieties were selected for in-depth transcriptomic and untargeted metabolomic analyses. Differential expression patterns of genes and metabolites between leaves and rhizomes of these selected varieties were explored. Results revealed that genes involved in the columbin biosynthesis pathway, namely, DXS, DXR, MCS, GPPS, and PMK, exhibited significantly higher expression in high columbin content varieties. At the same time, HMGR and HDR levels were notably lower. Furthermore, high columbin-content varieties contained increased isoprene levels, a crucial columbin precursor. These findings suggest gene expression and precursor content variations likely drive columbin content differences across *T. sagittata* varieties. This molecular insight into columbin biosynthesis clarifies the basis for content variation and provides valuable direction for breeding and cultivating high columbin-content *T. sagittata* varieties, amplifying its potential therapeutic applications.

Introduction

Tinospora sagittata, widely distributed in southern China, primarily thrives in moist habitats such as forest understories, edges, and bamboo groves. *T. sagittata* has demonstrated considerable potential for development and utilization due to its medicinal compounds, including diterpenoids and alkaloids (Hao et al., 2016; Liu et al., 2010; Qin et al., 2015; Shi et al., 2008; Zhong et al., 2022). Flavonoids (Abood et al., 2014; Chang et al., 2015; Xu et al., 2021), lignans (Huang et al., 2012), and steroids (Ahmed et al., 2006; Xu et al., 2021) also contribute to *T. sagittata*'s medicinal properties. The primary pharmacologically active alkaloids in *T. sagittata* are isoquinoline alkaloids with anti-inflammatory (Liu et al., 2010), analgesic (Yan et al., 2010), antibacterial (Yu et al., 2007), and antitumor effects like palmatine (Shi et al., 2006), jatrorrhizine (Zhong et al., 2022), and columbamine (Hao et al., 2016). Diterpenoids, another significant group of active compounds, mainly consist of clerodane diterpenes commonly found in the *Tinospora* genus (Atta-ur-Rahman and Ahmad, 1988; Dong et al., 2010; Li et al., 2007; Shi et al., 2006). Studies indicate that these clerodane diterpenoids, including columbin, possess anti-inflammatory properties (Zhu et al., 2022). Additionally, columbin has demonstrated hypoglycemic and antiviral effects (Nok et al., 2005). The content of columbin shows significant variation among *T. sagittata* varieties; for quality control and efficient production, it needs to be investigated.

Genetic and environmental factors play a significant role in the variability of columbin content across *T. sagittata* varieties. Columbin, a bitter diterpenoid lactone, is known for its therapeutic potential in traditional medicine, particularly in anti-inflammatory and anti-cancer applications. However, its levels can vary markedly due to a range of influences. Environmental conditions, such as soil properties, temperature (Alami et al., 2024a), and humidity, have been shown to directly impact the biosynthesis of secondary metabolites in medicinal plants, including columbin (Alami et al., 2024b, 2023). For instance,

soils with lower pH and high gravel content appear more conducive to columbin synthesis, a trend particularly notable in karst landscapes with well-drained soils in southwestern China (Huang et al., 2024; Meng et al., 2023). The unique microenvironment provided by these karst regions appears to facilitate nutrient profiles that enhance secondary metabolite production. Such findings highlight the importance of selecting specific growth environments to optimize columbin yield in wild and cultivated settings.

The influence of environmental factors extends to microclimates within different regions, where even slight differences in temperature or soil mineral composition can result in significant variability in metabolite concentrations (Che et al., 2021; Pereyra et al., 2023; Prévosto et al., 2020). This is particularly true for plants growing in nutrient-limited or drought-prone areas, where secondary metabolite production often serves as a protective mechanism. Studies have shown that plants exposed to these environmental stressors tend to synthesize more potent concentrations of pharmacologically active compounds, as part of a survival strategy (Nazari et al., 2023; Pichersky and Raguso, 2018; Yeshi et al., 2022). Understanding how these environmental factors interact to enhance columbin synthesis offers valuable insights into optimizing natural conditions for medicinal quality. This knowledge paves the way for selecting optimal habitats and soil compositions that could enhance the bioactive properties of *T. sagittata*, aligning with the standards of the Chinese Pharmacopoeia.

This study investigated the variation of columbin content among 32 *T. sagittata* varieties from five different locations and identified key genes in its biosynthetic pathway in high and low-columbin-content *T. sagittata* varieties to provide theoretical guidance for strain selection. The columbin content was initially measured in wild and cultivated *T. sagittata* varieties from southwestern China to screen and categorize high and low columbin content varieties. Subsequently, a systematic transcriptomic and metabolomic analysis was conducted to identify differentially expressed genes and metabolites, particularly within the clerodane diterpenoid biosynthetic pathway like columbin. This research aims to clarify the factors underlying columbin content variation in the medicinal parts of *T. sagittata*, thereby guiding the selection and cultivation of high columbin content varieties for enhanced pharmacological applications.

Materials and methods

Plant Materials

The *T. sagittata* varieties used in this study were sourced from five provinces: Hubei, Hunan, Yunnan, Sichuan, and Guizhou, including both wild and locally cultivated varieties. The *T. sagittata* varieties were grown in pots at Huazhong Agricultural University and are approximately two years old. All the 32 varieties SCCD (Huaxi Village, Tongji Town, Pengzhou City, Chengdu City, Sichuan Province), GZZY (Zunnan Avenue, Bozhou District, Zunyi City, Guizhou Province), SCLS (Taoxi Village, Laoheba Township, Mabian County, Leshan City, Sichuan Province), SCYB (Ganxi Village, Qianxi Town, Xuzhou District, Yibin City, Sichuan Province), YNZT (Taiping Village, Taiping Town, Shuifu City, Zhaotong City, Yunnan

Province), SCDY (Tianbao Village, Yinghua Town, Shifang City, Sichuan Province), and HBLC (Maotian Village, Tuanbao Town, Lichuan City, Enshi Prefecture, Hubei Province) were measured the columbin content in the rhizomes. Based on the columbin content, the samples were classified into high-content materials (H-type materials) and low-content columbin materials (L-type materials). H-type materials HBLC_12, HBLC_16, SCLS_3, SCLS_4, and YNCX and L-type materials HBLC_4, HBLC_9, HBLC_14, HBLC_19, and GZZY_1 were selected from both the rhizomes and leaves. The samples were washed and mixed uniformly, and each mixed sample was sampled randomly three times. The samples were then stored at -80°C for transcriptome sequencing and untargeted metabolomic analyses. In the paper, HL and HR represent leaves and rhizomes of H-type materials and LL and LR represent leaves and rhizomes of L-type materials, respectively.

Determination of columbin contents in *T. sagittata* rhizomes

Accurately weigh 10 mg of the columbin standard and dissolve it in chromatography-grade methanol, then dilute to a final volume of 10 ml to obtain a mother solution with a concentration of 1000.00µg/ml. Perform a gradient dilution of this mother solution and analyze and measure according to the methods described in the 2020 edition of the Chinese Pharmacopoeia. Regression analysis used columbin concentration as the independent variable (x-axis) and peak area as the dependent variable (y-axis), resulting in Regression Equation Table S1. The processing of *T. sagittata* rhizomes follows the guidelines outlined in the 2020 edition of the *Chinese Pharmacopoeia*.

The Agilent 1260 HPLC (high-performance liquid chromatography) system was used for metabolite determination with kromasil 100-5-C18 column(4.6*250mm, Φ5µm). The acetonitrile in chromatographic (A) and water (B) was used as the mobile phase (A: B = 40:60, v/v), and the detection wavelength was 210 nm. The flow rate is 1ml/min, the injection volume is 10 µL, and the column temperature is maintained at 25°C. The HPLC chromatograms of the columbin standard and the sample are shown in Figure S1. To validate the accuracy of the columbin content determination results, the measurement method's precision, stability, repeatability, and recovery rate were investigated. The results are summarized in Table S2, demonstrating that the measuring instrument exhibits strong precision. The extraction method maintains the stability of columbin, and it shows good repeatability. Moreover, the recovery rate of the extraction method is close to 100%.

Total RNA extraction, library preparation, and transcriptome sequencing

The total RNA extraction from H-type and L-type materials of plant tissues (rhizomes and leaves) followed the protocol outlined in the RNeasy Pure Plant Kit manual (Qiagen, Beijing, China). NanoDrop 2000 was used to check and measure the purity and concentration of the isolated RNA. The cDNA library construction and RNA-seq were performed by Biomarker Technologies (Qingdao, China). A total of 1 µg RNA per sample was used to generate a cDNA library using the TruSeq RNA Library Prep Kit for Illumina (Shanghai, China) according to the manufacturer's instructions, and index

codes were added to attribute sequences to each sample. The libraries were sequenced on an Illumina NovaSeq platform to generate 150 bp paired-end reads.

Data processing, transcriptome assembly, and functional annotation

Raw data (raw reads) in Fastq format were first processed through in-house perl scripts. Clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N, and low-quality reads from raw data. All the downstream analyses were based on clean, high-quality data. The clean reads were aligned to the *T. sagittata* genome using HISAT2 with default parameters (Kim et al., 2015). The StringTie was used to assemble the transcript, and the gene expression levels were estimated using fragments per kilobase of transcript per million fragments mapped (FPKM) (Pertea et al., 2015). An absolute fold change ≥ 2 and false discovery rate (FDR) < 0.01 were used as thresholds for the identification of differentially expressed genes (DEGs) between compared groups (LL vs HL and LR vs HR) using DESeq2 software (Love et al., 2014; Varet et al., 2016). Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the clusterProfiler packages [33]. KOBAS database (Bu et al., 2021) and clusterProfiler software (Yu et al., 2012) were used to test the statistical enrichment of differential expression genes in KEGG pathways.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

The validation was performed with the same RNA samples used for RNA-seq analysis. cDNA synthesis was performed using total RNA as the template with SynScript® III RT SuperMix for qPCR (+ gDNA Remover) from Vazyme, China. Twelve genes related to terpenoid biosynthesis were selected for qRT-PCR analysis to validate the reliability of transcriptome results. Primers were designed using Premier 3.0. and all primers used are listed in Table S3 (Koressaar and Remm, 2007). qRT-PCR was performed on a CFX96 Touch Real-Time PCR Detection System (Applied Biosystems, CA, USA). we used the ArtiCan^{ATM} SYBR qPCR Mix (Vazyme, China) according to the manufacturer's instructions for reaction volume. The reaction mixture contained 8.2 μ L of RNase-free ddH₂O, 0.4 μ L of primers (10 μ mol/L), 1 μ L of cDNA, and 10 μ L of ArtiCan^{ATM} SYBR qPCR Mix. The reaction conditions were as follows: 90 °C for 1min, followed by 95 °C for 10 s, and then 60 °C for 20 s for 40 cycles. The gene expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) from *T. sagittata* as the reference gene. The correlation between the qRT-PCR results and transcriptome data was assessed using Pearson's correlation test (Livak and Schmittgen, 2001).

UHPLCQ-TOF-MS analysis

The plant tissue samples consisted of rhizomes and leaves from two types of materials. 1000 μ L of extraction solvent containing an internal standard concentration of 20 mg/L (methanol/acetonitrile/water, 2/2/1, v/v/v) was added to each group (50 mg), then the mixture was

vortexed for 30 seconds. Steel beads were added, and the sample was ground for 10 min, followed by ultrasonication for 10 min. The mixture was then left to stand at -20°C for 60 minutes and centrifuged at 12,000 rpm for 15 minutes at a constant temperature of 4°C. Transfer 500 µL of the supernatant to a vacuum concentrator and dry it. Afterward, reconstitute the residue with acetonitrile\water (1\1, v\v, 160µL). Vortex the obtained extraction solution for 30 s, followed by ultrasonication for 10 min. Then, centrifuge at 12,000 rpm for 15 min at 4°C and collect the supernatant for UHPLC-Q-TOF-MS analysis. A pooled quality control (QC) sample was obtained by mixing all groups to monitor and evaluate the stability and reliability of the data.

The LC/MS system for metabolomics analysis comprises Waters Acquity I-Class PLUS ultra-high performance liquid tandem Waters Xevo G2-XS QToF high-resolution mass spectrometer. The column used is purchased from Waters Acquity UPLC HSS T3 column (1.8µm 2.1*100mm). Mobile phase A is 0.1% formic acid aqueous solution, and mobile phase B is 0.1% formic acid acetonitrile. The flow rate was 400 µL/min, with an injection volume of 1 µL. The gradient elution procedure was as follows: 0–0.25 min, 2% B; 0.25–10 min, linear increase of B to 98%; 10–13min, B maintained at 98%; 13–13.1 min, linear decrease of B to 2%; 13.1–15 min, B maintained at 2%. QC samples are inserted into the sample queue to monitor and evaluate the stability and reliability of the data. The MS conditions were set as follows: The low collision energy is 2V, the high collision energy range is 10 ~ 40V, and the scanning frequency is 0.2 s for a mass spectrum. The parameters of the ESI ion source are as follows: Capillary voltage: 2000V (positive ion mode) or -1500V (negative ion mode); cone voltage: 30V; ion source temperature: 150°C; desolvent gas temperature 500°C; backflush gas flow rate: 50L/ h; Desolventizing gas flow rate: 800L/h.

Data analysis

The raw data collected using MassLynx V4.2 is processed by Progenesis QI software for peak extraction, peak alignment, and other data processing operations based on the Progenesis QI software online METLIN database and Biomark's self-built library for identification (Wang et al., 2016). Principal component analysis was used to judge the repeatability of the samples within the group and the quality control samples. The identified compounds are searched for classification in the HMDB database (Wishart et al., 2018). Combining the difference multiple, the P and VIP values of the OPLS-DA model were adopted to screen the differential metabolites (Thévenot et al., 2015). The screening criteria are FC > 1.5, P value < 0.05, and VIP > 1. The difference metabolites of KEGG pathway enrichment significance were calculated using a hypergeometric distribution test (Kanehisa, 2000; Yu et al., 2012).

Results

Comparative Analysis of Columbin Content in *T. sagittata* varieties

We have collected the rhizome of *T. sagittata* from different locations to measure the content of columbin. A box plot (Fig. 1C) was created based on the distribution range of columbin content in different *T. sagittata* varieties. The variation in columbin content across different varieties was

significant. The concentration of columbin in rhizomes ranges from 5.40 mg/g to 46.09 mg/g, with an average value of 16.05 mg/g and a coefficient of variation of 55.39% (Table S4). According to the standards set by the 2020 edition of the *Chinese Pharmacopoeia*, the content of columbin must not be less than 1.0% (10 mg/g).

Out of 32 samples of *T. sagittata*, 22 samples met the *Pharmacopoeia* standards, resulting in a qualification rate of 68.75% for the herbal material, and among these, 7 samples achieved more than double the pharmacopoeial standard for columbin content, making them suitable for use as high-columbin content materials in the selection and breeding of high-quality *T. sagittata*. Cluster analysis based on columbin content (Fig. 1A) categorizes all tested materials into three groups. The first group consists of materials with extremely high columbin content, containing only one sample. The second group consists of materials with high columbin content, ranging from 14.96 mg/g to 29.70 mg/g. The third group includes materials with low columbin content, ranging from 5.40 mg/g to 11.90 mg/g, with the majority failing to meet the *pharmacopoeial* standards. Since the first group contains only one sample, it is classified as a high columbin content type (H-type material, more than 1.5 times the *Chinese Pharmacopoeia* standard). The third group is classified as a low columbin content type (L-type material, with concentrations ranging from 5.40 mg/g to 11.90 mg/g, with the highest content at the edge of the *pharmacopoeial* standard). The difference in columbin content between these two types is significant (Fig. 1B).

Transcriptomic analysis of *T. sagittata* with different columbin contents

In this study, twelve transcript libraries from rhizomes and leaves of two types of materials were constructed and further used for pair-end sequencing on an Illumina NovaSeq platform. 73.88 Gb clean reads were obtained after filtering reads with low-quality adapters. In particular, the size of the clean reads ranged from 5.71 Gb to 7.17 Gb, with an average of 5.71 Gb. The average Q30 percentages were 93.79%, and the GC content varied from 43.96–44.47%. These indicate that the sequencing quality is good. Then, these clean reads were then mapped to the *T. sagittata* reference genome, and the match rate was 89.25%-93.96% (Table S5). Principal component analysis (PCA) was performed using the FPKM value. The results showed that the three replicates for each sample group are grouped into separate units, indicating good reproducibility, and PC1 and PC2 accounted for 35.68% and 25.7%, respectively. (Fig. 2A)

Differentially expressed genes (DEGs) are selected using fold change ≥ 2 and FDR < 0.05 as the filter criteria for DEGs. DEGs are primarily identified through two comparisons: between the rhizomes of high columbin-content materials and low columbin-content materials (LR vs. HR) and between the leaves of the two groups (LL vs HL). In total, 11,615 DEGs were found in the present study. The LL vs. HL group had the most DEGs with 8,381 (4,926 upregulated, 3,455 downregulated), followed by the LR vs HR group with 6,038 (3,418 upregulated, 2,620 downregulated). In particular, 2,804 DEGs were common in the two comparison groups. To identify the biological function of the DEGs obtained from comparative transcriptome analysis, GO annotation and KEGG pathway analysis were performed. The DEGs are

extensively annotated for both comparison groups in the pathways related to biological processes and molecular functions, which are the two primary categories. Conversely, fewer pathways are annotated within the cellular component category. For LR vs. HR and LL vs. HL, the most significant number of DEGs enriched in the biological process category was cellular processes, with 2312 and 3100 DEGs, respectively, followed by metabolic processes, with 2167 and 2999 DEGs, respectively, and in the cellular component category, the most significant number of DEGs enriched in the cellular component is cell anatomical entity, with 2523 and 3528 DEGs respectively, followed by the cytoplasm, where there are 2139 and 3237 DEGs respectively. The most enriched molecular functions were binding and catalytic activity with 2329 and 2139 DEGs in the LR vs HR groups, respectively. For the LL vs. HL, in the molecular function category the LR vs. HR, the most significant number of DEGs enriched in molecular function were catalytic activity with 3237 DEGs followed by catalytic activity with 3164 DEGs (Fig. 2A, B, and C).

Next, KEGG enrichment analysis was further explored to identify the metabolic pathways of DEGs in the two types of *T. sagittata*. The results of the KEGG enrichment analysis show that most DEGs are enriched in the pathways involved in synthesizing secondary metabolites. For the LR vs. HR, there is a significant enrichment of DEGs in metabolic pathways such as phenylpropanoid biosynthesis, porphyrin and chlorophyll metabolism, benzoxazinoid biosynthesis, and carotenoid biosynthesis (Fig. 2E). In the LL vs HL comparison group, there is significant enrichment of differentially expressed genes (DEGs) in metabolic pathways such as sesquiterpene and triterpene biosynthesis, benzoxazinoid biosynthesis, phenylpropanoid biosynthesis, flavone and flavonol biosynthesis, steroid biosynthesis, flavonoid biosynthesis, and terpenoid backbone biosynthesis (Fig. 2F). Only a small number of DEGs are enriched in the diterpene biosynthesis pathway, and this enrichment did not reach a significant level in either comparison group.

To verify the reliability of the RNA-seq results and the transcriptomic findings' consistency and reliability, 12 genes with significant expression differences between samples were selected for qRT-PCR analysis. 12 genes selected for validation are all related to terpene synthesis and include *DXR*, *DXS*, *HMGR*, *HMGs*, *TPS*, *MVD*, and *GGPPS*. The correlation between the relative expression levels measured by qRT-PCR and the FPKM values from the transcriptomic data was calculated. All selected genes exhibited a high correlation between qRT-PCR and transcriptomic data sets, with the coefficient of determination (R^2) consistently exceeding 0.65 (Fig. 3). These indicate that the transcriptome data are reliable, and the analysis conducted on the dataset obtained from transcriptome sequencing in this study is robust and dependable.

Untargeted Metabolomic Analysis of *T. sagittata* with Different Columbin Contents

In addition to DEGs, the composition and number of metabolites also varied. Untargeted metabolomic profiling of rhizomes and leaves of different material types was conducted using both positive and negative ionization modes to identify differential metabolites. In both negative electrospray ionization (Figure S2 A) and positive electrospray ionization (Figure S2 B) modes, the total ion chromatograms (TIC) of the QC samples displayed consistent peak intensities and retention times, indicating good

instrument stability and affirming the reliability of the results. In summary, a total of 4481 metabolites were detected from all samples. All detected metabolites were grouped into 14 superclasses according to the HMDB database shown in Figure. 4E. Of all the metabolites, the most abundant superclass was lipids and lipid-like molecules with 752 metabolites, including 47 diterpenoids and 76 terpenoid derivatives, following

These organic acids and derivatives were the most abundant compounds, totaling 312, and organoheterocyclic compounds, totaling 263. Principal component analysis (PCA) showed a distinct separation among samples (Fig. 4A). The first main component explained (55.72%) of the total variability, while the second principal component accounted for (14.52%) of the total variability of the data set, which indicated a clean separation of the samples by the components. The results of the PCA indicate significant differences between the rhizomes and leaves of different types of *T. sagittata* materials.

To identify specific differential metabolites (DAMs), the selection criteria include fold-change ≥ 1.5 or fold-change ≤ 0.67 , $P \leq 0.05$, and $VIP \geq 1$. These criteria are applied to compare the combinations of LR vs. HR and LL vs. HL for the screening of differential metabolites, and the results are shown in a Venn diagram and histogram. A total of 495 (227 upregulated and 265 downregulated), 1999 (834 upregulated and 1165 downregulated) were significantly different in LR vs. HR and LL vs. HL, respectively (Fig. 4B). Based on the annotation from the KEGG database, an enrichment analysis of differential metabolite pathways was conducted (Fig. 4C, D). The DEMs in LR vs. HR were significantly enriched in KEGG terms pentose and glucuronate interconversions, amino sugar and nucleotide sugar metabolism, and isoquinoline alkaloid biosynthesis, which contains the highest number of differential metabolites among all enriched metabolic pathways, with 15 differential metabolites. In the LL vs. HL comparison group, the significantly enriched secondary metabolism pathways include limonene and pinene degradation, monoterpene biosynthesis, flavonoid and flavonol biosynthesis, aflatoxin biosynthesis, as well as cutin, suberine, and wax biosynthesis. Both comparison groups showed enrichment in hormone signal transduction, terpenoid biosynthesis, and ABC transporter pathways, although they did not reach significance ($P > 0.05$).

Columbin Synthesis Analysis

Columbin is a diterpenoid compound with a lactone structure. The biosynthetic pathway of diterpenoid compounds can be divided into three parts. Thus, the biosynthetic pathway for columbin can also be divided into three steps: the formation of GGPP, the precursor of all diterpenoids as well as all terpenoids; the construction of carbon skeletons of columbin, clerodane skeleton; and the post-synthesis modification of the molecular skeletons. The biosynthesis of the second and third parts is currently unclear, but the enzymes primarily catalyzing these parts are terpene synthases (TPSs) and cytochrome P450s (CYP450s) (Fig. 5).

According to the KEGG annotation results, 121 genes related to the biosynthesis of columbin were identified in the transcriptome data, and genes differentially expressed between two types of materials were further screened. As shown in Fig. 5, the average expression levels of the five types of genes—DXS, DXR, MCS, GGPPS, and PMK—are significantly higher in the H-type material than in the L-type material. Conversely, HMGR and HDR expression levels are significantly higher in the L-type material than in the H-type material. The expression patterns of MVD in the leaves and rhizomes show opposite results. Many genes encode the same enzyme but in different expression directions. For example, even though five DXS genes (including *TsA02G016310*, *TsB07G004490*, *TsB07G004540*, *TsA07G010890*, and *TsA07G011790*) are more specifically expressed in H-type material, the other two DXS genes (including *TsB02G013160* and *TsA07G011790*) upregulated in L-type material, indicating that the direction and magnitude of these genes transcriptional responses were not conservative. Despite this, the overall expression level of genes encoding the same key enzymes (including DXS, DXR, PMK, MCS, GGPPS) of H-type material was significantly higher than that of L-type material, which was consistent with the accumulation pattern of columbin. These findings suggested that the columbin biosynthesis of *T. sagittata* was accomplished through the collaborative function of many classes of enzymes, which would explain why the columbin content in H-type material was higher than that in L-type material. Isoprene, as the basic unit for biosynthesizing diterpenoid substances, is also present in higher concentrations in H-type materials compared to L-type materials. This indicates a correlation between the amount of the isoprene unit and the final concentration of columbin.

Discussion

Germplasm resources are crucial for the research and cultivation of medicinal plants. Studying these resources lays the foundation for breeding superior varieties and promotes the development of the traditional Chinese medicine industry (Gao et al., 2020). The current study provides a comprehensive analysis of columbin content across various *T. sagittata* varieties, revealing significant variability in its accumulation, which has implications for both the quality control of *T. sagittata* as a medicinal herb and the potential to develop high columbin varieties. The findings confirm that most varieties meet the Chinese Pharmacopoeia standards, yet only a subset demonstrates sufficiently high columbin content to be deemed superior for breeding purposes. This highlights the importance of selecting and propagating high columbin-content varieties to optimize the therapeutic quality of *T. sagittata*. The results showed that the qualification rate of the medicinal materials was 68.75%. Among them, the columbin content in SCLS was the highest, four times the pharmacopeia's standard. However, its agronomic traits are poor, with fewer branches. It can be considered an alternative superior germplasm, and the yield can be further increased by improving its agronomic traits.

Transcriptomic analyses further elucidate differences between high and low columbin-content varieties at the molecular level. The differential gene expression patterns between H-type and L-type materials indicate that the biosynthesis of columbin is likely controlled by a suite of genes involved in terpenoid synthesis, such as DXS, DXR, and GGPPS, which play essential roles in the biosynthetic pathway (Alfieri et al., 2018; You et al., 2020; Zhang et al., 2015). Interestingly, the variation in expression patterns of

these genes suggests a complex regulation mechanism governing diterpenoid accumulation. In the H-type material, genes involved in the early steps of terpenoid synthesis pathways (e.g., DXS and GGPPS) are highly expressed, indicating a more efficient synthesis of GGPP, the precursor of diterpenoids, thus leading to enhanced columbin biosynthesis. Conversely, genes like HMGR and HDR, which exhibit higher expression in L-type materials, might be linked to alternative metabolic routes or feedback mechanisms that limit columbin synthesis in these varieties.

The untargeted metabolomic analysis supports this transcriptional evidence, revealing significant metabolic differences between H-type and L-type materials. The distinct clustering of metabolites and enriched pathways, especially those associated with secondary metabolisms like flavonoid, terpenoid, and phenylpropanoid biosynthesis, suggests that high-columbin content varieties are characterized by active secondary metabolite production (Dixon and Dickinson, 2024; Reshi et al., 2023; Twaij and Hasan, 2022). This may enhance the plants' adaptive response to environmental stresses and potentially contribute to the efficacy of *T. sagittata* in traditional medicine (Dixon and Dickinson, 2024). Moreover, identifying differentially accumulated metabolites associated with terpenoid biosynthesis corroborates the gene expression data, establishing a clear metabolic signature for high columbin varieties. Specifically, enriched pathways like flavonoid biosynthesis and steroid biosynthesis in H-type varieties might reflect co-regulated biosynthetic pathways that support columbin accumulation. Such coordinated biosynthesis of related secondary metabolites could be an adaptive trait selected for in high columbin content varieties, potentially conferring additional medicinal properties.

The KEGG pathway enrichment highlights the intricate metabolic landscape differentiating high and low columbin varieties. Enrichment in pathways related to terpenoid biosynthesis, especially those involving diterpenoids, aligns with the elevated columbin levels in H-type varieties. Pathways such as porphyrin, chlorophyll metabolism, and carotenoid biosynthesis are also enriched, which may have additional implications for the photosynthetic and stress response capacities of H-type varieties (Li et al., 2022; Liu et al., 2023; Luo et al., 2019; Sun et al., 2023). The increased expression of terpene synthase (TPS) genes and cytochrome P450 enzymes, essential for the structural diversity of diterpenoids, further underscores the metabolic commitment of H-type varieties to columbin synthesis.

Finally, the validation of transcriptomic data through qRT-PCR confirms the reliability of our findings and provides a robust basis for future functional studies aimed at understanding columbin biosynthesis regulation. Given that columbin is synthesized through several catalytic steps involving enzymes like TPS, diTPS, and P450s, further research on the specific roles of these enzymes in *T. sagittata* could reveal precise regulatory mechanisms. The variation in gene expression across multiple genes coding for the same enzymes also suggests potential regulatory diversification, which might be exploited in future breeding efforts.

This study establishes a foundation for columbin biosynthesis research in *T. sagittata* and identifies genetic and metabolic markers that can facilitate the selection of high columbin-content varieties. Such

insights are critical for cultivating *T. sagittata* as a high-quality medicinal resource and optimizing its therapeutic potential through breeding and biotechnological strategies.

Conclusion

This study identifies high columbin *T. sagittata* varieties that meet and exceed the Chinese Pharmacopoeia standard, making them ideal for breeding superior medicinal plants. Transcriptomic analysis reveals key genes—like DXS, DXR, and GGPPS—are upregulated in high columbin varieties, enhancing GGPP production, a precursor to columbin biosynthesis. Metabolomic profiling supports these findings, with enriched pathways for terpenoid, flavonoid, and phenylpropanoid biosynthesis, suggesting these high columbin varieties may offer enhanced therapeutic benefits. The validation of transcriptomic data with qRT-PCR confirms the reliability of these genetic markers. This research provides a foundation for breeding high-quality *T. sagittata* varieties with optimized columbin content, promising applications in medicinal and agricultural fields.

Declarations

Conflicts of interests

The authors declare no conflict of interest.

Author Contributions

Yonghui Sang and Mohammad Murtaza Alami contributed equally: Writing – review & editing, Writing an original draft, Software, Resources, Methodology, Investigation, Formal analysis, and Data curation. Haiqian Si: Visualization, Resources, Data curation. Shaohua Shu and Shengqiu Feng: Validation, Software, Resources, Data curation. Zhinan Mei: Validation, Supervision, Project administration, Data curation, Conceptualization. Xuekui Wang: Writing review & editing, Visualization, Validation, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Figures

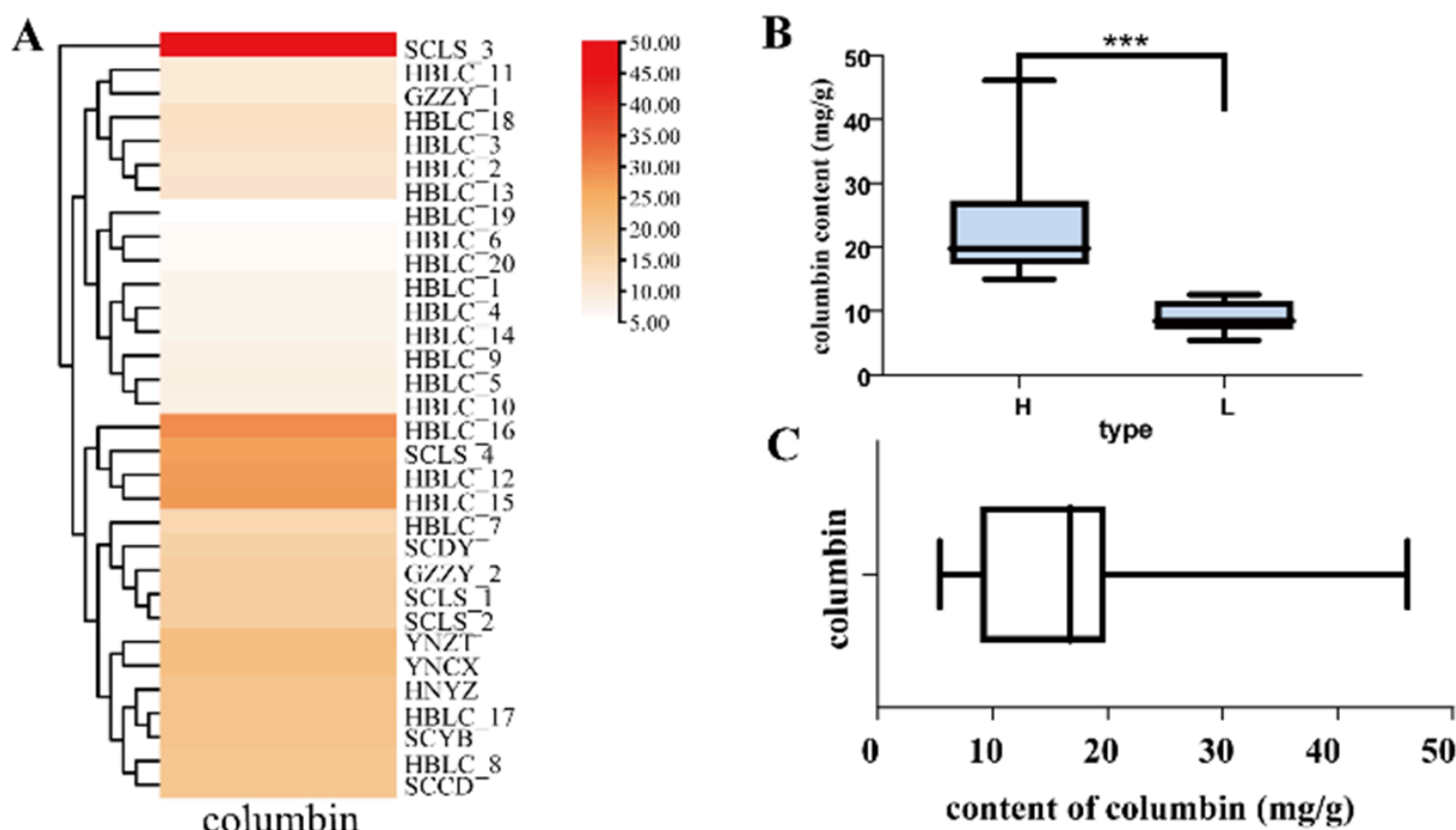


Figure 1

(A) Cluster analysis of columbin in the rhizome of different *T. sagittata* varieties; (B) The significant analysis of the content of columbin between the two types of materials; (C) Variation range of columbin in the rhizome of different *T. sagittata* varieties. SCCD; Huaxi Village, Tongji Town, Pengzhou City, Chengdu City, Sichuan Province. GZZY; Zunnan Avenue, Bozhou District, Zunyi City, Guizhou Province. SCLS; Taoxi Village, Laoheba Township, Mabian County, Leshan City, Sichuan Province. SCYB; Ganxi Village, Qianxi Town, Xuzhou District, Yibin City, Sichuan Province. YNZT; Taiping Village, Taiping Town, Shuifu City, Zhaotong City, Yunnan Province. SCDY; (Tianbao Village, Yinghua Town, Shifang City, Sichuan Province. HBLC; Maotian Village, Tuanbao Town, Lichuan City, Enshi Prefecture, Hubei Province.

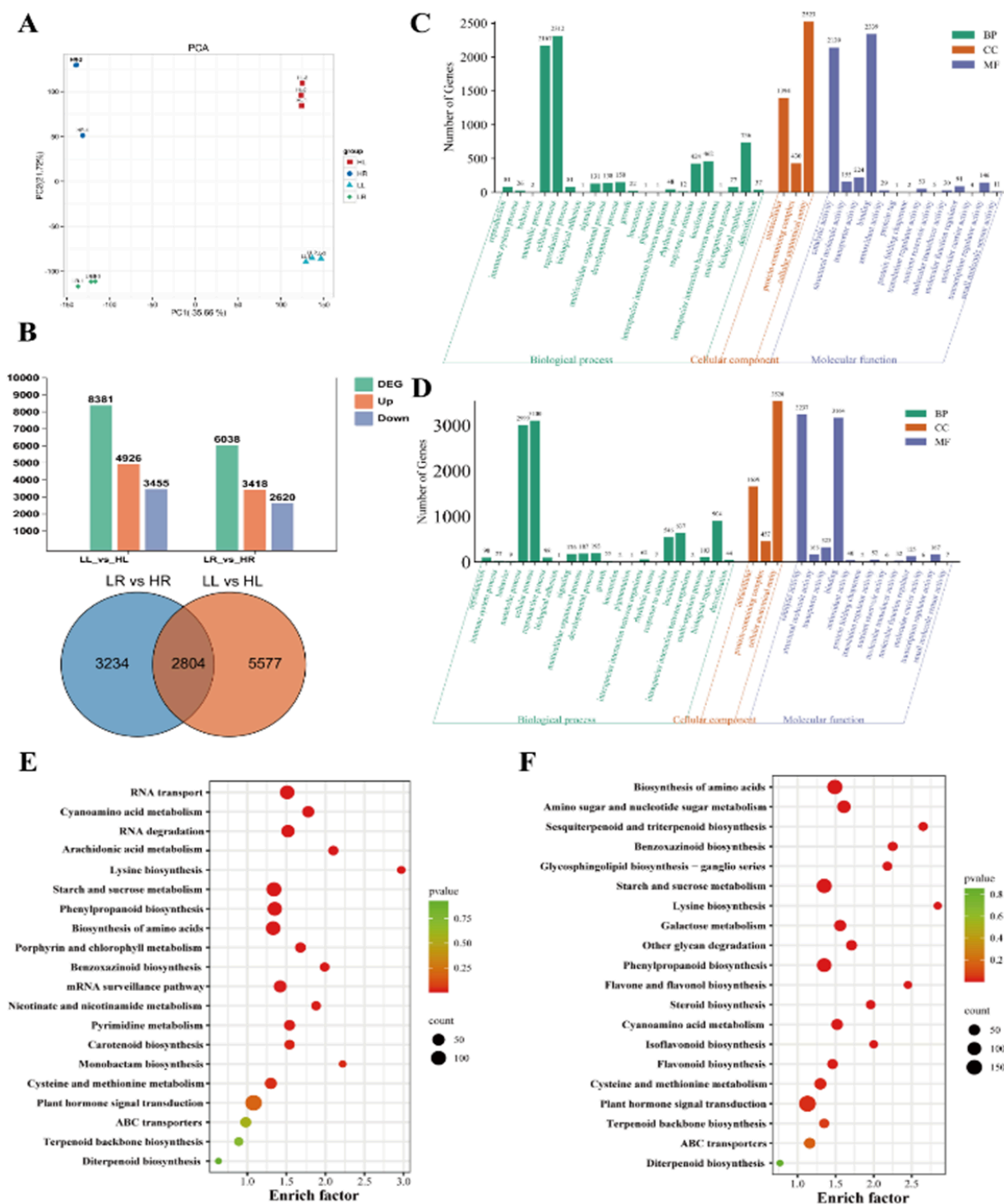


Figure 2

Identification and enrichment analysis of differentially expressed genes for the LL vs. HL and LR vs. HR groups; (A) Principal component analysis of the transcriptome data from the LL, HL, LR and HR samples; (B) Statistical analysis of upregulated and downregulated DEGs for the LL vs. HL and LR vs. HR groups, and the Venn diagram of DEGs for the S LL vs. HL and LR vs. HR groups; (C) GO enrichment analysis of DEGs for the LR vs. HR groups; (D) GO enrichment analysis of DEGs for the LL vs. HL groups; (E) KEGG

enrichment analysis of DEGs for the LR vs. HR groups; (F) KEGG enrichment analysis of DEGs for the LL vs. HL groups. The abscissa is the enrichment factor, representing the ratio of the proportion of genes annotated to a pathway in the differential gene to the proportion of genes annotated to the pathway in all genes. A more prominent enrichment factor indicates a more significant enrichment level of the differentially expressed genes in this pathway.

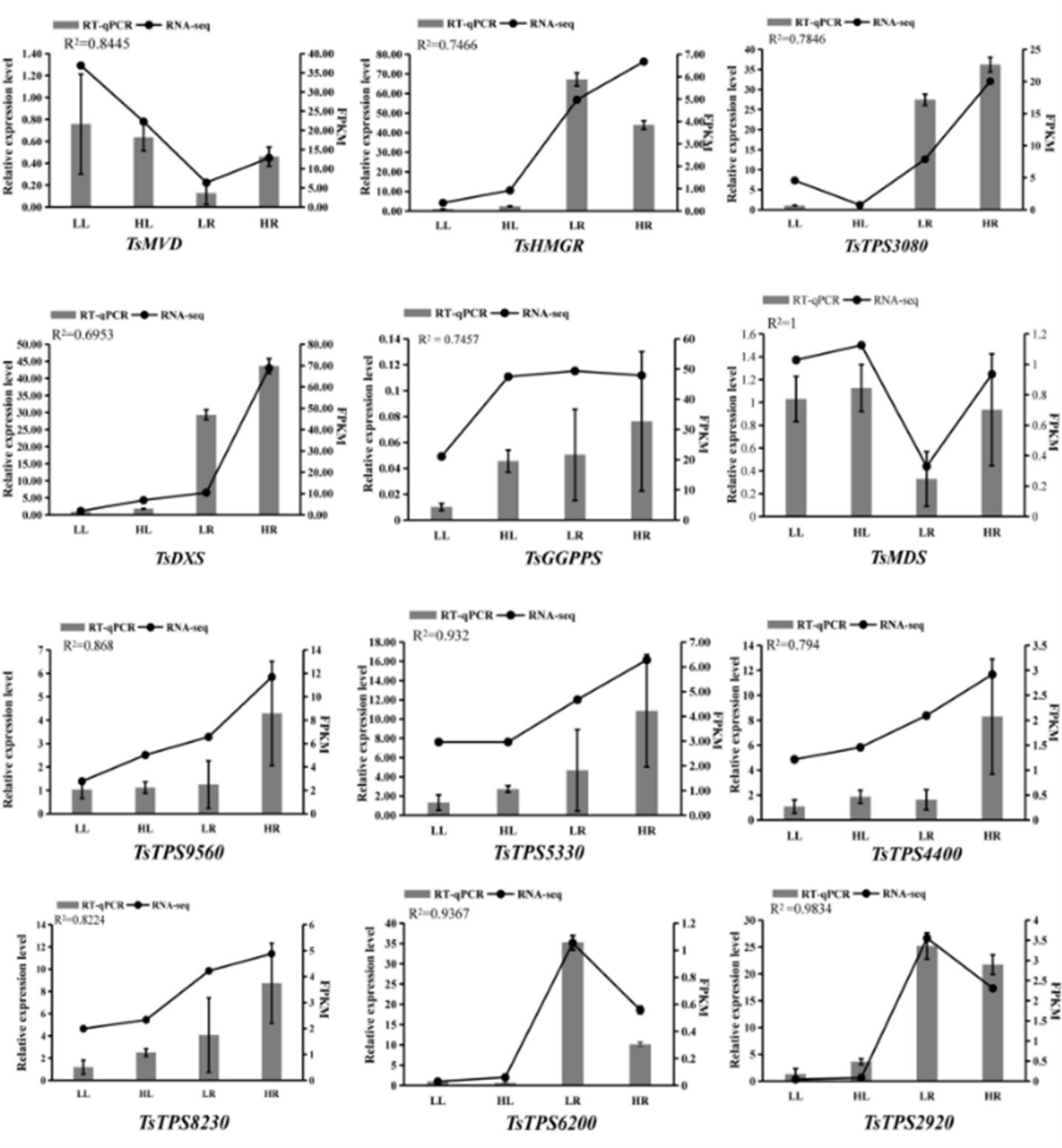


Figure 3

Verification of 12 genes by qRT-PCR. The gray histogram shows the qPCR results, and the black line shows the RNA-seq data results.

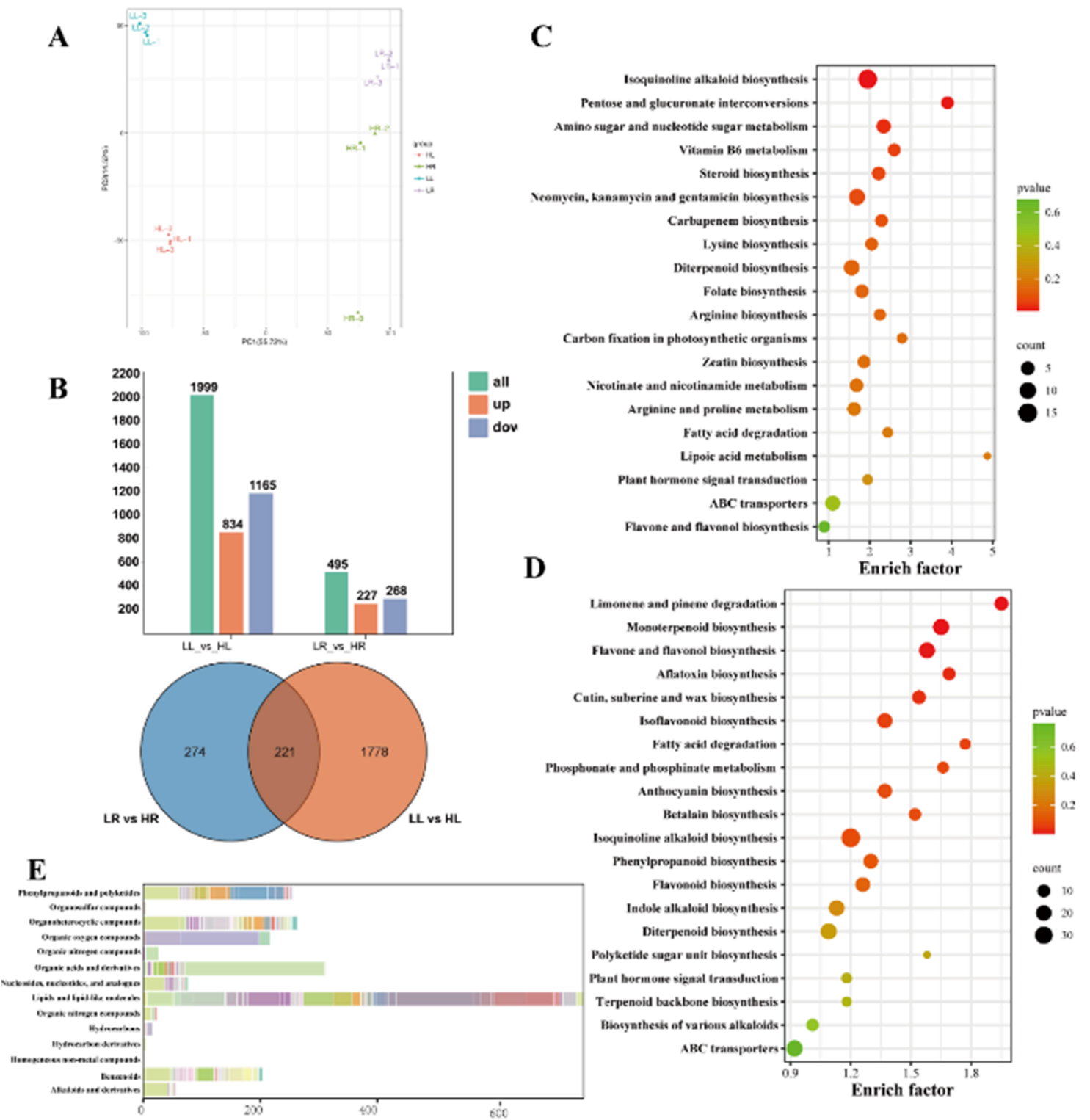


Figure 4

Identification and enrichment analysis of differentially accumulated metabolites; (A) PCA scores plot of four samples; (B) Statistical analysis of upregulated and downregulated DEMs for the LL vs. HL and LR vs. HR groups, and the Venn diagram of DEMs for the LL vs. HL and LR vs. HR groups; (C) KEGG

enrichment analysis of DEMs for the LR vs. HR groups; (D) KEGG enrichment analysis of DEMs for the LL vs. HL groups. (E) Identified metabolites classification histogram according to HMDB database.

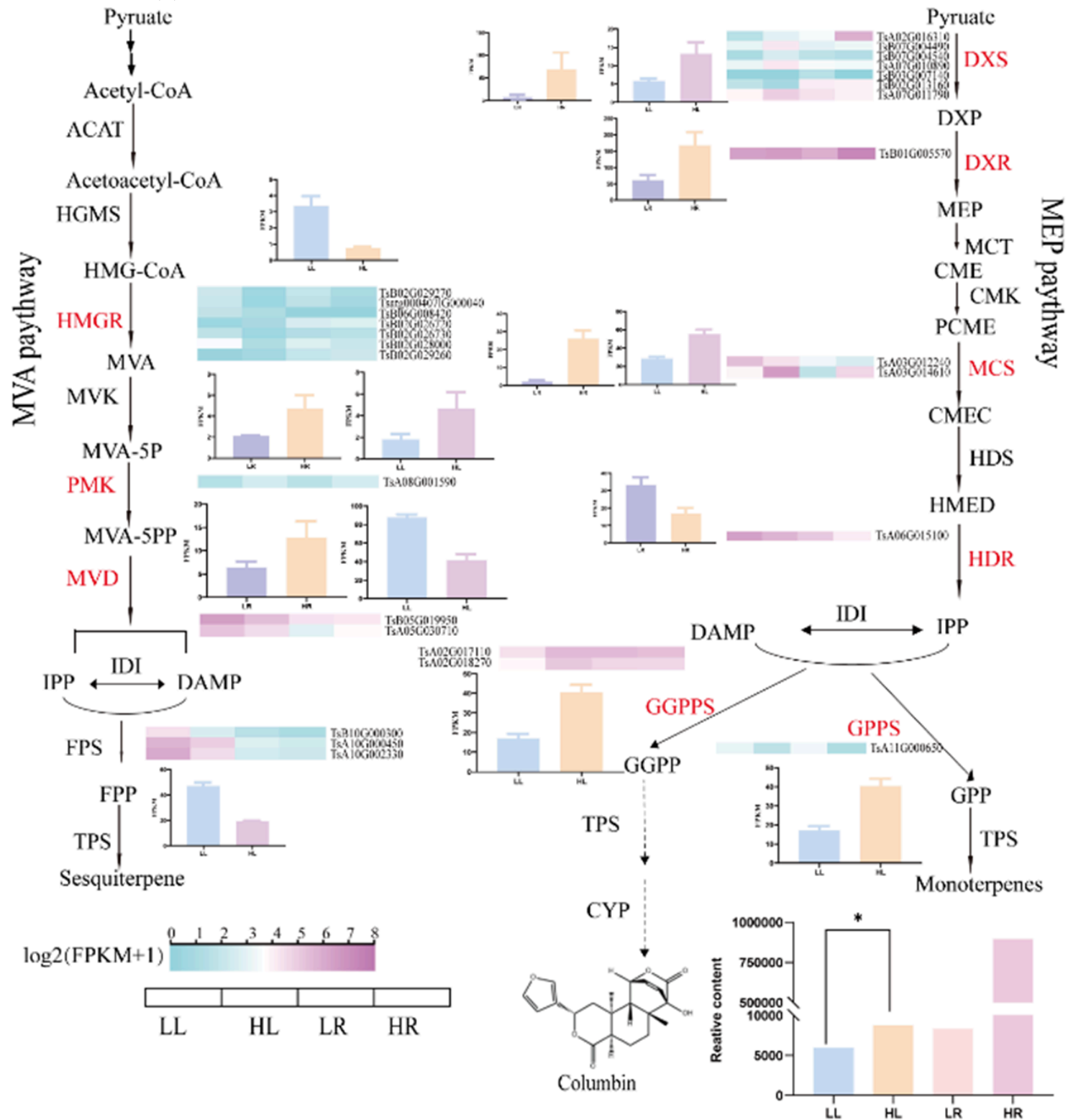


Figure 5

Schematic models for the biosynthesis of columbin. The heatmap represents genes with differential expression levels in the three comparison combinations, the expression levels in four samples, and the bar box near the heatmap represents the average FPKM of these genes. The solid arrows represent

known reactions and one-step reactions, and the dotted arrow represents speculative and multi-step reactions.

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