

Metabolomic and Transcriptomic Analyses Reveals Candidate Genes and Pathways Involved in Secondary Metabolism in *Bergenia purpurascens*

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Abstract

Bergenia purpurascens is an important medicinal, edible and ornamental plant. The lack of omics information hinders the study of its metabolic pathways and related genes. In order to investigate candidate genes and pathways involved in secondary metabolism in *B. purpurascens*, roots, stems and leaves of *B. purpurascens* were subjected to metabolomic and transcriptomic analyses in this study. A total of 351 differentially accumulated secondary metabolites were identified. We identified 111 candidate enzyme genes in phenylpropanoid and flavonoid biosynthesis pathway. 22 candidate key genes related to phenylpropanoid and flavonoid synthesis were obtained by WGCNA analysis, from which 14 structural genes in phenylpropanoid and flavonoid synthesis and five transcription factors related to flavonoid synthesis were identified. This study provides data resources and new insights for further studies on the biosynthesis of major active components in *B. purpurascens*.

INTRODUCTION

Bergenia purpurascens (Hook.f.et Thoms.) Engl., also called *Purple Bergenia*, is a perennial herb of the genus *Bergenia* in the family Saxifragaceae, grows under forests, shrubs, alpine meadows, and alpine gravel gaps at an altitude of 2700–4800 meters, distributed in southwestern Sichuan, northern Yunnan, southern and eastern Tibet of China, as well as the northern region of South Asia.[1] *B. purpurascens* is considered an important medicinal, edible and ornamental plant.

The *Bergenia* plant have a variety of bioactive substances, including coumarins, flavonoids, terpenoids, polysaccharides, alkaloids, polyphenols, organic acids, et. The rhizomes and leaves of the *Bergenia* plants are used as a remedy for different diseases in the folk medicine system in Russia and Asia.[2] It has a wide range of pharmacological activities. *Bergenia crassifolia* meets the criteria of being adaptogens, and increases the power of resistance against multiple (physical, chemical or biological) stressors[3–5]. *B.purpurasceus* extracts has anti-inflammatory and anti-bacterial effects[6]–[7]. Treating wounds with *Bergenia ciliate* rhizome extract can promote healing [8, 9]. *Bergenia ligulata* extract has antilithiatic and antioxidant properties[10, 11]. The polysaccharides from *Bergenia emeiensis* exhibited a strong antioxidant capacity[12, 13]. *Bergenia ligulata* has an anticancer effects and it induces apoptosis[14]–[15]. Zhao et al. identified dozens of volatile compounds in *B. purpurascens*[16]. Qu et al. identified 43 chemical constituents in *B. purpurascens*, mainly flavonoids (18), phenolic glycosides (13) and organic acids (7)[17]. So far, more than 100 compounds were isolated and identified from the genus *Bergenia*.

Bergenia contains complex active ingredients that have a variety of important pharmacological effects. The current research on the genus *Bergenia* is mainly focused on pharmacological effects and chemical composition identification. It has become particularly important to understand the pathways of secondary metabolite synthesis in *Bergenia*. According to studies, the accumulation of secondary metabolites in plants is influenced by various factors including growth and development, temperature, light and water[18]. Considering the lack of high-throughput omics analysis of *Bergenia*, we collected

roots, stems and leaves of *B. purpurascens* for transcriptome sequencing and metabolomic analysis to investigate candidate genes and pathways involved in secondary metabolism in *B. purpurascens*. Through comparative analysis of transcriptome and metabolome of different organs, we obtained a large number of differentially expressed genes (DEGs) with differentially accumulated metabolites (DAMs), and the key metabolic pathways of phenylpropanoids and flavonoids were identified. These results provide data resources and new insights for further studies on the molecular mechanisms of the synthesis of the main active components of *B. purpurascens*.

MATERIALS AND METHODS

Plant Materials

The *B. purpurascens* in this study was collected from Jiulong County, Sichuan Province, China. Its species information was confirmed by morphological identification and DNA barcode technology with *ITS2* and *psbA-trnH* sequences [19]. Three-year-old *B. purpurascens* roots, stems and leaves were collected for transcriptomic and metabolomic analysis, and all experiments in this study were performed in three biological replicates.

Quasi-Targeted Metabolomics

We performed a quasi-targeted metabolomic analysis of three biological replicate samples of *B. purpurascens* roots, stems and leaves to explore the differences in transcriptional and metabolic in different organs. The metabolomic analysis was executed by Novogene Co. Ltd. (Beijing, China). The experimental samples were detected using MRM (Multiple Reaction Monitoring) based on novogene in-house database. The data files generated by HPLC-MS/MS were processed using the SCIEX OS Version 1.4. These metabolites were annotated using the KEGG database (<http://www.genome.jp/kegg/>), HMDB database (<http://www.hmdb.ca/>) and Lipidmaps database (<http://www.lipidmaps.org/>). Principal components analysis (PCA) and Partial least squares discriminant analysis (PLS-DA) were performed at metaX. We applied univariate analysis (t-test) to calculate the statistical significance (P-value). The metabolites with VIP > 1 and P-value < 0.05 and |log2fold change| ≥ 1 were considered to be DAMs.

RNA-Seq and Data Analysis

We performed RNA-seq of three biological replicate samples of *B. purpurascens* roots, stems and leaves. The RNA-seq was executed by Majorbio Ltd. (Shanghai, China). Total RNA was extracted from each sample, and the purity and concentration of RNA were assayed by Nanodrop2000. RNA integrity was detected by agarose gel electrophoresis, and RIN (RNA integrity number) were determined with DNF-471 detection kit and Agilent2100. The mRNA was isolated from total RNA using magnetic beads with Oligo (dT), while cDNA libraries were generated using mRNA from each sample. Sequencing was performed using the Illumina Novaseq 6000 platform. Gene annotation was performed based on NR, Swiss-Port, Pfam, eggNOG, GO, and KEGG databases. DEGs in different organs of *B. purpurascens* were screened

with DESeq2. GO and KEGG enrichment analysis of DEGs was performed based on Majorbio cloud platform (<https://cloud.majorbio.com/>).

qRT-PCR

To verify the RNA-seq data, qRT-PCR analysis was performed on an qTower³G real-time PCR system (Analytik Jena GmbH). A housekeeping gene, *GAPDH*, was used to normalize the expression of the target gene; it was synthesized according to the primer sequence described by Yin et al.[20]. The primers used for qRT-PCR were designed using NCBI Primer blast (Table S1). To ensure reproducibility, qRT-PCR reactions for each sample were performed in three technical replicates and three biological replicates. The relative expression levels of ten selected regulatory genes were examined using qRT-PCR. The expression patterns of the DEGs obtained from RNA-seq and qRT-PCR were consistent (Figure S1), indicating the reliability of the RNA-seq results. The $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression.

Weighed gene co-expression network analysis

For coexpression network analysis, we conducted weighted gene coexpression network analysis with the default settings on the online tool of Majorbio Cloud Platform (<https://cloud.majorbio.com/page/tools/>). To obtain the genes related to main secondary metabolites biosynthesis, all DEGs (TPM > 2) and metabolomics data detected in the three organs of *B. purpurasceus* were subjected to WGCNA to construct trait-related modules. The Cytoscape software (version 3.9.1) was used to visualize the co-expression network.

RESULTS

3.1 Transcriptomic Identification of DEGs in the Comparisons between Different Tissues of *B. purpurasceus*

Based on the transcriptomic analysis of root, stem and leaf of *B. purpurasceus*, a total of 65.66 Gb of clean reads were obtained from nine kernel samples; each sample contained ≥ 6 Gb of data with Q20 quality scores of $\geq 97.49\%$ and Q30 quality scores of $\geq 93.03\%$ (Table S2).

Under the parameters of “ $|\log_2 \text{ fold change}| \geq 1$ ” and “ $p \leq 0.05$ ”, 33994 DEGs (21416 and 12578 up-regulated and down-regulated, respectively) were identified by comparing root and leaf, 18132 DEGs (14252 and 3880 up-regulated and down-regulated, respectively) were identified by comparing root and stem, 24224 DEGs (14005 and 10219 up-regulated and down-regulated, respectively) were identified by comparing leaf and stem (Table S3 and Fig. 1A). Among them, there were 534 DEGs associated with secondary metabolism (Figure S2A). An overview of the expression profiles of all identified DEGs after filtration ($|\log_2 \text{ fold change}| \geq 1$, $p < 0.05$, $\text{TPM} \geq 2$) is shown in the heatmap in Fig. 1B. We compared the up-regulated and down-regulated DEGs from different comparisons using Venn diagram; including 12514

up-regulated and 1609 down-regulated DEGs in the root, 1782 up-regulated and 957 down-regulated DEGs in the stem, 10156 up-regulated and 6087 down-regulated DEGs in the leaf (Fig. 1C, D).

3.2 GO and KEGG Pathway Enrichment Analyses of DEGs in the Comparisons between Different Tissues of *B. purpurascens*

To determine the function of the identified DEGs, we performed GO analysis, which shows GO term enrichment in three major functional categories: molecular function (MF), cellular component (CC), and biological process (BP) (Table S4_Sheet 1–3). The top 10 enriched GO terms from all DEGs across different comparisons are shown in Figure S3.

To further analyze the biological functions of DEGs, we performed KEGG enrichment analysis. The DEGs of root vs. stem comparison were mainly related to flavonoids biosynthesis, Pentose and glucuronate interconversions, Monoterpenoid biosynthesis and Phenylpropanoid biosynthesis ($p < 0.05$) (Figure S4A; Table S5_Sheet1). The DEGs in the root vs. leaf comparison were mainly related to biosynthesis of cofactors, and others enriched were mostly related to nutrition metabolism, such as starch and sucrose metabolism and amino acid metabolism ($p < 0.05$) (Figure S4B; Table S5_Sheet2). The DEGs in the leaf vs. stem comparison were mainly related to flavonoid biosynthesis, phenylpropanoid biosynthesis, glutathione metabolism, and other plant secondary metabolism ($p < 0.05$) (Figure S4C; Table S5_Sheet3). A total of 139, 141 and 139 pathways were enriched in root vs. stem, root vs. leaf and leaf vs. stem comparisons. Flavonoid biosynthesis, Monoterpenoid biosynthesis and Phenylpropanoid biosynthesis pathways were enriched in root vs. stem and leaf vs. stem comparisons.

3.3 Metabolomic Identification of DAMs in the Comparisons between Different Tissues of *B. purpurascens*

Based on the quasi-targeted metabolomics analysis of root, stem and leaf of *B. purpurascens*. A total of 948 metabolites were identified, including amino acid and derivatives (180), flavonoids (161), carbohydrates and its derivatives (106), organic acid and its derivatives (97), nucleotide and its derivatives (59), organoheterocyclic compounds (50), lipids (49), phenolic acids (45), phenylpropanoids and polyketides (37), terpenoids (33), phenols and its derivatives (31), amines (22), alkaloids and derivatives (22), etc (Figure S5A). PLS-DA revealed significant biochemical changes in three groups (Figure S5B, C, D). This suggested that VIP analysis could be used to screen for DAMs.

Under the parameters of “VIP > 1, $|\log_2 \text{fold change}| \geq 1$ ” and “ $p < 0.05$ ”, a number of (DAMs) were identified in the different comparisons, including 125 up-regulated and 138 down-regulated DAMs in the root vs. stem comparison, 161 up-regulated and 206 down-regulated DAMs in the root vs. leaf comparison, 192 up-regulated and 189 down-regulated DAMs in the leaf vs. stem comparison (Fig. 2A, Table S6). Among them, there were 351 differentially accumulated secondary metabolites (Figure S2B). An overview of the metabolite profiles of the three tissues is shown in Fig. 2B. We compared the up-regulated and down-regulated DAMs from different comparisons using a Venn diagram; including 37 up-regulated and 17 down-regulated differentially accumulated secondary metabolites (DASMs) in the root,

31 up-regulated and 14 down-regulated DASMs in the stem, 53 up-regulated and 50 down-regulated DASMs in the leaf (Fig. 2C, D, Figure S6).

3.4 KEGG Pathway Enrichment of DAMs in the Comparisons between Different Tissues of *B. purpurasceus*

Based on KEGG annotation, the DAMs in different comparisons were enriched in many pathways, including biosynthesis of secondary metabolites, flavonoid biosynthesis, phenylpropanoid biosynthesis, and amino acid metabolism (Table S7). The top 20 KEGG terms are shown in Figure S7, of which the most significantly enriched terms in the root vs. stem comparison were flavonoid biosynthesis and phenylpropanoid biosynthesis (Figure S7A). In the root vs. leaf and leaf vs. stem, biosynthesis of secondary metabolites was the most enriched term (Figure S7B, C).

3.5 Weighed gene co-expression network analysis

In order to explore phenylpropanoid and flavonoid biosynthesis related genes, we performed WGCNA analysis on all DEGs and seven modules were identified (Figure S8A). As shown in Fig. 3A, the correlation analysis between modules and traits revealed that ME-A and ME-F modules demonstrated significant correlations with the biosynthesis of the majority of phenylpropanoid metabolites. The ME-A module (contains 334 genes) exhibited a positive correlation with a total of 25 metabolites, while the ME-F module (contains 920 genes) displayed a negative correlation with the same set of 25 metabolites. Among them, 746 genes exhibited significant correlations with coumarins (Fig. 3B; Table S8_Sheet3). 921 genes in ME-A and ME-F exhibited significant correlations with the synthesis of flavone and flavonol (Figure S9; Table S8_Sheet4).

KEGG analysis was carried out to annotate the gene function in these two modules (Figure S8B, C). Genes in ME-A module were mainly enriched in phenylpropanoid and flavonoid biosynthesis pathways. Genes in ME-F module were mainly enriched in flavonoid biosynthesis pathways. The structural and regulatory genes play important roles in phenylpropanoid and flavonoid synthesis (Fig. 6, 7). In the ME-A module, four structural genes (*CCR*, *CAD*, *COMT*, and *CCoAOMT*) involved in the phenylpropanoid biosynthesis pathway and one structural gene (*PGT1*) associated with the flavonoid biosynthesis pathway were identified. Conversely, the ME-F module exhibited the presence of two structural genes (*PAL* and *4CL*) involved in the phenylpropanoid biosynthesis pathway, along with seven structural genes (*CHS*, *CHI*, *F3H*, *DFR*, *LAR*, *ANS*, and *ANR*) associated with the flavonoid biosynthesis pathway. The presence of numerous structural genes associated with phenylpropanoid and flavonoid biosynthesis in the two modules provided further evidence to suggest that the coexpression genes in these modules were involved in the accumulation of phenylpropanoids and flavonoids in *B. purpurasceus*. It was found that the genes in the two modules were also significantly enriched for plant hormone signal transduction pathways.

Transcription factors play crucial roles in regulating genes involved in various aspects of plant growth and development, encompassing secondary metabolism. In this study, the transcription factors in ME-A

and ME-F modules include four *bHLHs*, ten *MYBs*, one *NAC*, seven *WRKYs*, four *LBDs*, nine *ZINC-FINGERS*, ten *AP2/ERFs*, two *MADSs* and five *bZIPs*. Among them, *DN643_c0_g1* (*GLABRA3*, a bHLH transcription factor) might involve in anthocyanin biosynthesis. Additionally, *DN1014_c1_g4* (*MYBD*), *DN1283_c0_g1* (*MYB7*), *DN13711_c0_g1* (*MYB4*) and *DN4322_c0_g2* (*MYB111*) were related to flavonol and anthocyanin biosynthesis.

The top 20 hub genes associated with phenylpropanoid and flavonoid biosynthesis, which exhibiting the highest connectivity values in the ME-A and ME-F modules, were subjected to gene correlation analysis with Cytoscape software (Fig. 4A, B). Two genes related to lignin biosynthesis (*DN1716_c0_g1*, *KNAT7* and *DN1657_c0_g1*, *CRL2*) in the ME-A module. A gene related to coumarin synthesis (*DN44914_c0_g1*, *COSY*) and a MYB transcription factor (*DN13711_c0_g1*, *MYB4*) related to flavonoid biosynthesis in the ME-F module. Other genes might also be involved in phenylpropanoid and flavonoid biosynthesis, which needs further verification.

3.6 Phenylalanine Biosynthesis in Different Tissues of *B. purpurasceus*

At least 25% of photosynthetic products in plants are stored in phenylpropanoids (e.g., lignans, flavonoids) derived from phenylalanine, and multiple classes of secondary metabolites associated with this pathway were identified in *B. purpurasceus* associated with this pathway. We therefore analyzed the key DEGs and DAMs involved in the phenylalanine biosynthetic pathway. We identified 19 candidate enzyme genes for phenylalanine biosynthesis among all DEGs, including *AcoC* (chorismate synthase), *CM* (chorismate mutase), *PAT* (prephenate aminotransferase), and *ADT* (arogenate dehydratase), whose expression levels were mostly up-regulated in leaf (Table S9_Sheet1). The accumulation of phenylalanine was higher in stem and the expression level of *ADT* was relatively high in the stem (Fig. 5B). This pathway is also the upstream pathway for the synthesis of several alkaloids, and Fig. 5A shows the accumulation pattern of 22 alkaloids identified in *B. purpurasceus* (Fig. 5A).

3.7 Phenylpropanoid Biosynthesis in Different Tissues of *B. purpurasceus*

Comparative analysis of the transcriptome and metabolome of different organs of *B. purpurasceus*, DEGs and DAMs in different comparisons were significantly enriched in the phenylpropanoid biosynthesis pathway. We analyzed the major DEGs and DAMs involved in this pathway. We identified 89 candidate enzyme genes related to phenylpropanoid biosynthesis (Table S9_Sheet2). Figure 6 shows that *PAL* (phenylalanine ammonia-lyase), *4CL* (4-coumarate-CoA ligase), and *CAD* (cinnamyl-alcohol dehydrogenase) genes were up-regulated in leaves, *E1.11.1.7* (peroxidase) gene were mainly up-regulated in stems and roots, and *CYP73A* (trans-cinnamate 4-monooxygenase) was highly expressed in all tissues. A total of 12 metabolites were enriched in the phenylpropanoid biosynthesis pathway. The accumulated levels of p-coumaraldehyde, p-coumaryl alcohol, coniferyl alcohol were highest in the leaf. The accumulated levels of phenylalanine and caffeyl aldehyde were highest in the stem. The accumulated levels of p-coumaric acid, ferulic acid, coniferyl aldehyde and coniferin were highest in the root. (Fig. 6, Table S10_Sheet2)

3.8 Flavonoid Biosynthesis in Different Tissues of *B. purpurasceus*

DEGs and DAMs in all comparisons were significantly enriched in the flavonoid biosynthesis pathway. We identified 22 candidate enzyme genes for flavonoid biosynthesis, mostly up-regulated in stems, including *E5.5.16* (chalcone isomerase), *CHS* (chalcone synthase), *CYP73A* (trans-cinnamate 4-monooxygenase), *ANS* (anthocyanidin synthase), *F3H* (naringenin 3-dioxygenase), *F3'H* (flavonoid 3'-monooxygenase) and *DFR* (dihydroflavonol 4-reductase) (Fig. 7, Table S9_Sheet3). Nineteen flavonoid metabolites were identified in flavonoid biosynthesis pathway, mostly up-regulated in root, such as epicatechin and catechol. Naringenin chalcone and naringenin were up-regulated 215.3-fold and 8.8-fold in the root vs. leaf, up-regulated 23.8-fold and 24-fold in the root vs. stem. (Fig. 7, Table S10_Sheet3)

DISCUSSION

B. purpurasceus exhibits a high abundance of secondary metabolites, but their biosynthesis and regulatory mechanisms are unclear. This study provides a new perspective for comprehending candidate genes and pathways involved in secondary metabolism in *B. purpurasceus*, and provide data resources and new insights for further studies on the molecular mechanisms of major active ingredient synthesis in *B. purpurasceus*.

In the wake of genomics and proteomics, metabolomics is a new technology emerging in histology[21]. In metabolomics, utilizing high-throughput methods, many metabolites can be analyzed qualitatively as well as quantitatively, and the relationship between metabolite levels and physiological changes can be discovered.[22] In this study, a quasi-targeted metabolomics analysis was performed on the roots, stems and leaves of *B. purpurasceus*, and a total of 948 metabolites were detected in three groups of samples. These metabolites were significantly enriched in the secondary metabolite biosynthesis pathway, flavonoid biosynthesis pathway, and phenylpropanoid biosynthesis pathway. Additionally, the coumarins compound bergenin was used as a quality indicator for the medicinal herbs of *B. purpurasceus*. Therefore, we evaluated the major secondary metabolites related to the phenylpropanoid metabolic pathway in *B. purpurasceus*.

Phenylalanine is the initiator of the phenylpropanoid biosynthetic pathway, which is synthesized in chloroplasts using chorismate from the shikimate pathway as precursors, mainly through a three-step reaction involving CM, PAT, and ADT. This pathway is conserved in photosynthetic bacteria and plants, with ADT being the key enzyme that catalyzes the final step of the reaction. Its activity is typically inhibited by feedback from phenylalanine.[23] From the transcriptomic data, seven *ADT* genes were identified, with most being up-regulated in the leaf, while metabolomics showed that phenylalanine was more abundant in the stem. This may be due to the feedback inhibition of phenylalanine on *ADTs* or that it is transported between different organs (Fig. 4).

Phenylpropanoid metabolism yields more than 8,000 metabolites, which is one of the main pathways of plant secondary metabolism and plays an important role in plant development and plant-environment interplay. At present, terrestrial plants have evolved several branches of this pathway and produce

metabolites including lignin, flavonoids, lignans, phenylpropanoid esters, hydroxycinnamic acid amides, and sporopollenin. The first three steps of the phenylpropanoid pathway are catalyzed by phenylalanine ammonia lyase (PAL), cinnamic acid 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL), which provide precursors for all of the downstream metabolites.[24]

Through WGCNA analysis, 22 candidate genes associated with phenylpropanoid and flavonoid were identified. Among them, six structural genes for phenylpropanoid synthesis were disclosed, of which the *PAL*, *CCR*, *CAD* and *COMT* were identified as the key genes for lignin synthesis and have high expression level in leaves. Multiple lignin synthesis precursors accumulate at higher levels in leaves. Meanwhile, we identified two lignin synthesis-related genes, *KANT7* and *CRL2*, among the candidate hub genes associated with phenylpropane synthesis. *KNAT3* synergizing with *KNAT7* functions positively for the synthesis of S-lignin, *KNAT3* and *KNAT7* play a synergistic role in regulating the growth and development of *Arabidopsis thaliana* secondary cell wall. [25] *AtCRL2* is involved in the synthesis and/or maintenance of vascular tissue. [26]

The coumarins is a kind of the main biological active substance of *B. purpurasceus*. Bergenin possesses a wide range of pharmacological activities such as anti-cancer, anti-inflammatory, antimicrobial, immunosuppressive, antioxidant and skin-protective activities.[27] Warfarin continues to be used as a first-line drug in clinical practice.[28] Coumarins play important roles in plants including acting as antagonists of auxin involved in growth regulation;[29] absorbing ultraviolet radiation, protecting young plants from excessive sunlight radiation; preventing viral diseases in plants. *B. purpurasceus* roots and stems accumulate large amounts of coumarins, which may be one reason of its slow root and stem growth. Induced systemic resistance (ISR) is one important defense mechanism of plants, conferring plants with rapid, whole-body immune response to phytopathogen attack, which can be triggered by beneficial microbes.[30] The ISR-activated plants-microbiota association can reinforce the defense intensity by recruiting ISR-related strains[31] that are chemoattracted by root exudates containing plant secondary metabolites, such as coumarins and benzoxazinoids.[32, 33] We identified a coumarin synthase gene, *COSY*, among the candidate hub gene related to phenylpropanoid synthesis. *COSY* is a key enzyme in the biosynthesis of coumarins.[34] *Arabidopsis thaliana cosy* mutants have strongly reduced levels of coumarin and accumulate o-hydroxyphenylpropanoids instead, *cosy* mutants have reduced iron content and show growth defects when grown under conditions with limited availability of iron. [35]

Flavonoids are one important class of plant secondary metabolite, and widely distributed in plants. Flavonoids have been reported to possess anti-cancer, anti-tumour and antioxidant effects.[36, 37] Flavonoid biosynthesis pathway is relatively conserved and most key enzyme genes have been cloned and their functions validated in some model plants.[38] Eight structural genes associated with flavonoid synthesis were disclosed in the ME-A and ME-F modules, of which the *CHS*, *CHI*, *F3H*, *DFR*, *LAR*, *ANS* and *ANR* gene was identified as the key gene for flavonoid synthesis and have high expression level in stems. Five associated transcription factors genes (*GLABRA3*, *MYBD*, *MYB4*, *MYB7*, *MYB111*) have high expression levels in the stem. *GLABRA3* is an important regulator of the anthocyanin pathway in

Arabidopsis thaliana. In the absence of nitrogen, the *GLABRA3* and *PAP1/PAP2* (production of anthocyanin pigment 1/2) are strongly induced and anthocyanin synthesis is activated in seedlings, while the anthocyanins synthesis is defect in *glabra3* loss-of-function mutant.[39] In *Arabidopsis thaliana*, MYBD is a positive regulator of anthocyanin biosynthesis.[40] *Arabidopsis thaliana* MYB4 can inhibit flavonoid accumulation by repressing expression of *ADT6* (*Arogenate Dehydratase 6*). MYB4 and its homologs MYB7 and MYB32 interact with the bHLH transcription factors TT8, GL3 and EGL3 and thereby interfere with the transcription activity of the MBW complexes.[41, 42] *AtMYB111* expression in transgenic tobacco enhances expression of genes of the phenylpropanoid pathway leading to an elevated content of flavonols.[43]

Flavonol biosynthesis is one of the important branches of the flavonoid biosynthesis pathway, and *FLS* is a key gene in flavonol biosynthesis, whose expression level is significantly up-regulated in leaf. Metabolomic analysis showed that half of flavones and flavonols were significantly up-regulated in leaf (Figure S10). Previous studies have shown that flavonoid biosynthesis is stimulated by oxidative stress, which is triggered by reactive oxygen species (ROS). Flavonoids not only inhibit the production of ROS, but also protect against damage caused by UV radiation.[44] Flavonols protect plants from UV damage.[45] Half of the 161 flavonoids were glycosylated (Table S11). Glycosylated flavonoids have higher water solubility, prevent the autoxidation of hydroxyl groups and facilitate their transport to the cell membrane.[46]

Transcription factors play a crucial role in the regulation of genes involved in various aspects of plant growth and development, encompassing secondary metabolism. In recent years, an increasing number of transcription factors and the underlying mechanisms of plant secondary metabolism regulation have been elucidated.[47] In addition, plant hormones also play an important role in each stage of plant growth and development, and also regulate the synthesis of flavonoids, terpenoids, alkaloids and other secondary metabolites in plants.[48, 49] Jasmonates (JA) is a plant hormone which induce biosynthesis of many secondary metabolites. JA-responsive transcription factors that regulate the JA-induced accumulation of secondary metabolites belong to different families including AP2/ERF, bHLH, MYB and WRKY.[50] When *Artemisia argyi* leaves were stimulated by exogenous hormones, the metabolic direction of secondary metabolites was changed and the biosynthesis of multiple secondary metabolites including phenylpropanoids, flavonoids, terpenoids, alkaloids and others were facilitated.[51] A significant enrichment of 21 genes were identified to be involved in the plant hormone signal transduction pathway, alongside a notable presence of numerous transcription factors (Figure S8B, C). The investigation of the molecular mechanisms underlying the interplay between hormonal signals and transcription factors involved in the control of secondary metabolite biosynthesis holds significant importance.

In recent decades, according to the physiological analysis of different plants tissues, plants at different stages of development, and plants grown in different environments, there is increasing evidence that plants can store spatial and temporal secondary metabolites for growth-development and environmental adaptation.[52] Thus, the high level of accumulation of secondary metabolites in plants may be an indicator of the enrichment of metabolic pathway or high expression of related genes in cells, while in

most cases the biosynthetic site is restricted to one organ and transported to the storage or action site, depending on the property of metabolite, through vascular vessels or symplastic/apoplastic transport to different parts of plants.[53]

Based on the integrative analyses of transcriptomics and metabolomics, the expression levels of some genes involved in phenylalanine, phenylpropanoid and flavonoid biosynthesis pathways did not show significant correlation with the accumulation of metabolites in different tissues. The secondary metabolites or intermediates in *B. purpurasceus* might be transported from the synthetic sites to the storage or action sites, and the intermediates can mediate final metabolite synthesis.[54] Due to the harsh environment in which *B. purpuraceus* grows, the transport of secondary metabolites to different sites might be as a physiological response to stress and defense stimuli for environmental adaptation.

Many plant secondary metabolites are highly valuable to humans. However, direct extraction of these metabolites is costly due to slow plant growth rates or low concentrations in plant tissues, and the heterologous synthesis of them is fraught with challenges due to the unknown or complexity of their biosynthesis pathways. Increasing metabolite production by engineering transcription factors is a promising alternative. By ectopic expression or over-expression of regulatory proteins, we can expect to up-regulate the entire pathway.

Declarations

Availability of data and materials

The raw data of RNA-seq were submitted under BioProject accession number PRJNA987807.

Competing interests

We declare that all authors have no conflict of interest.

Author Contributions

Q.Z. and F.Y. designed the project and managed the project. F.Y., X.Z., N.X, J.C., X.L., Y.W, H.Z ran the experiments. F.Y. and Q.Z. analyzed the results. F.Y. and Q.Z. wrote the manuscript.

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Ethics approval and consent to participate

Not applicable

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Figures

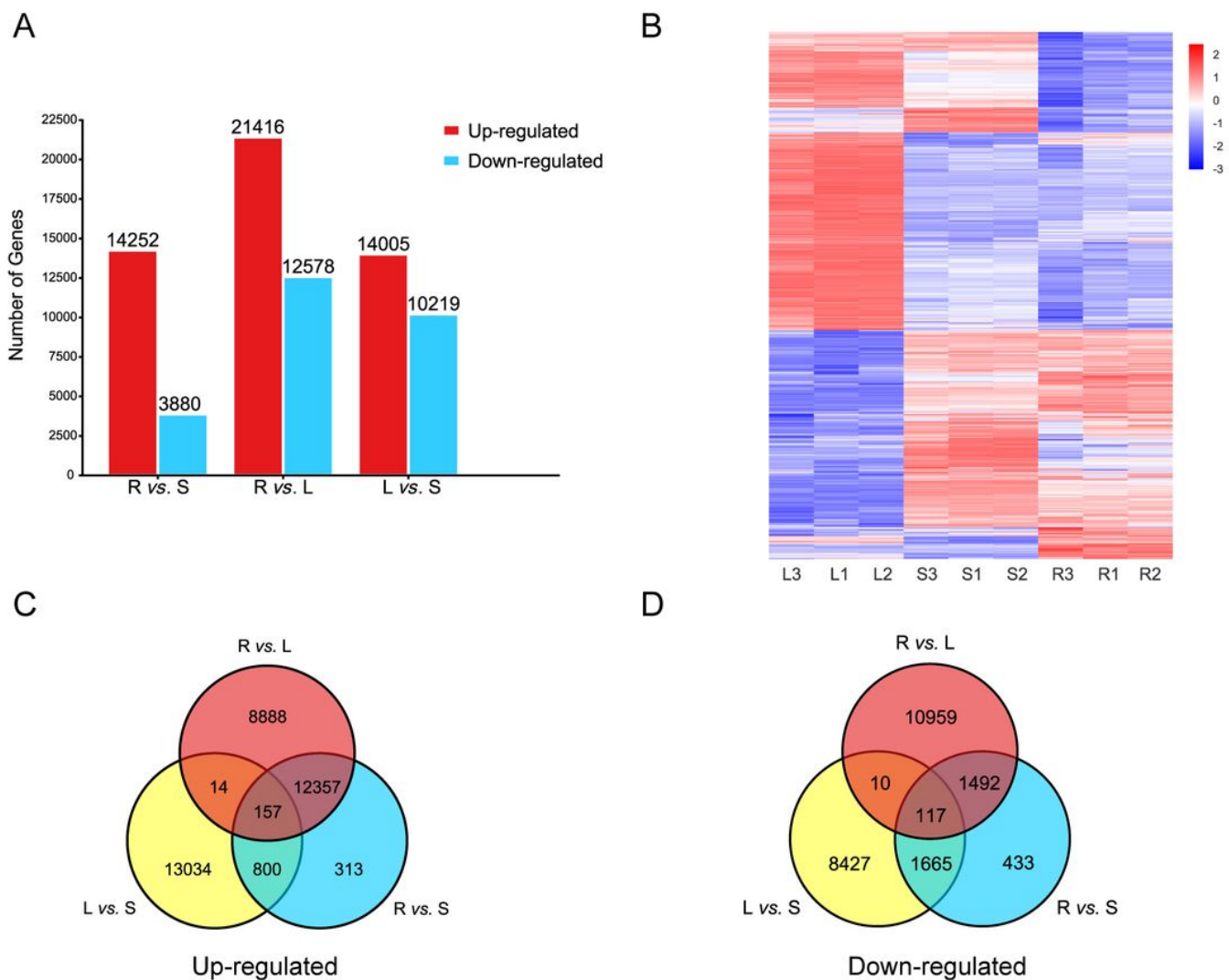


Figure 1

Overview of DEGs in different organs of *B. purpurascens*. (A) Number of up-regulated and down-regulated DEGs in different comparisons. (B) Clustering heatmap of DEGs in different organs. (C) Venn diagram of up-regulated DEGs in different comparisons. (D) Venn diagram of down-regulated DEGs in different comparisons. R: root, S: stem, L: leaf.

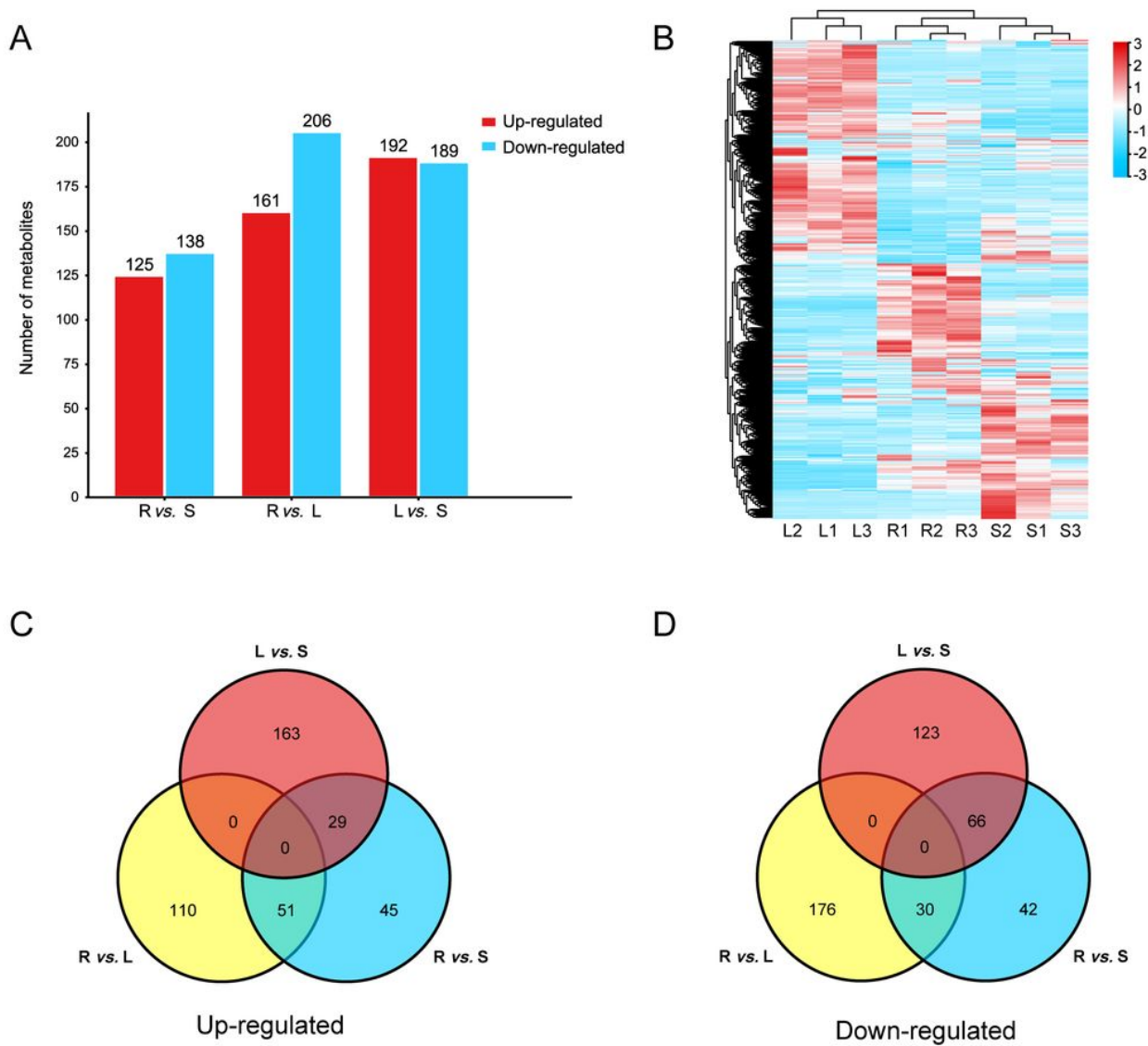


Figure 2

Overview of DAMs in different organs of *B. purpurascens*. (A) Number of DAMs in different comparisons. (B) Cluster heatmap of DAMs in different organs. (C) Venn diagram of up-regulated DAMs in different comparisons. (D) Venn diagram of down-regulated DAMs in different comparisons.

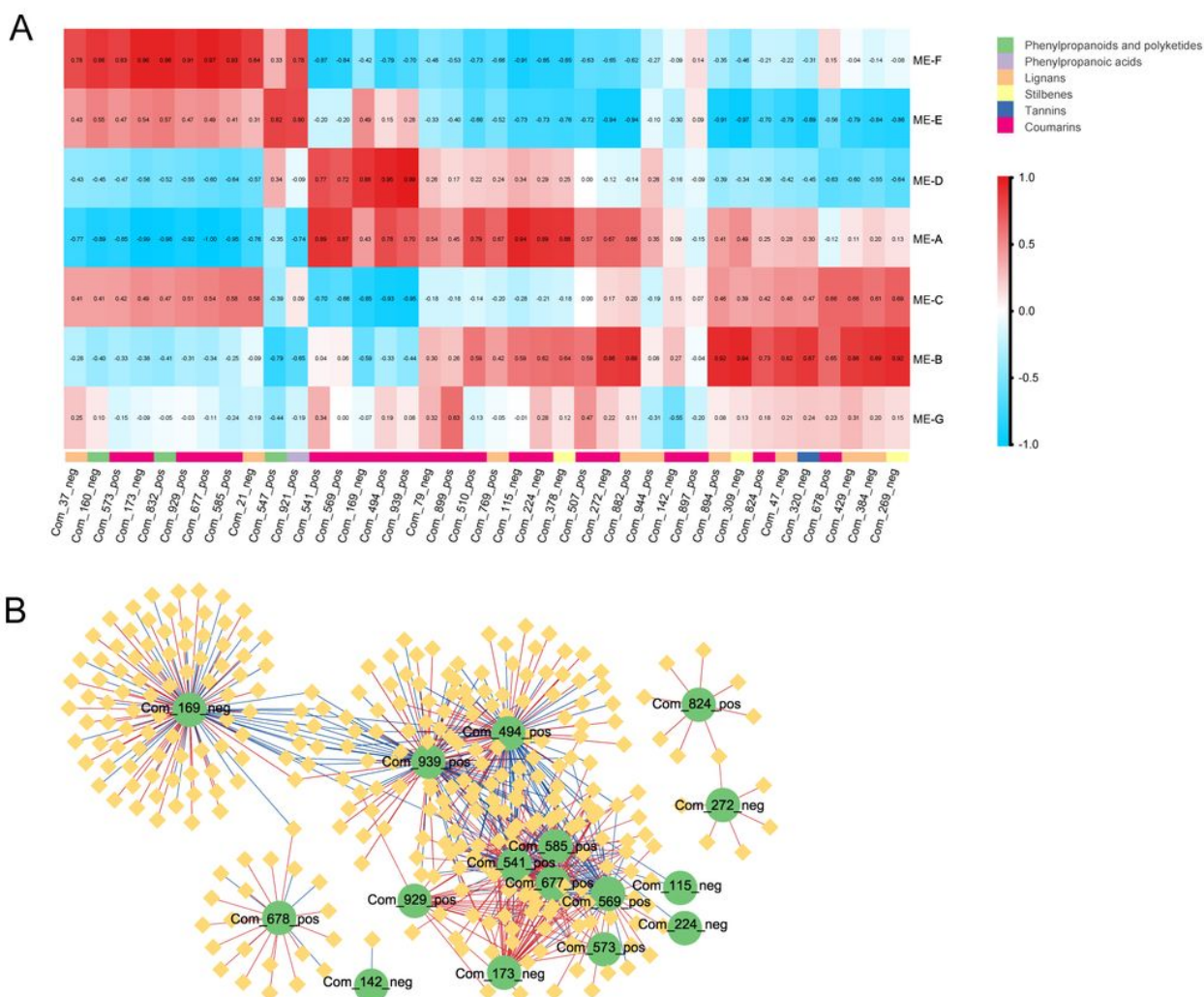


Figure 3

Weighted gene co-expression network analysis. (A) Weighted gene coexpression network analysis of phenylpropanoids. Each row corresponds to a module. Each column represents a specific phenylpropanoid. (B) Correlation network diagram of DEGs in ME-A and ME-F modules and coumarins. The top 50 genes with the highest correlation coefficients (r) with each coumarins are shown in the figure. Yellow diamonds indicate genes, green circles indicate metabolites, red lines indicate positive correlations, and blue lines indicate negative correlations. Com_115_neg, Bergenin; Com_142_neg, Ayapin; Com_169_neg, Warfarin; Com_173_neg, Esculin; Com_224_neg, N-p-Coumaroyl hydroxydehydroagmatine; Com_272_neg, Smyrindioloside; Com_494_pos, p-coumaroyl tomatidine; Com_541_pos, N-p-Coumaroyl spermidine; Com_569_pos, Fraxetin; Com_573_pos, 4-Methylumbelliferyl phenylphosphonate; Com_585_pos, 6-Hydroxymethylherniarin; Com_677_pos, Phellodenol H; Com_678_pos, Phellodenol H O-hexoside; Com_824_pos, columbianetin acetate; Com_929_pos, Skimmin; Com_939_pos, Marmesin.

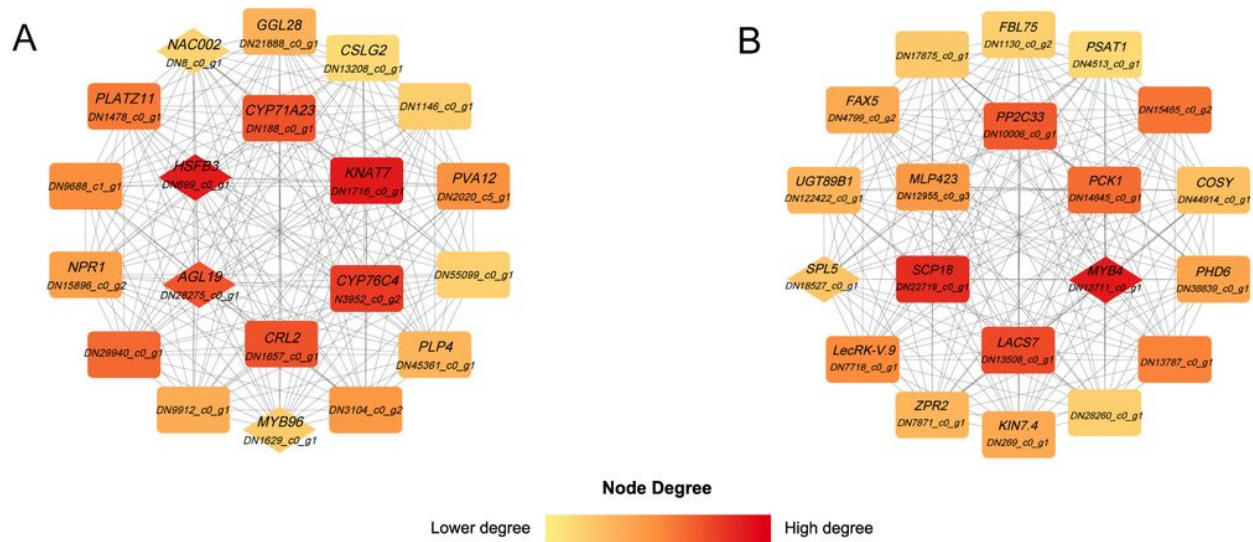


Figure 4

Candidate hub genes in Me-A and ME-F module. (A) The first 20 gene network in the ME-A module. The diamond represents the transcription factor. (B) The first 20 gene network in the ME-F module. Diamond represents the transcription factor. The more forward ranking is represented by a redder color.

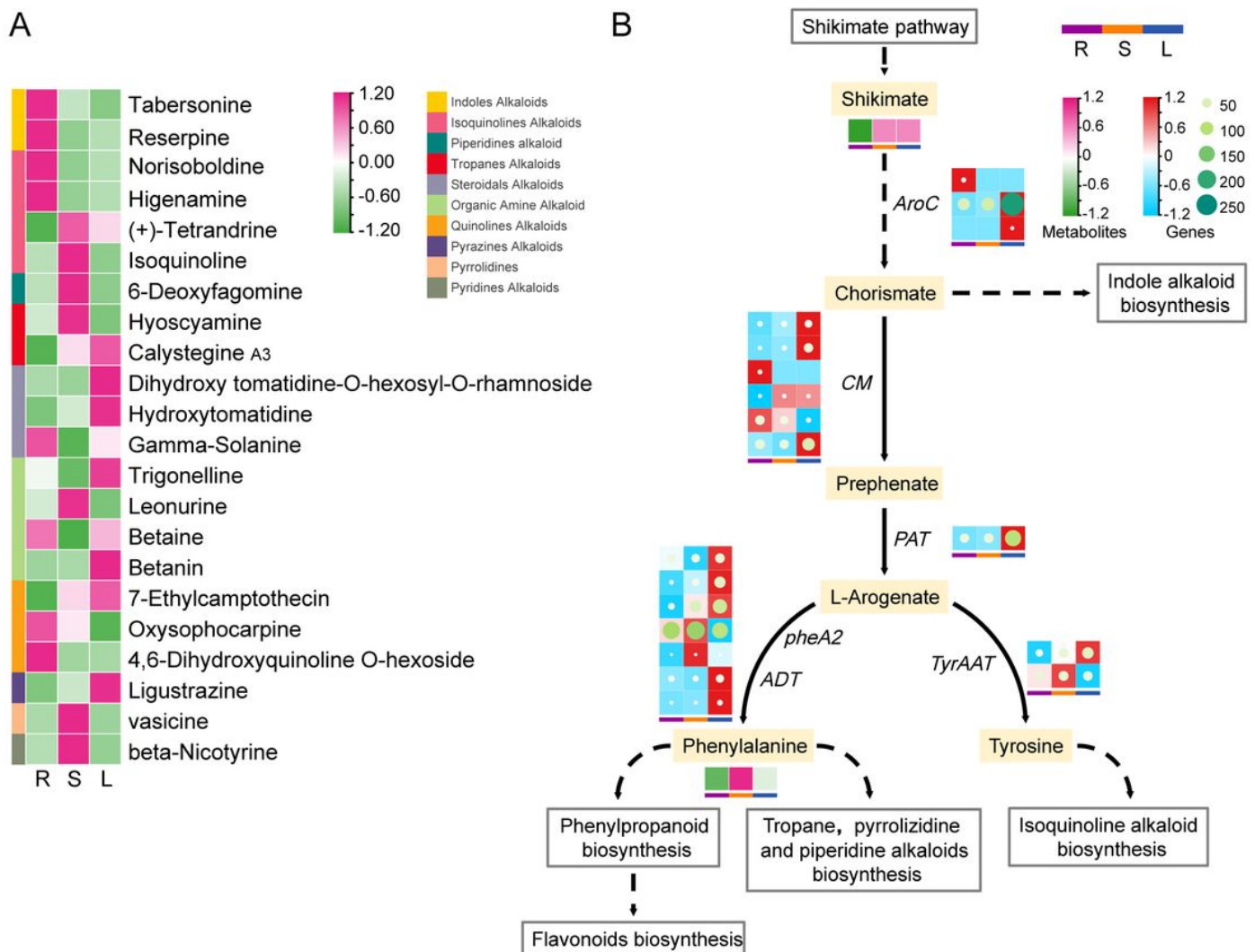


Figure 5

Integrated transcriptomic and metabolomic analysis of the phenylalanine biosynthesis pathway. (A) Accumulation pattern of alkaloids identified in *B. purpurascens*. (B) Phenylalanine biosynthesis pathway. The heatmap shows the expression patterns of DEGs and DAMs during phenylalanine biosynthesis. The color scale indicates the relative amounts of DEGs and DAMs. Darker colors indicate higher expression. Pink represents up-regulated DAMs, and green represents down-regulated DAMs. Red represents up-regulated DEGs and blue represents down-regulated DEGs. The size of the circle indicates the TPM of the DEGs, and a larger circle size indicates a higher level of gene expression. Key enzyme gene abbreviation: AroC, chorismate synthase; CM, chorismate mutase; PAT, prephenate aminotransferase; ADT, arogenate dehydratase.

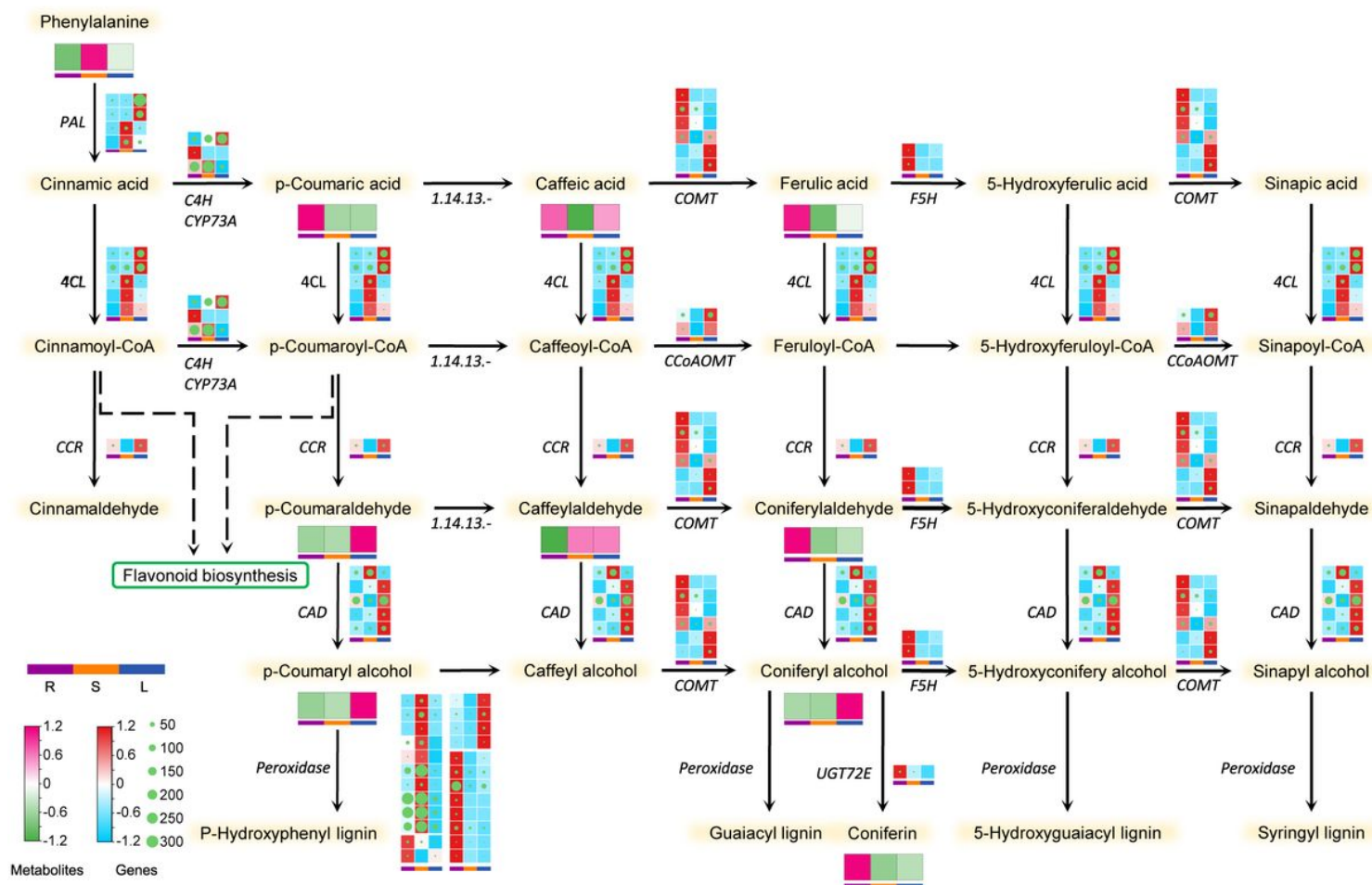


Figure 6

Integrated transcriptomic and metabolomic analysis of the phenylpropanoid biosynthesis pathway. This is the phenylpropanoid biosynthesis pathway. The heatmap shows the expression patterns of DEGs and DAMs during phenylalanine biosynthesis. The color scale indicates the relative amounts of DEGs and DAMs. Darker colors indicate higher expression. Pink represents up-regulated DAMs, and green represents down-regulated DAMs. Red represents up-regulated DEGs and blue represents down-regulated DEGs. The size of the circle indicates the TPM of the DEGs, and a larger circle size indicates a higher level of gene expression. Key enzyme gene abbreviation: PAL, phenylalanine/tyrosine ammonia-lyase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; C4H/CYP73A, trans-cinnamate 4-monooxygenase; COMT, caffeic acid 3-O-methyltransferase; F5H, ferulate-5-hydroxylase; CCoAOMT, caffeoyl-CoA O-methyltransferase; UGT72E, coniferyl-alcohol glucosyltransferase.

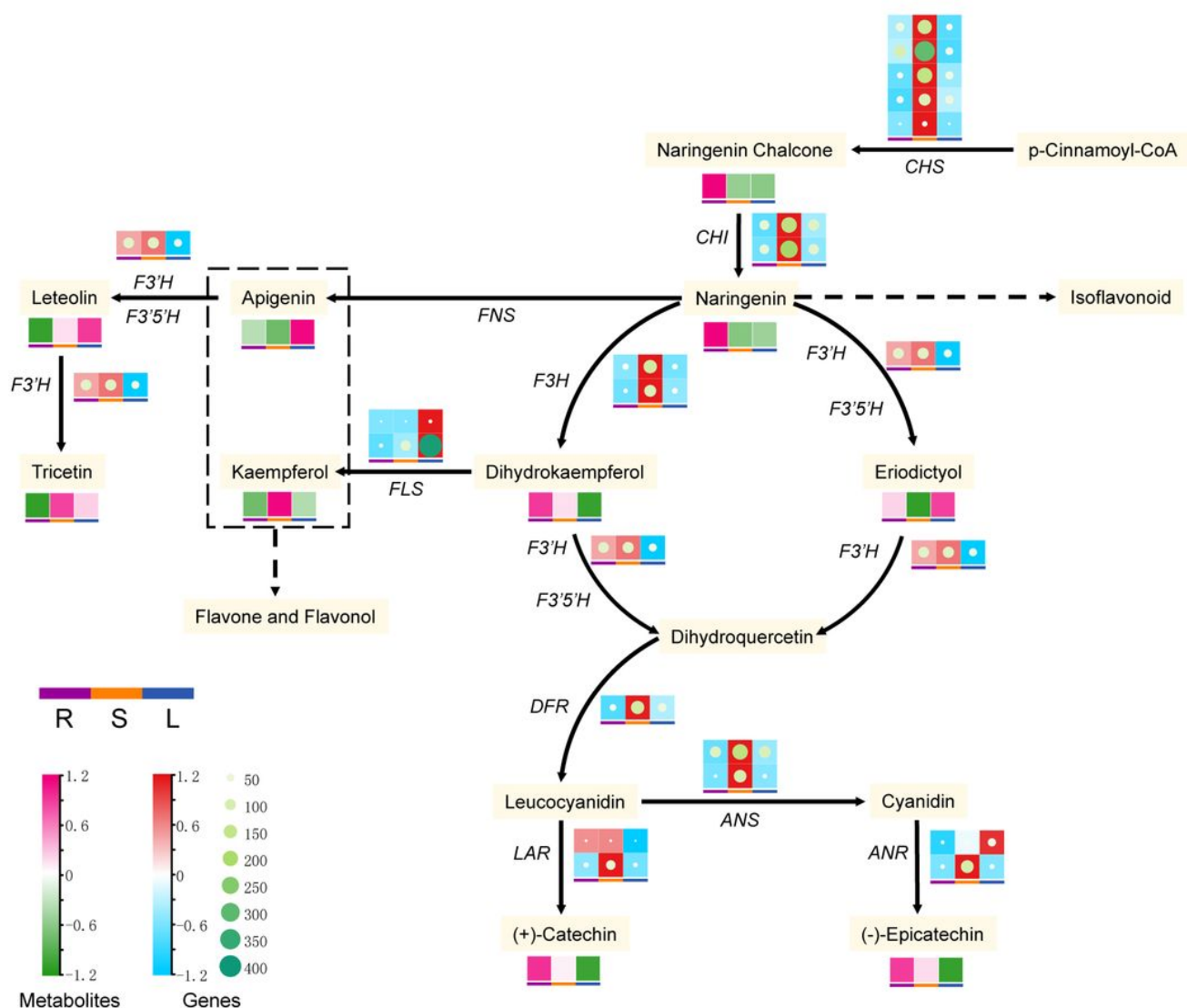


Figure 7

Integrated transcriptomic and metabolomic analysis of the flavonoid biosynthesis pathway. This is the flavonoid biosynthesis pathway. The heatmap shows the expression patterns of DEGs and DAMs during phenylalanine biosynthesis. The color scale indicates the relative amounts of DEGs and DAMs. Darker colors indicate higher expression. Pink represents up-regulated DAMs, and green represents down-regulated DAMs. Red represents up-regulated DEGs and blue represents down-regulated DEGs. The size of the circle indicates the TPM of the DEGs, and a larger circle size indicates a higher level of gene expression. Key enzyme gene abbreviation: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavonoid 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; FLS, flavonol synthase; FNS, flavone synthase; DFR, dihydroflavonol 4-reductase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; ANR, anthocyanidin reductase.

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