

Integrated Metabolomic and Transcriptomic Analyses Provide New Perspectives into the Discoloration of Hawk Tea Tender Leaves

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Abstract

Background

Litsea coreana (commonly known as hawk tea) is a spring-color foliage plant within the Lauraceae family. Its leaves are processed into “hawk tea” a distinctive traditional beverage in Southwest China that serves as a vital cultural and socio-economic resource within local communities. Its leaves typically exhibit red or green coloration, gradually transitioning to common green during maturation. In recent years, non-conventional tea cultivars with high anthocyanin content, particularly those displaying atypical leaf colors, have gained significant agricultural attention due to their potential advantages in tea quality. Investigating the overall pigment metabolism characteristics and associated biosynthetic pathways in hawk tea leaves exhibiting different initial colors across various maturation stages holds strong research significance and substantial application value.

Results

This study applied both targeted metabolomics and transcriptomics to investigate the metabolite accumulation and molecular mechanisms in the leaves of *L. coreana* with different leaf colors. Three anthocyanins, namely cyanidin-3-*O*-glucoside, cyanidin-3-*O*-rutinoside, and pelargonidin-3-*O*-glucoside significantly accumulated in the red tender leaves of *L. coreana*. Metabolic pathways of the various pigments were mapped, and through the combined analysis of metabolomics and transcriptomics, key enzymes involved in their synthesis were identified. Additionally, nine transcription factors, including 3 MYB, 2 bHLH, 3 C2C2 zinc finger proteins, and 1 GRAS, were predicted to directly or indirectly regulate anthocyanin biosynthesis in response to endogenous substances such as nitrogen, hormones, and sugars.

Conclusion

Our data reveal the core metabolites and regulatory networks involved in the coloration of *L. coreana* tender leaves, providing new insights for the comprehensive utilization of this resource.

1. Instruction

The Lauraceae family includes important economic trees that play a significant role in forestry, medicine, light industry, food, and ornamental landscaping [1]. Currently, *Cinnamomum camphora* and *C. burmannii* are the most commonly used species in landscaping, though other plant species are occasionally used with limited diversity. Many species within the Lauraceae family also have promising prospects for landscaping development largely due to their vibrant new leaf red and orange colors and their attractive tree forms, making them highly ornamental [2, 3]. Spring-colored leaf species within the Lauraceae family are primarily from genera such as *Litsea*, *Machilus*, and *Neolitsea* and are characterized by unique colors in their new shoots and tender leaves and perform well in suitable viewing environments [4–7].

Among the species in the genus *Litsea*, *Litsea coreana* var. *lanuginosa* commonly known as hawk tea is an evergreen plant whose tender leaves exhibit various colors, including red (or purple) and green just like its other relative species [8]. The tree is a unique tea raw material native to China and is primarily distributed in Chongqing, with other populations found in Sichuan and Guizhou [9, 10]. The leaves of *L. coreana* are rich in flavonoids, polyphenols, polysaccharides, and coumarins, which regulate blood sugar, protect the liver, and have anti-inflammatory, and antioxidant properties [11–13]. As a result, tea made from these natural leaves is highly favored by the local population.

The leaf color of plants results from the combined effects of various pigments, and the differences in coloration are primarily due to the varying accumulation of anthocyanins, chlorophylls, and carotenoids. Chlorophyll imparts a green color, while carotenoids contribute yellow hues, and anthocyanins provide red coloration. These colors are typically determined by the types of pigments present and their relative concentrations [14]. Anthocyanins are natural secondary metabolites soluble in water that function as antioxidants. Since they enhance the immune and have anti-inflammatory, anticancer and antitumor effects, they have received increasing attention in the fields of dietary supplements, beverages, and pharmaceuticals [15–17]. For instance, Gao et al demonstrated the exhibition of antihypertensive, hypoglycemic, antioxidant, antiproliferative, antibacterial, and lipid metabolism-modulating effects of the high-anthocyanin Zijuang tea through pharmacological activity experiments [18]. Anthocyanins also improve vision, prevent cardio-cerebrovascular diseases, and protect the stomach [19–21]. Therefore, the red-leaf *L. coreana* is often regarded as of superior quality in local traditions and preferably produced due to its higher market price for red tender leaves.

Although research on its chemical composition [22], pharmacological effects [23], genomics [24], and ecological distribution [25] has been conducted, the substances affecting the color of *L. coreana* tender leaves and the associated biosynthetic pathways remain unclear. The application of omics technologies in the study of *L. coreana* leaf coloration has also been limited. Integrated metabolomics and transcriptomics analyses provide a systematic approach for studying the metabolites and regulatory networks in many plants. For example, Kang et al. detected a significant accumulation of lipids, especially fatty acyls and glycerophospholipids, as well as six amino acid derivatives in tea seeds, and predicted the regulatory roles of CsRAP2.10, CsWRKY2.1, CsbLHL18, and CsET2 [26]. Zhang et al. identified 47 metabolites that differ in the accumulation between yellowing and green leaves, suggesting that key genes such as *UDPG*, *HCT*, and *CsGSTF1* may play crucial roles in the process [27]. Song et al. discovered that transcription factors (TFs) like NAC008 and MYB23 may be responsible for the accumulation of flavonoids and anthocyanins in the purple tea varieties, leading to differences in leaf color compared to green leaves [28]. Zheng et al. found that the decline in chlorophyll and carotenoid content in albino tea leaves was due to changes in gene expression within the methyl erythritol 4-phosphate pathway [29]. Rothenberg et al. also used metabolomics to identify at least 12 anthocyanins in pink tea flowers and combined it with transcriptomics, revealing the molecular mechanisms underlying the rare pink flower coloration in anthocyanin-rich tea plants [30].

This study employed a combination of metabolomics and transcriptomics approaches to investigate the metabolic regulatory network involved in the color fading process of *L. coreana* tender leaves. The results will contribute to a systematic understanding of the accumulation characteristics and molecular mechanisms of metabolites related to leaf color in *L. coreana*. This study will provide theoretical support for the in-depth development and utilization of anthocyanin metabolites in *L. coreana*. It will also lay a solid research foundation for future efforts to regulate leaf color, as well as for breeding *L. coreana* varieties with superior traits, excellent quality, unique functional components, and ornamental value, specifically high anthocyanin content and stable red-leaf phenotypes.

2. Materials and methods

2.1 Plant materials

Fresh tender leaves with red (R) green (G) color were collected from six 3-year-old disease-free *L. coreana* trees grown in the nursery of the Chongqing Forestry Science Research Institute (Chongqing, China) under natural condition (20°C, 80% relative humidity, and an 8-hour photoperiod). Sampling took place from mid-March to early April 2022 (Fig. 1A, S1). The tender leaves were harvested and classified into three different developmental stages based on their color, including red (S1), semi-red (S2), and green (S3) stages. The collected leaves were rapidly frozen in liquid nitrogen and then stored at -80°C for subsequent analysis. All experiments were repeated, including 3 biological replicates with a total of 18 samples (labeled as R-S1-1, R-S1-2, R-S1-3; R-S2-1, R-S2-2, R-S2-3; R-S3-1, R-S3-2, R-S3-3; G-S1-1, G-S1-2, G-S1-3; G-S2-1, G-S2-2, G-S2-3; G-S3-1, G-S3-2, G-S3-3).

2.2 Pigment content measurement

The contents of chlorophyll and carotenoids were extracted 0.2 g from the leaves using 95% ethanol, followed by measuring their optical density (OD) at 665, 649, and 470 nm using a UV-Vis spectrophotometer (JINGHHUA 721 – 100 UV-Vis spectrophotometer, Shanghai). The chlorophyll a content (C_{Chla}) was calculated as $13.95 OD_{665} - 6.88 OD_{649}$, C_{Chlb} as $24.96 OD_{649} - 7.32 OD_{665}$, the carotenoid content (C_{Car}) as $(1000 OD_{470} - 2.05 C_{Chla} - 114.8 C_{Chlb}) / 245$, and the total chlorophyll content as $C_{Chla} + C_{Chlb}$. The pigment content was expressed as mg of pigment per gram of fresh leaf tissue (mg/g). The leaves were cut into 1–2 mm pieces, and 0.03 g was mixed with 10 mL of 0.1 mol/L hydrochloric acid (HCl) solution. Seal the flask and shake thoroughly to ensure the leaves are fully submerged in the extraction solution. Incubate in the dark for 24 hours until the leaves become colorless, yielding the anthocyanin extract. The resulting extract was then analyzed using a UV spectrophotometer at wavelengths of 600 nm and 530 nm. One unit of anthocyanin is denoted as "U" and the change in OD per gram of fresh leaf extract is calculated as: $OD_{530} - OD_{600} = 0.1 U$.

2.3 Analysis of the anthocyanin metabolites

The anthocyanin metabolites were determined using the AB Sciex QTRAP 6500 LC-MS/MS platform (MetWare, <http://www.metware.cn/>). Stock solutions of $1 \text{ mg} \cdot \text{mL}^{-1}$ standards were prepared in 50% methanol (MeOH) and stored at -20°C . Before analysis, the stock solutions were diluted with 50% MeOH to working concentrations. Leaf samples were freeze-dried, ground at 30 Hz for 1.5 min, and stored at -80°C . Exactly 50 mg of powdered leaf sample was mixed with a solution of 0.5 mL MeOH, water and HCl prepared in the ratio of 500:500:1 (V/V/V). The mixture was vortexed and sonicated for 5 min each and then centrifuged at 12,000 g for 3 min at 4°C . The supernatant was filtered using $0.22 \mu\text{m}$ filter paper and analysed using the ultra high performance liquid chromatography tandem mass spectrometry (UPLC-ESI-MS/MS) system (UPLC, ExionLC™ AD; MS, Applied Biosystems 6500 Triple Quadrupole). The UPLC conditions included Waters ACQUITY BEH C18 column ($1.7 \mu\text{m}$, $2.1 \text{ mm} \times 100 \text{ mm}$), solvent system consisting of water with 0.1% formic acid, methanol with 0.1% formic acid, and gradient of 95:5 at 0 min, 50:50 for 6 min, 5:95 for 12 min, hold for 2 min, 95:5 for 14 min, hold 2 min, flow rate of 0.35 mL min^{-1} , temperature of 40°C , and injection volume of $2 \mu\text{L}$. Mass spectrometry was performed using QTRAP® 6500 + LC-MS/MS with ESI Turbo Ion-Spray in positive mode. The ESI parameters included a source temperature of 550°C , ion spray voltage of 5500 V, and curtain gas of 35 psi. Anthocyanins were analyzed using scheduled multiple reaction monitoring (MRM), with optimized transitions for each compound. Data were processed using Analyst 1.6.3 and Multiquant 3.0.3 software for quantification. Principal Component Analysis (PCA) conducted by R package factoextra (<https://cloud.r-project.org/package=factoextra/>) [31]. To display clusters of samples with the first two components. The differential accumulated metabolites (DAM) between different groups were determined based on the following criteria: $|\log_2(\text{fold change})| \geq 2$, P value < 0.05 .

2.4 Illumina sequencing

Total RNA was extracted using a plant RNA extraction kit (Huayueyang Biotechnology Co., Ltd.). The concentration and purity were detected using NanoDrop assay (Thermo Scientific NanoDrop 2000). Total RNA with a quantity of $\geq 1 \mu\text{g}$ was selected and processed using the NEBNext Ultra II RNA Library Prep Kit for Illumina. mRNA with polyA tails was enriched using Oligo (dT) magnetic beads, followed by random fragmentation of the mRNA using divalent cations to induce ion disruption. The fragmented mRNA served as a template for cDNA synthesis, with random oligonucleotides used as primers. The resulting double-stranded cDNA was purified, then subjected to end repair and the addition of an "A" base at the 3' end, followed by ligation of sequencing adapters. cDNA fragments of approximately 400–500 bp were selected using AMPure XP beads (BECKMAN Agencourt), followed by PCR amplification. The PCR products were further purified using AMPure XP beads to obtain the final library. Library quality was assessed using the Agilent 2100 Bioanalyzer (Agilent, 2100) with the Agilent High Sensitivity DNA Kit (Agilent, 5067 – 4626). The total concentration of the library was quantified using PicoGreen (Quantiflux ST fluorometer, Promega, E6090; Quant-iT PicoGreen dsDNA Assay Kit, Invitrogen, P7589), and the effective library concentration was determined by qPCR (Thermo Scientific StepOnePlus Real-Time PCR System). Multiple DNA libraries were pooled in equal volumes, diluted, and quantified. The pooled library was sequenced in PE150 mode on an Illumina sequencer.

2.5 RNA-Seq data analysis

The raw sequencing data in FASTQ format were filtered using fastp software (version 0.18.0) to obtain high-quality and clean reads by removing the reads containing adapters, reads with more than 10% unknown nucleotides (N), and low-quality reads with over 50% bases with a quality score (Q-value) ≤ 20 [32]. The De novo transcriptome assembly was then performed using Trinity software and the gene and transcript expression levels were quantified using the RSEM software, with Transcripts Per Million (TPM) as the quantification metric. The TPM is based on the number of transcript reads, and accounts for both transcript length and the number of genes expressed in the sample. The DESeq2 was used to perform differential expression analysis between multiple samples (R-S1 vs R-S2, R-S2 vs R-S3, R-S1 vs G-S1, R-S2 vs G-S2, R-S3 vs G-S3, G-S1 vs G-S2, and G-S2 vs G-S3). The false discovery rate (FDR) was calculated using the p-adjusted values, with thresholds set as p-adjusted < 0.05 and $|\log_2(\text{fold change})| \geq 1$ to identify differentially expressed genes (DEGs). Statistical enrichment analysis of all DEGs was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<https://www.kegg.jp/kegg/>).

2.6 Protein-protein interaction (PPI) network analysis

By analyzing the domain information in gene transcription products, transcription factor prediction and family classification can be conducted. We utilized PlantTFDB 5.0 (<http://planttfdb.gao-lab.org/>) to perform transcription factor analysis on genes derived from plants. The PPI network analysis of the genes of interest was performed using the STRING database (<http://string-db.org/>) [33]. We used the well-established model organism *Arabidopsis thaliana* with comprehensive protein interaction data and directly extracted the interaction relationships corresponding to the genes of interest from the database to construct the PPI network. Subsequently, the Pearson correlation and Euclidean distance algorithms were applied to calculate the Pearson correlation coefficients (R^2) and p-values by integrating transcriptome TPM data with the anthocyanin metabolome data. A correlation coefficient > 0.7 was selected to construct a visual co-expression network, which was visualized using Cytoscape software version 2.8 [33].

2.7 qRT-PCR analysis

The qRT-PCR experiments were performed in triplicates according to the instructions of the TB Green Premix Ex Taq II (Tli RNaseH Plus) kit on a Bio-Rad iCycler thermal cycler (Bio-Rad, Hercules, CA, USA). The qPCR primers (Table S1) for *L. coreana* genes were designed using Primer 5.0 software (Premier Biosoft International, Palo Alto, California, US). The *actin* gene was selected as the internal reference gene [34]. The melting curve exhibited a single peak (Fig. S2), indicating that specific amplification products were melting at a specific temperature. Relative gene expression levels were calculated using the $2^{-\Delta\Delta CT}$.

2.8 Statistical analyses

Line charts, bar graphs, and heat maps were generated using GraphPad 8.0. Statistical significance was assessed using one-way ANOVA with SPSS 25 software (IBM, Chicago, IL, USA). A p-value less than 0.05 was considered statistically significant.

3. Results

3.1 Leaf color changes at different developmental stages

In this study, the newly collected *L. coreana* leaves transitioned from red to semi-red and eventually to fully green during the spring foliage stage (Fig. 1A), with the highest anthocyanin content in the red than the green leaves ($p < 0.05$). The anthocyanin content was not significantly different in the green leaves during the entire leaf development process but was significantly lower than those in the red leaves at all stages (Fig. 1B). The chlorophyll content increased as the leaf color transitioned from red to green, with the green leaves showing significantly higher levels of chlorophyll than the red and semi-red leaves ($p < 0.05$). The chlorophyll content also increased throughout leaf development in the green leaves (Fig. 1C) but was similar between the red and semi-red leaves. The carotenoid content was also not significantly different between red and semi-red leaves at different developmental stages (Fig. 1D).

3.2 Identification of anthocyanin metabolites in *L. coreana* leaves

The corresponding heatmap demonstrated a strong correlation between the three biological replicates used for each sample (Fig. 2A). The principal component analysis (PCA) revealed a clear separation between red and green leaves along the first principal component (PC1), which explained 49.65% of the variation (Fig. 2B). The distinct separation between red and green leaves in the PCA indicates significant differences in metabolite profiles between leaf color variants, as well as across the three developmental stages of the leaves. All the analyses confirmed the reliability of the metabolomic data.

A total of 42 metabolites were identified, including 31 anthocyanins, 3 proanthocyanidins and 8 other flavonoids (Fig. 2C; Table S2). Differentially accumulated metabolites (DAMs) were analyzed in pairwise comparisons across seven groups including R-S1 vs R-S2, R-S2 vs R-S3, R-S1 vs G-S1, R-S2 vs G-S2, R-S3 vs G-S3, G-S1 vs G-S2, and G-S2 vs G-S3. Thirteen, 23, 22, 19, 20, 12, and 1 DAMs were identified in each respective comparison, with a total of 29 DAMs across the groups (Fig. 2D; Table S3). The analysis of the absolute content of significantly accumulated anthocyanins in leaves with different developmental stages and leaf colors revealed that cyanidin-3-*O*-glucoside (C3G), cyanidin-3-*O*-rutinoside (C3R) and pelargonidin-3-*O*-glucoside (P3G) were the predominant anthocyanins in the red *L. coreana* tender leaves. The contents of these anthocyanins were significantly higher in red than green tender leaves but gradually decreased as the leaf developed (Fig. 2E). The expression patterns of these anthocyanin metabolites were also consistent with the phenotypic changes, suggesting that C3G, C3R and P3G may be key metabolites associated with the formation of red tender leaves in *L. coreana*.

3.3 Transcriptome data quality and DEGs analysis

To further investigate the color development mechanism in *L. coreana* leaves, transcriptomic analysis was performed. A total of 18 samples yielded 121.05 GB of clean data, with each sample generating more than 5.76 GB, with a Q30 base percentage exceeding 93.01% (Table S4). Further filtering of sequencing data, data filtering Clean Data range from 92.90 to 93.38% (Table S5). The Pearson correlation coefficients (R^2) derived from the heatmap analysis were greater than 0.9, indicating high sample reproducibility (Fig. 3A). Additionally, the PCA revealed clustering within groups and clear separation between different groups, with PC1 accounting for 24.37%, primarily distinguishing green from red tender leaves and PC2 accounting for 14.93%, with a strong separation between visually distinct red and green leaves (Fig. 3B). These PCA results suggest high reproducibility of the data within groups and significant variation between groups.

A total of 21,445 DEGs were identified using DESeq2. Among these, 2,004 DEGs were detected in the comparison between the S1 and S2 developmental stages of red tender leaves, with 1,211 genes up-regulated and 793 down-regulated. A total of 8,285 DEGs were identified between the S2 and S3 developmental stages of red tender leaves, with 4,519 genes up-regulated and 3,766 down-regulated. A comparison of the first developmental stage of red tender leaves with the S1 developmental stage of green tender leaves revealed 6,252 DEGs, with 3,462 genes up-regulated and 2,790 down-regulated. A comparison of the S2 developmental stage of red tender leaves with the second developmental stage of green tender leaves, identified 5,181 DEGs, with 2,558 genes up-regulated and 2,623 down-regulated. The comparison between the S3 developmental stage of red tender leaves and the third developmental stage of green tender leaves revealed 14,167 DEGs, with 7,519 genes up-regulated and 6,648 down-regulated. The comparison of the S1 and S2 developmental stages of green tender leaves showed 1,967 DEGs, with 1,144 genes up-regulated and 829 down-regulated, while the comparison between the S2 and S3 developmental stages of green tender leaves revealed 2,732 DEGs, with 1,370 genes up-regulated and 1,362 down-regulated (Fig. 3C).

3.4 Analysis of key structural genes associated with the accumulation of three pigment types

The KEGG pathway enrichment analysis (Fig. 3D) identified porphyrin and chlorophyll metabolism, carotenoid biosynthesis, flavonoid biosynthesis, and anthocyanin biosynthesis as the major metabolic pathways related to leaf color (Table 1). The analysis of the DEGs showed the enrichment of 36 genes in the flavonoid biosynthesis pathway, 3 genes in the anthocyanin biosynthesis pathway, 28 genes in the porphyrin and chlorophyll metabolism pathway, and 21 genes in the carotenoid biosynthesis pathway. The analysis also identified 39 DEGs encoding 12 structural genes in the anthocyanin biosynthesis (Fig. 4A), including 2 *C4H*, 7 *CHS*, 1 *F3H*, 1 *F3'H*, 2 *ANS*, 1 *FLS*, 2 *LAR*, 1 *ANR*, 2 *C3'H*, 4 *CCOAMT*, 13 *HCT*, 3 *3-GT* genes. Additionally, we identified 28 genes encoding 23 enzymes involved in three chlorophyll metabolic pathways (Fig. 4B), including chlorophyll biosynthesis, chlorophyll cycle and chlorophyll degradation pathways. In the chlorophyll biosynthesis pathway, 1 *HEMA*, 1 *HEML*, 1 *HEMC*, 2 *HEMD*, 2 *HEME*, 1 *HEMF*, 2 *HEMH*, 1 *CHLH*, 1 *CHLE*, 1 *CHLI*, 1 *CHLP*, and 1 *POR* genes were found. In the chlorophyll cycle pathway, we identified 1 *CHLG*, 1 *HCAR*, 2 *NOL*, 1 *CAO*, and 2 *CLH*, while in the

chlorophyll degradation pathway, 1 *EARS*, 1 *cobA*, 1 *HO*, 1 *RCCR*, 1 *SGR*, and 1 *UGT* gene were identified. We also identified 21 DEGs encoding 10 enzymes related to carotenoid biosynthesis (Fig. 4C), including 3 *crtB*, 2 *CCD7*, 1 *CYP97C1*, 1 *crtZ*, 3 *ABA1*, 1 *CCS1*, 3 *NCED*, 1 *ABA2*, 1 *AAO*, and 5 *CYP707A* genes.

Table 1. Leaf color-related metabolic pathways in <i>L. coreana</i> .			
Number	Pathway	Gene count	Pathway ID
1	Flavonoid biosynthesis	36	ko00941
2	Anthocyanin biosynthesis	3	ko00942
3	Porphyrin and chlorophyll metabolism	28	ko00860
4	Carotenoid biosynthesis	21	ko00906

3.5 Identification of transcription factors involved in pigment synthesis metabolism

The transcription factor (TF) analysis performed on the assembled genes identified 928 TFs belonging to 21 TF families (Fig. 5A), with v-myb myeloblastosis viral oncogene homolog (MYB), APETALA2/ethylene-responsive element binding factor (AP2), and basic helix-loop-helix (bHLH) being the top three most abundant TF families. To precisely select TFs involved in the synthesis of the three pigment types, we performed a PPI network analysis of 88 genes, including the 39 genes related to anthocyanin synthesis, 28 genes related to chlorophyll metabolism, and 21 genes involved in carotenoid synthesis with the 928 TFs (Fig. 5B). Using *A. thaliana* as a reference species, we selected interactions with a combined score greater than or equal to 0.4 and the top 300 interacting pairs for Cytoscape visualization. After excluding those that did not form clusters with structural genes, we identified 42 TFs (Fig. 5C), including 12 that encode MYB-type TFs and five that encode bHLH-type TFs.

Looking at the expression of biosynthetic pathway genes (Fig. 6), during leaf development, limited precursors in green tissue preferentially direct flux toward lignin biosynthesis, while red tissue favors anthocyanin biosynthesis.

3.6 Correlation Analysis between Metabolomics and Transcriptomics

To further identify genes involved in pigment synthesis in *L. coreana* leaves, we conducted a correlation network analysis by evaluating the Spearman correlations among DEGs, DAMs, and pigment content (Table S6). A threshold of $p < 0.05$ and $|r| > 0.7$ was used to filter the network (Fig. 5D). Based on this, we identified six structural genes (*ANR*, *3GT-3*, *HCT-7*, *HO*, *AAO*, *ABA2*) and seven (*CCOAMT-1*, *CCOAMT-4*, *HCT-2*, *hemD-2*, *CCS1*, *CYP707A-3*, *crtZ*) anthocyanin metabolites that positively and negatively correlated with anthocyanin content, respectively. Additionally, three TFs (*MYB-9*, *bHLH-4*, *GRAS-4*) were positively correlated with anthocyanin content (Fig. 5D). Similarly, six structural genes (*CLH-2*, *hemH-1*, *NOL-1*, *NCED-2*, *ABA1-3*, *HCT-11*) and one TF (*C2C2-3*) were positively correlated with chlorophyll content, while

two structural genes (*C3'H-1*, *HCT-7*) and five TFs (*MYB-1*, *MYB-5*, *bHLH-3*, *C2C2-2*, *C2C2-4*) were negatively correlated with chlorophyll content (Fig. 5D). No genes meeting the correlation criteria were identified for carotenoid content. However, the contents of the total anthocyanin, C3G and P3G showed significant positive correlations with the *3GT-3* gene involved in anthocyanin biosynthesis, *HCT-7* gene in the flavonoid biosynthesis pathway, and the TFs *MYB-9* and *bHLH-1*. Interestingly, *HCT-7* was also significantly negatively correlated with chlorophyll content, and the TFs *MYB-1*, *MYB-5*, and *bHLH-1*. Therefore, these genes may regulate pigment formation in *L. coreana* leaves.

3.7 qRT-PCR verification of RNA-Seq analysis

We selected eight key genes from the correlation analysis to validate the transcriptomics data using the qRT-PCR. The results showed that the qRT-PCR data were consistent with the TPM values, confirming the reliability of the transcriptomic data (Fig. 7). We also observed significantly higher levels of *MYB-5* and *HCT-7* genes in the red tender leaves compared to the green tender leaves at all developmental stages, further supporting the involvement of *MYB-5* and *HCT-7* as key factors in the pigment formation in *L. coreana* leaves.

4. Discussion

4.1 Anthocyanin content as the key factor determining leaf color in *L. coreana*

Chlorophyll, carotenoids, and anthocyanins are the pigments that primarily influence leaf color in plants. In our study, the balance of chlorophyll, carotenoids, and anthocyanins was disrupted in red tender leaves of *L. coreana* compared to its green leaves. The total chlorophyll levels in green and red leaves increased with development, while carotenoid levels remained stable. In contrast, anthocyanin accumulated more in red than in green tender leaves early in development, with levels gradually decreasing but remaining higher than in green leaves at the same stages. Similar findings have been reported in other plants. For instance, in *Alternanthera bettzickiana*, the green-leaved varieties are characterized by low anthocyanin content, with mature chloroplasts giving the leaves a green color, while the red varieties have low chlorophyll content, deformed chloroplasts, and higher anthocyanin content, resulting in red leaved plants [35]. In the *Anthurium andraeanum*, the formation of leaf color is largely influenced by chloroplast development and pigment biosynthesis activity [36], while in *Brassica juncea*, the purple leaf phenotype is due to an increase in anthocyanins and suppression of chlorophyll synthesis [37]. In *Triadica sebifera*, green-leaved plants primarily contain chlorophyll and carotenoids, which decrease during the purple-leaf phase as the anthocyanin levels increase, enhancing the purple appearance of the leaves [38]. Therefore, compared to these plants, *L. coreana* exhibits a distinct leaf coloration pattern in which the final red color of its tender leaves is less related to chlorophyll and carotenoids but is primarily driven by the anthocyanin content.

4.2 The major anthocyanin products in *L. coreana*

Leaf color is one of the most desirable traits in urban trees that is determined by anthocyanins [14]. Over 650 distinct anthocyanins have been identified in nature, with the most common being cyanidin, delphinidin, petunidin, peonidin, malvidin, and pelargonidin. Among the anthocyanins, cyanidin and pelargonidin are associated with a red or deep red coloration [17, 39]. Consistent with these findings, we identified two cyanidins C3G and C3R, and a pelargonidin P3G as the predominant anthocyanins with significantly higher concentrations in the red tender leaves of *L. coreana* than in green leaves, indicating that these anthocyanins play key roles in accumulating red pigments in the leaves of *L. coreana*.

4.3 The differences between red and green tender leaves in *L. coreana*

The role of anthocyanin biosynthesis in leaf development has been extensively studied across various plant species, including monocotyledonous ornamental plant *Caladium* [40, 41], vegetable *Brassica* [37, 42], tea tree *Camellia sinensis* [28], as well as autumn leaf tree species such as *Acer pictum* subsp. *mono* [43], *A. palmatum* [44], and *Liquidambar Formosana* [45]. In our current study, several key structural genes involved in early anthocyanin biosynthesis in *L. coreana* leaves, including *C4H*, *CHS*, *CHI*, *F3H*, and *F3'H* genes and late anthocyanin biosynthetic genes such as *ANS* and *3GT*, were differentially expressed during leaf development. The expression of *3GT* and *ANR* genes strongly correlated with anthocyanin content in young red leaves of *L. coreana*, suggesting their critical role in accumulating anthocyanin. The two genes share the same substrate, producing colored anthocyanins using anthocyanidin synthase. The produced anthocyanins are unstable without glycosylation, potentially forming proanthocyanidins via *ANR* or stable anthocyanins via *3GT*.

Lignin and anthocyanin compete for common precursors, which may explain the differences in the leaf color between red and green tender leaves in *L. coreana*. Therefore, based on the expression of biosynthetic pathway genes during leaf development, limited precursors in green tissue preferentially support lignin biosynthesis, while red tissue favors anthocyanin biosynthesis. In *Populus*, a multi-omics analysis of transgenic trees overexpressing *miR156* revealed that *miR156* regulates the biosynthesis of anthocyanins, flavonoids, and flavonols through miRNA, TFs, and structural genes, but also suppresses lignin biosynthesis, which competes for common precursors [46]. The overexpression of *MYB6* in transgenic poplars also upregulated flavonoid biosynthetic genes, leading to a significant accumulation of anthocyanins and proanthocyanidins and a reduction in lignin content and secondary cell wall deposition [47]. Conversely, the overexpression of *ZmMYB42* increased H- and G-type lignin content but decreased the S-type lignin, flavonols, and anthocyanin [48, 49]. These findings highlight the regulation of the competitive relationship between lignin and anthocyanin biosynthesis in plants by upstream TFs and epigenetic modifications.

4.4 The fading of red color in *L. coreana* leaves

The downregulation of anthocyanin biosynthesis-related TFs and structural genes may contribute to the fading of red color in *L. coreana* leaves. For example, in our study, the expression levels of structural genes involved in anthocyanin biosynthesis, including *C4H-1*, *CHS-1* to *CHS-7*, *F3'H*, *F3H*, *ANS-1*, and *ANS-2*, gradually decreased during leaf development and could have led to the reduced accumulation of anthocyanins, resulting in the gradual fading of the leaf color. Anthocyanin biosynthesis is regulated by key structural genes and the MBW complex, which interacts with the cis-elements in the promoters of these genes [15-17]. The MBW complex consists of R2R3-MYB, bHLH, and WD40 TFs, but its function is primarily controlled by the activity of R2R3-MYB genes, which promote or inhibit the transcription of structural genes [38, 47]. In our study, the expression levels of three R2R3-MYB genes, including *MYB-1*, *MYB-5*, and *MYB-9* gradually decreased in the color-fading leaves of red tender leaves, with *MYB-1* and *MYB-5* showing relatively high expression in green leaves and significant but negative correlation with chlorophyll content. This suggests that *MYB-1* and *MYB-5* may require bHLH-type TFs, such as bHLH-4 identified in this study, to efficiently regulate anthocyanin accumulation in *L. coreana* leaves. However, further research should be conducted to validate whether these genes play a similar role in anthocyanin regulation.

4.5 Endogenous factors influencing anthocyanin accumulation and leaf coloration in *L. coreana*

Though anthocyanin is the main pigment that influences the leaf color of *L. coreana* tender leaves, a combined transcriptomic and metabolomic analysis revealed a weak correlation between the expression of genes involved in the anthocyanin biosynthetic pathway and anthocyanin levels. However, a significant number of genes related to lignin, chlorophyll and carotenoid biosynthetic pathways were strongly correlated with the accumulation of anthocyanin. The accumulation of anthocyanin is influenced by endogenous factors such as nitrogen, hormones, and sugars (). In our research, we identified TFs that respond to anthocyanin levels in *L. coreana*, including MYB and bHLH-type TFs, as well as C2C2 zinc finger and DELLA proteins. In *Arabidopsis*, the *AtLSD1* gene encoding a C2C2-type zinc finger protein regulates cell death and is involved in the formation of aerenchyma under waterlogged conditions [50]. In rice, the nitrogen-efficient cultivar Yangdao 6 has well-developed aerenchyma and demonstrates superior nitrate absorption and utilization compared to the nitrogen-inefficient cultivar Nongxing 57 [51]. The C2C2 zinc finger protein gene family may share a conserved function in plant-programmed cell death by influencing aerenchyma formation and nitrogen utilization. On the other hand, DELLA proteins, a subfamily of the GRAS TF family, regulate various hormonal signals, including gibberellic acid (GA), auxin, abscisic acid (ABA), and ethylene [52, 53], with the GA negatively regulating anthocyanin synthesis [54, 55], similar to our findings. For instance, in our study, the expression levels of the *GRAS-4* gene, which negatively regulates GA biosynthesis, were significantly higher at all developmental stages in red tender leaves compared to green tender leaves, and its expression correlated positively with anthocyanin content. Additionally, GA-related pathways such as diterpenoid biosynthesis (ko00904) and plant hormone signal transduction (ko04075) were significantly enriched in KEGG analysis, suggesting that the

endogenous gibberellins in *L. coreana* may directly or indirectly influence the accumulation of anthocyanin through the *GRAS-4* gene, thereby affecting the leaf coloration.

In the plant hormone signal transduction pathway, the precursors for ABA biosynthesis originate in the carotenoid biosynthesis pathway, supporting the induction of anthocyanin synthesis by ABA [56]. This also explains the significant correlation of genes involved in carotenoid biosynthesis with anthocyanin content in *L. coreana* leaves. Thus, the accumulation of ABA precursors could influence the ABA and anthocyanin biosynthesis. Furthermore, the regulatory effects of ABA on anthocyanin synthesis may be enhanced by sugars, since combined ABA treatment with sugars significantly promotes the expression of many anthocyanin-related genes in *Arabidopsis* [55]. These findings highlight the crucial role of sugars in regulating anthocyanin accumulation. Sugars contribute to anthocyanin biosynthesis through glycosylation leading to the formation of stable anthocyanins and provision of the precursors for anthocyanin biosynthesis via the shikimate pathway, which relies on pentose sugar metabolism. Thus sugars are essential for the respiratory processes required for anthocyanin synthesis [57]. Besides, their sugars in the anthocyanin synthesis pathway at the material level, sugars also regulate the signal mechanism to affect anthocyanin synthesis [58]. In our study, pathways related to sugar metabolism, such as glyoxylate and dicarboxylate metabolism (ko00630) and starch and sucrose metabolism (ko00500) were enriched, supporting the participation of endogenous sugars in the accumulation of anthocyanin in *L. coreana* leaves.

The synthesis of plant sugars is also closely linked to photosynthesis, which involves chlorophyll and carotenoids absorbing the light energy [59]. Therefore, chlorophyll and carotenoid content in *L. coreana* may influence the accumulation or degradation of anthocyanin through the photosynthesis-sugar-hormone pathway, leading to the red coloration of tender leaves and the gradual fading to green as the leaves mature. Thus, the regulation of anthocyanin synthesis in *L. coreana* is a complex system, where endogenous factors, including environmental influences play significant roles. However, the exact mechanisms and interactions within this regulatory network need further elucidation. In summary, we have outlined the expression changes of key TFs and anthocyanin biosynthetic genes during the development of leaves with different colors, providing a comprehensive diagram of anthocyanin accumulation in *L. coreana* at various developmental stages (Fig. 8).

Conclusion

This study comprehensively explored the metabolic and regulatory pathways involved in leaf color change in *L. coreana* using targeted metabolomics and transcriptomics of leaves with different colors at three developmental stages. Anthocyanins, which are the primary metabolites responsible for the color change from red to green in tender leaves, gradually decreased in content during leaf development, while a total of 31 anthocyanin-derived DAMs were identified. We also mapped the metabolic pathways of major pigments, including chlorophyll, carotenoids, and anthocyanins and, through the integrated metabolomic and transcriptomic analysis, identified key enzymes involved in the accumulation of the pigments. Additionally, nine TFs, comprising 3 MYB, 2bHLH, 3 C2C2 zinc finger proteins, and 1 GRAS TF

were predicted to respond to endogenous substances and regulate anthocyanin synthesis in tender leaves of *L. coreana*, which warrant further investigation. Overall, this study provides new insights into the core metabolic products and regulatory networks of *L. coreana* leaves, offering guidance for the breeding and cultivation of ornamental and edible tea plants, and contributing to the sustainable use of tender leaf color resources.

Declarations

Ethics approval and consent to participate

The methods involved in this study were carried out in compliance with local and national regulations.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author Contribution

Hengxing Zhu, Qianli Dai and Ximeng Yang conceived and designed the project. Feiyi Huang, Min Lu, Chenggong Lei and Xueping Hu, Chen Benwen performed the experiments. Xin Huang, Xiaolong Nie, Daojing Chen, Sicheng Huang and Ximeng Yang analyzed data. Hengxing Zhu and Ximeng Yang wrote the manuscript. Qainli Dai, Benwen Chen and Ximeng Yang supervised and revised the manuscript.

Data Availability

The raw RNA-Seq data of Illumina sequences have been deposited in the NCBI Sequence Read Archive under accession numbers PRJNA1235469. Metabolomics datasets and other supporting data are included in the supplementary materials of this article.

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Figures

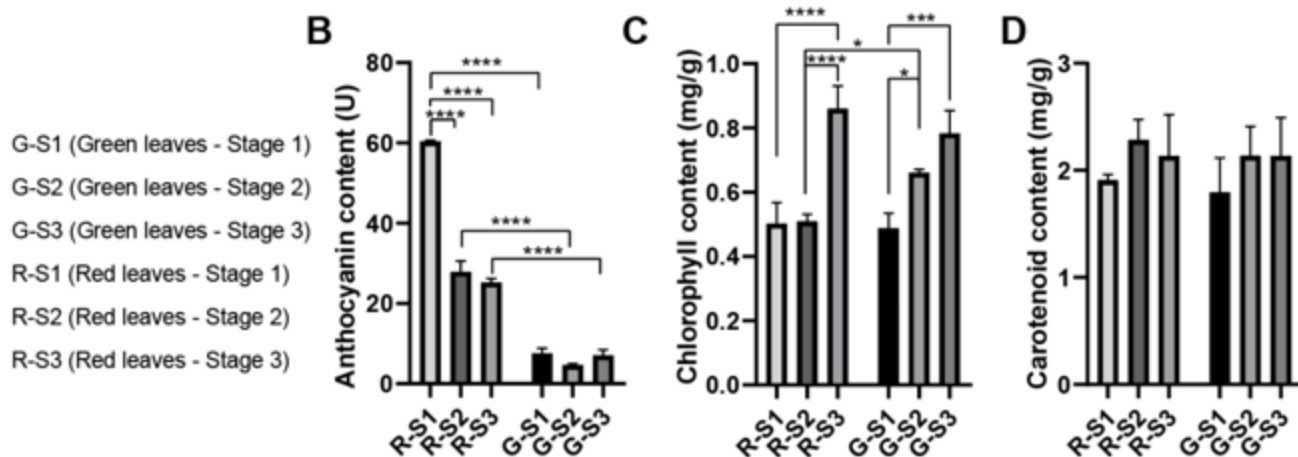
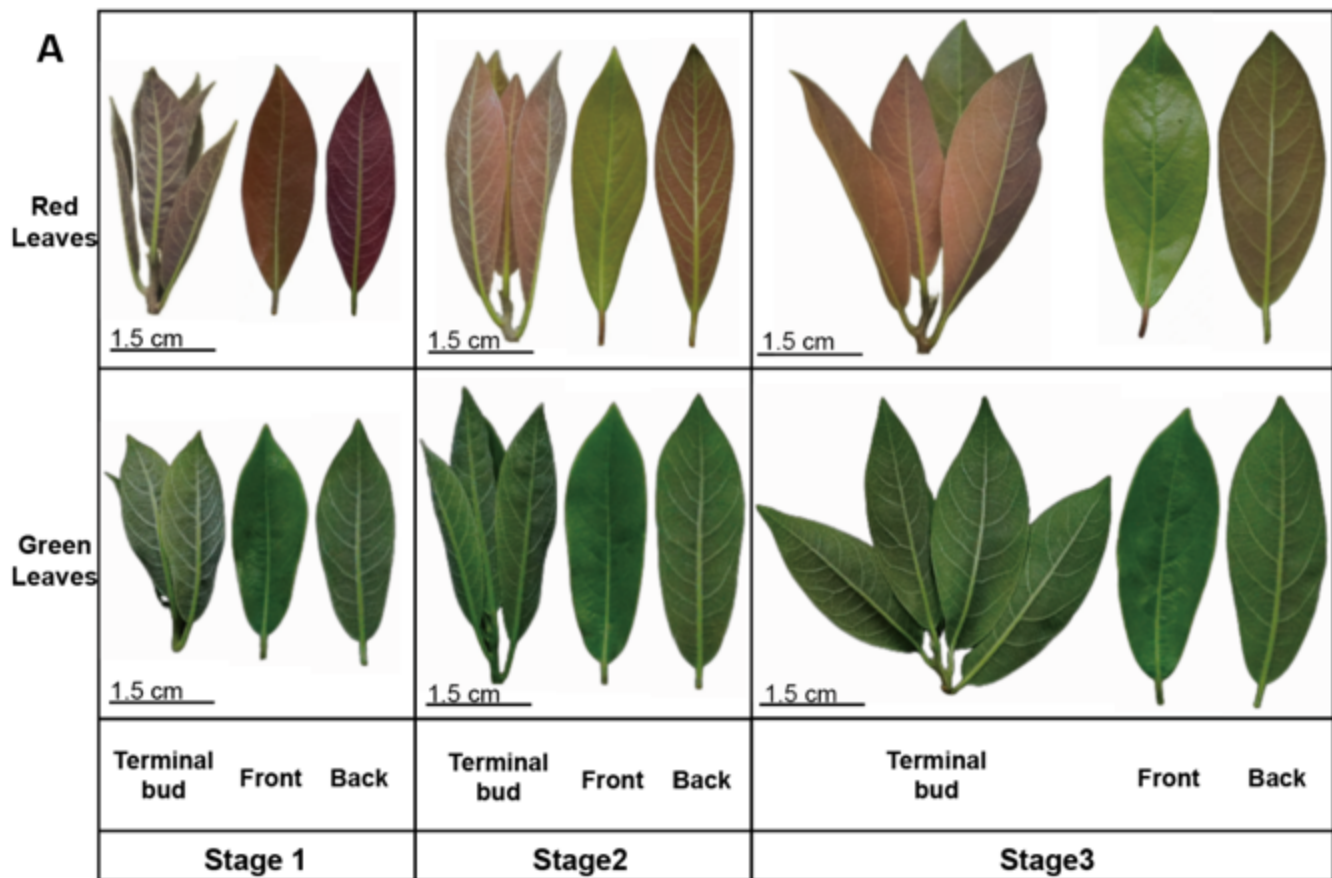


Figure 1

Phenotypic observations and pigment content changes in *L. coreana* leaves. (A) Morphological observations of red-leaves (R) and green-leaves (G) *L. coreana* at three developmental stages (S1-S3). Content of anthocyanins (B), chlorophylls (C), and carotenoids (D) in red-leaves (R) and green-leaves (G) *L. coreana* at three developmental stages (S1-S3). (n = 3) Values represent the mean of three independent trees, each consisting of leaves with similar coloration. Error bars represent the standard deviation of the mean. Asterisks on the bar charts indicate significant differences ($p < 0.05$), * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$, analyzed by Ordinary one-way ANOVA.

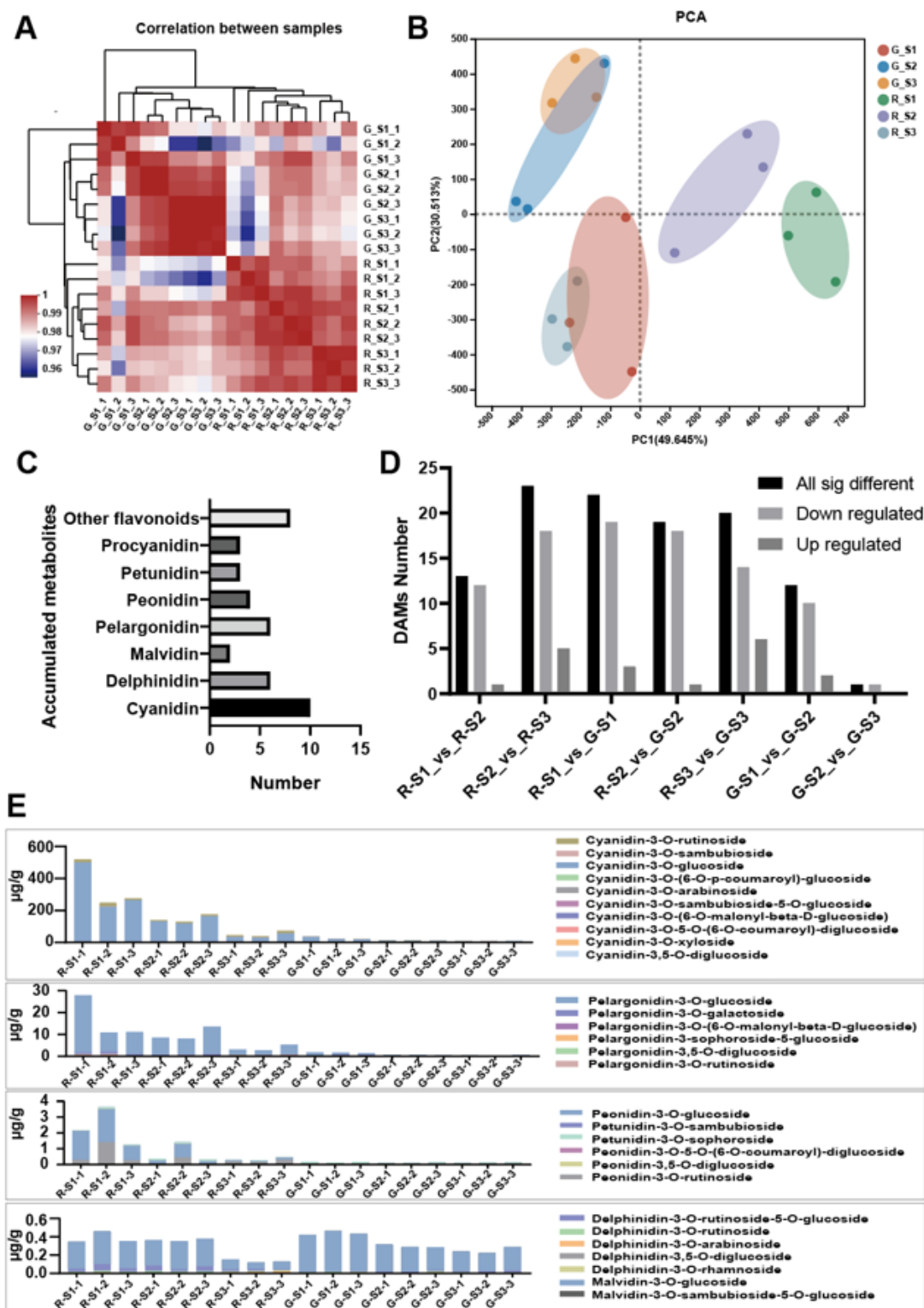


Figure 2

Metabolomic Analysis of tender leaves in *L. coreana*. (A) Sample correlation analysis. The closer the correlation coefficient is to 1, the stronger the correlation. (B) Principal component analysis of metabolomic data from leaves of different colored *L. coreana*. (C) Classification of metabolites in the leaves. The x-axis represents the quantity of each flavonoid metabolite. (D) Differentially accumulated metabolites (DAMs) in the seven comparisons: R-S1 vs R-S2, R-S2 vs R-S3, R-S1 vs G-S1, R-S2 vs G-S2, R-S3 vs G-S3, G-S1 vs G-S2, and G-S2 vs G-S3.

S3 vs G-S3, G-S1 vs G-S2, and G-S2 vs G-S3. (E) Analysis of the absolute content of significantly differentially accumulated anthocyanin metabolites.

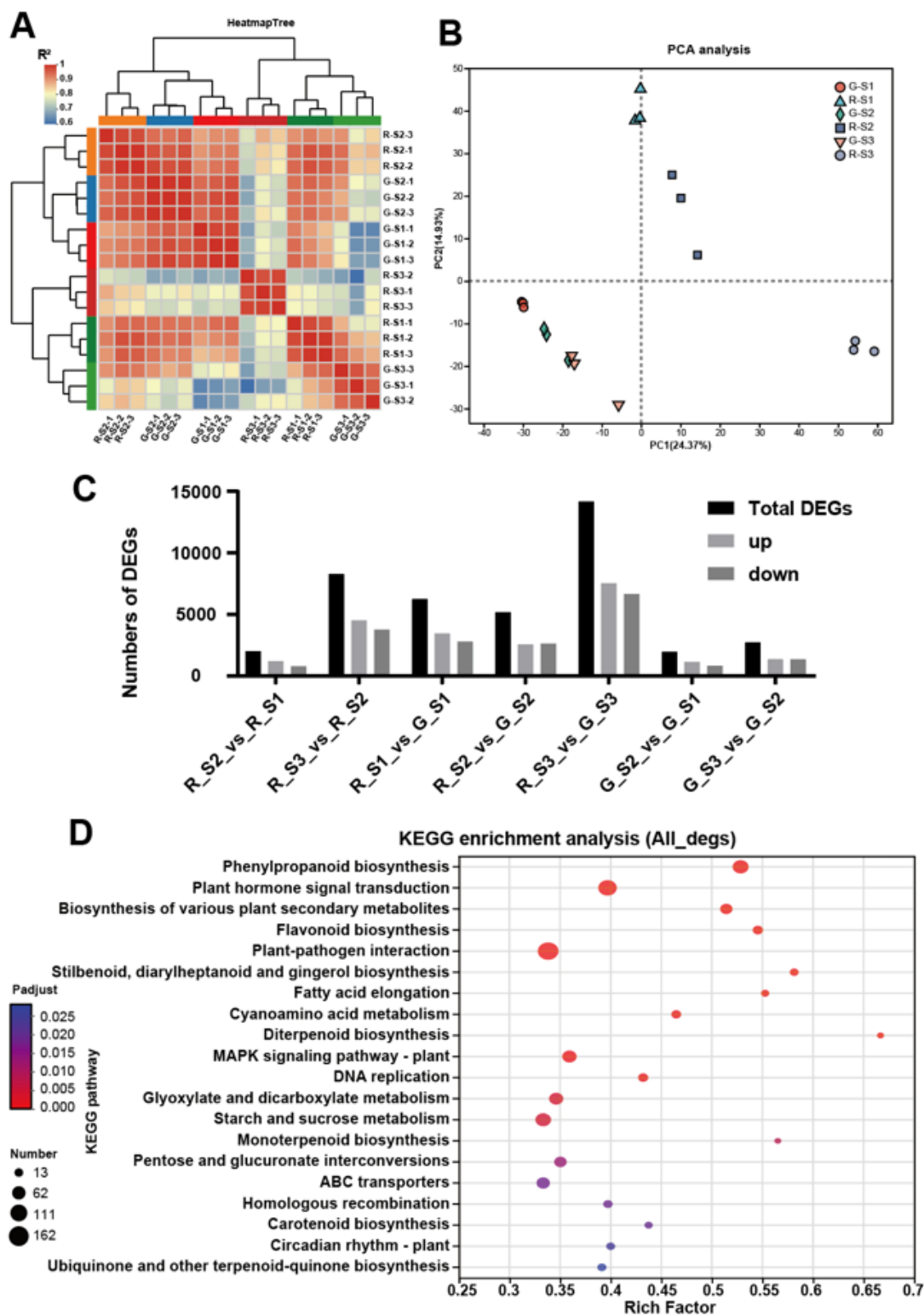


Figure 3

Transcriptomic analysis of *L. coreana* tender leaves. (A) Sample correlation heatmap based on transcriptomic data from 18 samples with different leaf colors and developmental stages. (B) Principal

component analysis (PCA) of transcriptomic data from 18 samples. (C) Differentially expressed genes (DEGs) statistics for different comparisons. (D) KEGG enrichment pathways for all DEGs in the various comparisons.

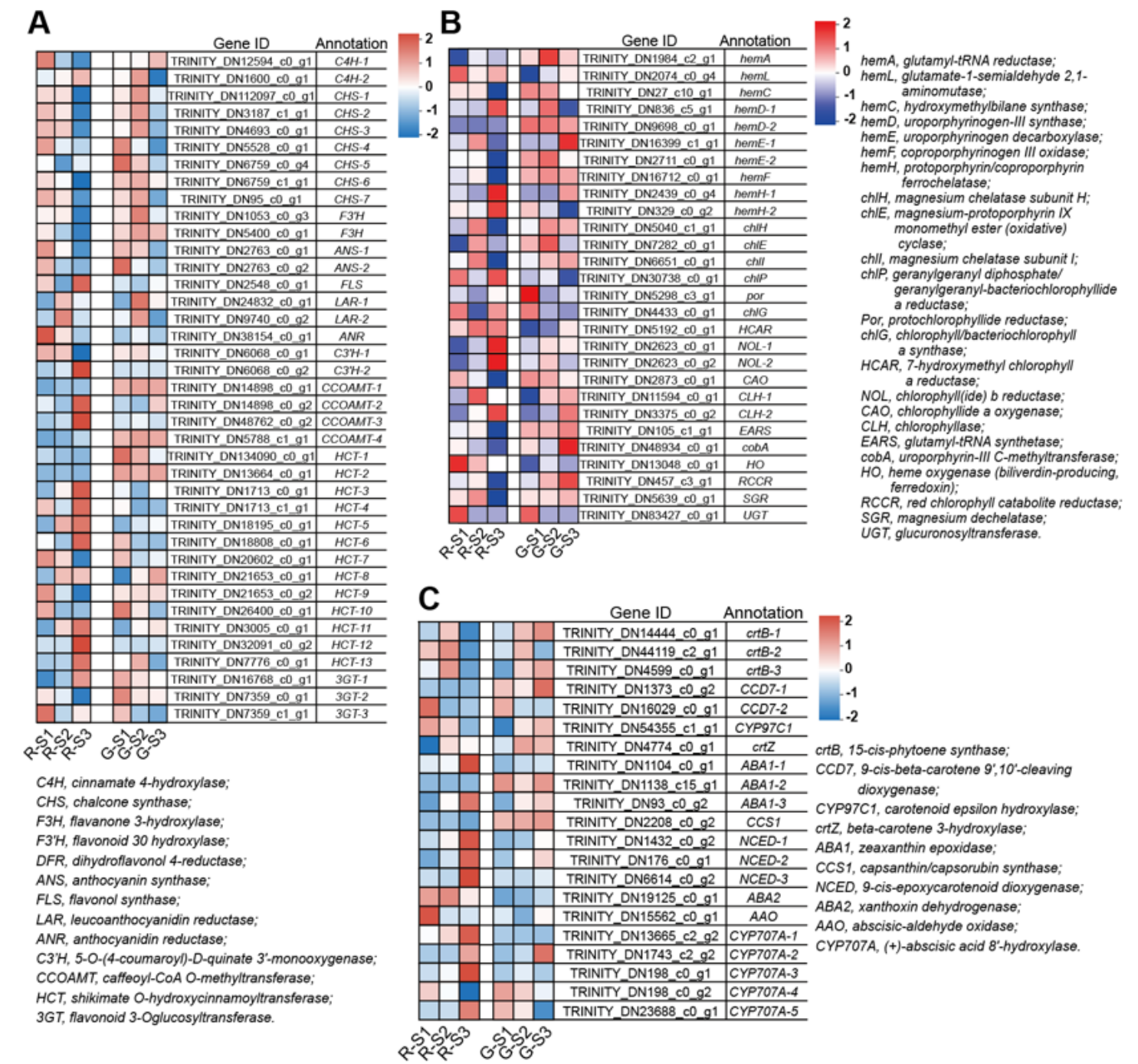


Figure 4

Heatmaps of differentially expressed genes in the three pigment biosynthesis pathway. (A) DEGs in the anthocyanin biosynthesis pathway. (B) DEGs in the porphyrin and chlorophyll metabolism pathway. (C) DEGs in the Carotenoid biosynthesis pathway. The red represents up-regulated genes and the blue represents downregulated genes.

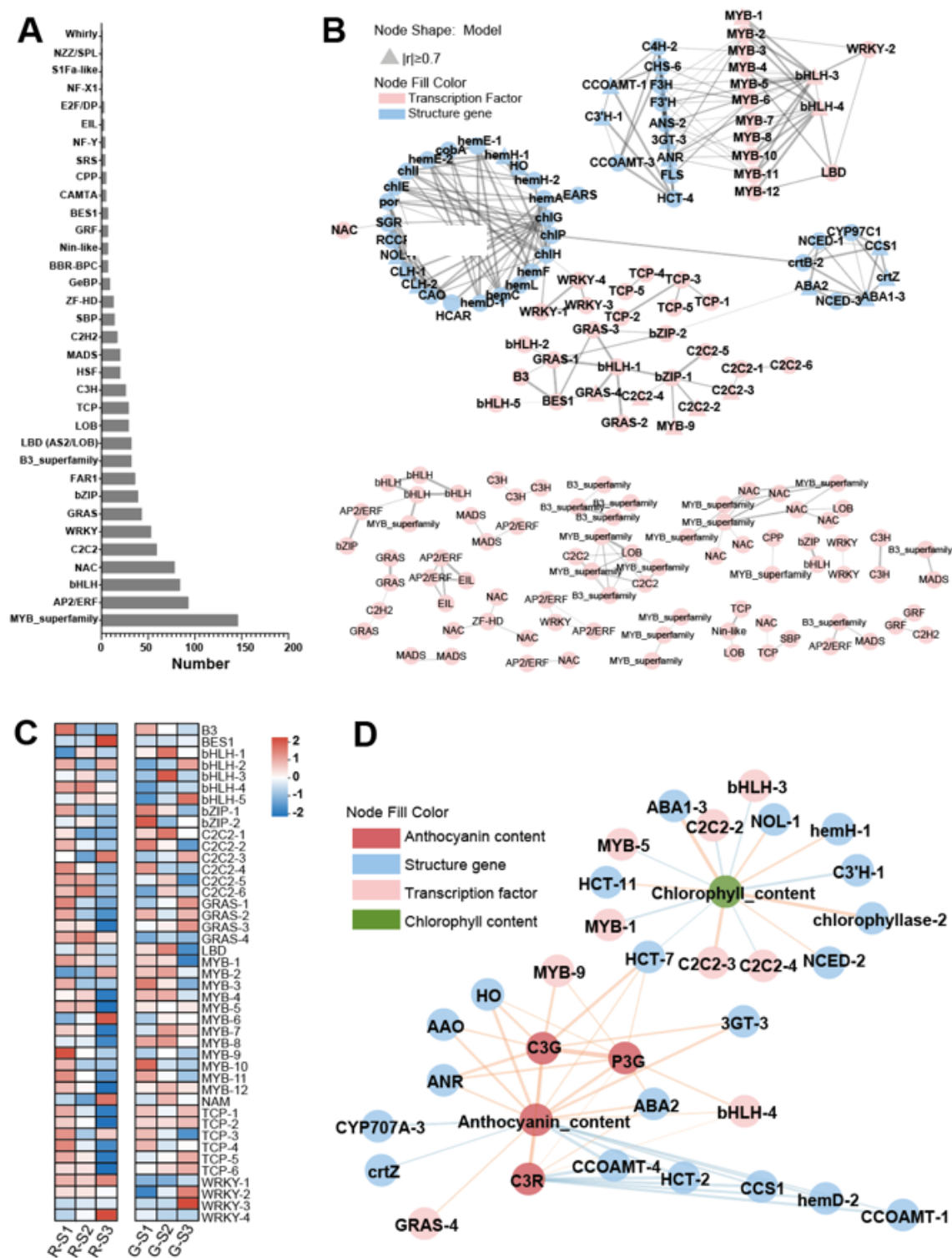


Figure 5

Transcription factors involved in the synthesis of three pigment types. (A) The number of genes of different transcription factor types within the differential genes. (B) Protein-protein interaction (PPI) network analysis between 88 pigment-related genes and 928 transcription factor-encoding genes. The thickness of the connecting lines represents the combined score of interactions between gene-encoded proteins, with thicker lines indicating stronger interactions. Genes marked with triangles are significantly

correlated with pigment content ($|r| > 0.7$). **(C)** Heatmap of the differential expression patterns of the 42 transcription factors, after excluding those that did not form clusters with structural genes. **(D)** Correlation network of pigment synthesis-related genes and metabolites in *L. coreana* Leaves. The thickness of the lines represents the strength of the correlation coefficient, with thicker lines indicating higher absolute values. The line color represents the type of correlation: red lines indicate positive correlations, and blue lines indicate negative correlations.

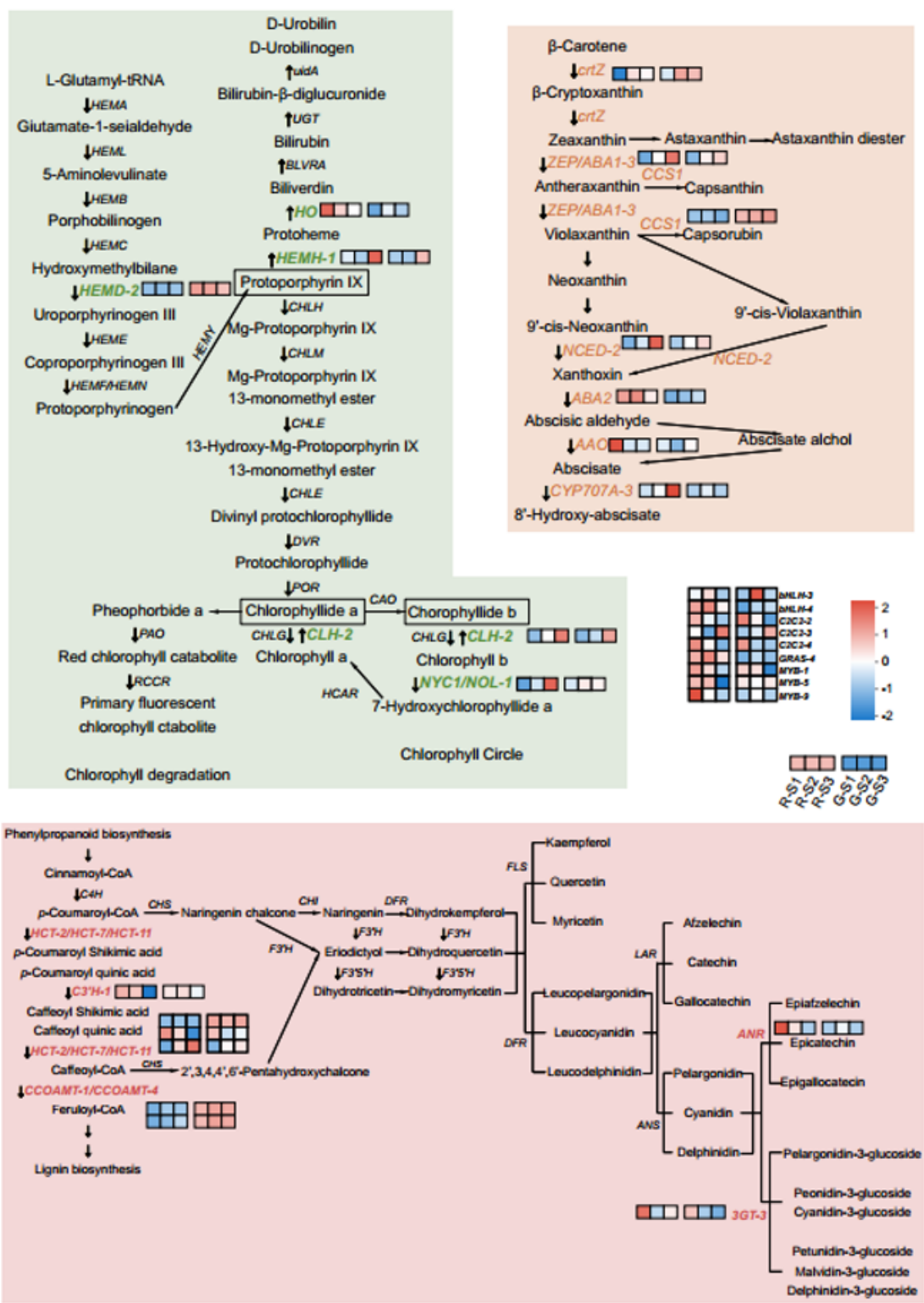


Figure 6

Heatmap of differentially expressed genes significantly correlated with pigment content. The green box highlights the Porphyrin and Chlorophyll Metabolism pathway, the orange box highlights the Carotenoid Biosynthesis pathway, the red box highlights the Anthocyanin Biosynthesis pathway.

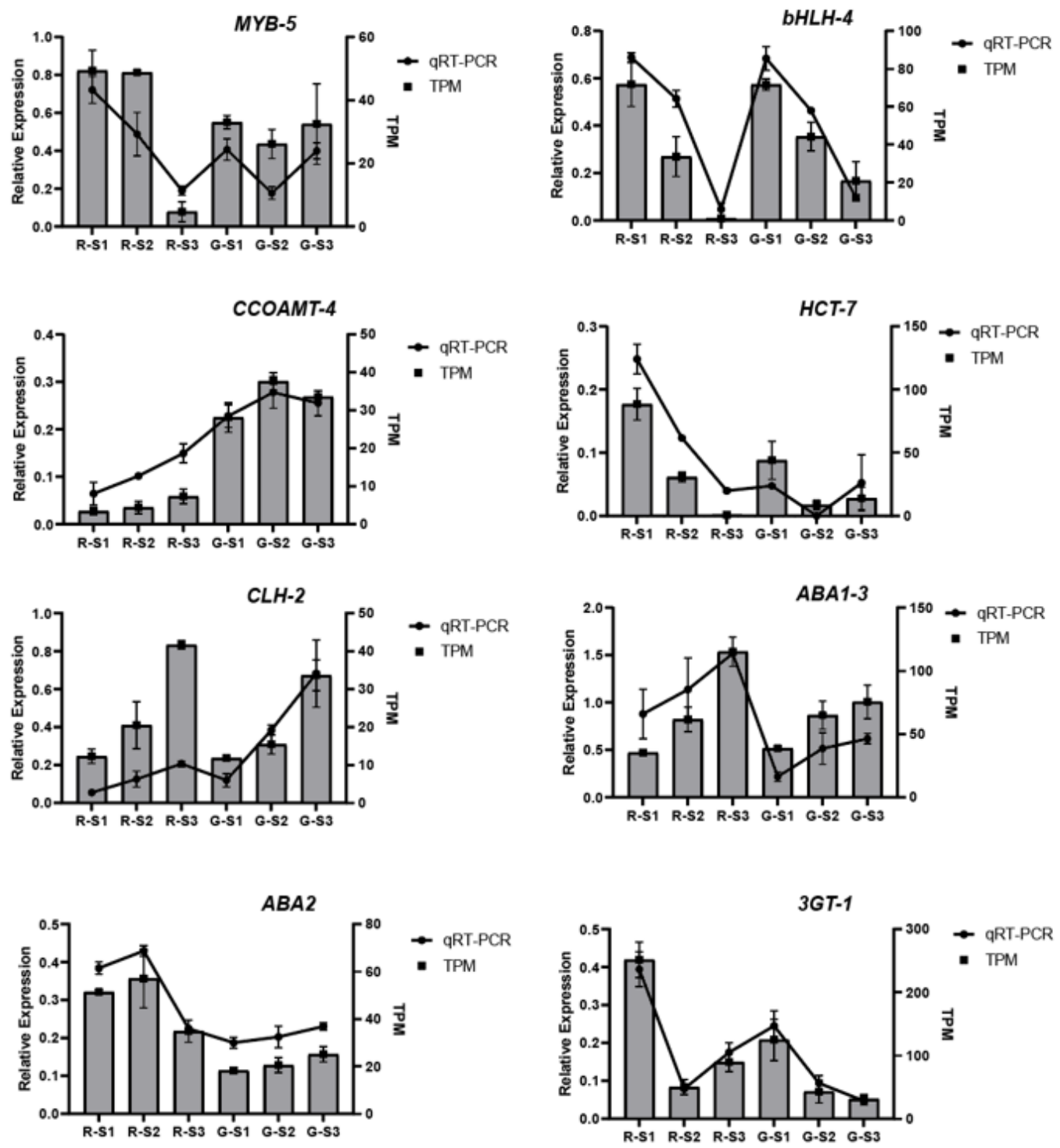


Figure 7

qRT-PCR validation of RNA-Seq data. The right y-axis represents the TPM values obtained from RNA-Seq. The left y-axis shows the relative gene expression levels measured by qRT-PCR. Error bars represent the standard error of the mean (SE) (n = 3).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1.xlsx](#)
- [TableS2.42flavonoidmetabolitesintheleavesofthesampledL.coreana.xlsx](#)
- [TableS3.29anthocyanindifferentialaccumulatedmetabolitesinthegroup.xlsx](#)
- [TableS4.TranscriptomeDataQualityAnalysis.xlsx](#)
- [TableS5.Datafilteringstatistics.xlsx](#)
- [TableS6.SpearmanccorrelationmatrixbetweenDEGsandDAMsandpigmentcontent..xlsx](#)
- [Fig.S1.png](#)
- [Fig.S2.png](#)