

Transcriptome sequencing and anthocyanin metabolite analysis involved in leaf red color formation of *Cinnamomum camphora*

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Abstract

Cinnamomum camphora, a key multifunctional tree species, serves primarily landscaping. Leaf color, crucial for its ornamental appeal, undergoes a transformation to red. However, the molecular mechanisms remain largely unexplored in *C. camphora*. In this study, green leaf (GL), color turning red leaf (RL) and whole red leaf (WRL) were obtained to measure pigment contents, GL and RL were analyzed transcriptomic alterations. A decline in chlorophylls and a rise in anthocyanins were observed during leaf color from green translate to red. Using LC MS/MS, 11 kinds of anthocyanins showed significant accumulative differences, with Cyanidin-3,5-O-diglucoside exhibiting the greatest disparity. Comparative RNA-seq identified 22,948 genes against reference genes, revealing 544 novel genes. Of these, 4,065 genes were up-regulated and 8,152 were down-regulated. Among them, 22, 4, and 31 differentially expressed genes (DEGs) were associated with chlorophyll biosynthesis, chlorophyll degradation, and anthocyanin biosynthesis, respectively. Additionally, differential expression was noted in 64 bHLH, 54 MYB, and 21 MYB-like transcription factors. These findings suggest a strong correlation between pigment metabolism and transcriptome data to release the mechanism with the leaf color translated to red of *C. camphora*.

Introduction

Leaf color, as vital as flower and fruit color, is a primary characteristic of ornamental plants that captures attention. Unlike flowers and fruits, which appear only briefly, leaf colors constitute a prominent feature contributing to the unique landscapes in the plant kingdom¹. In deciduous species, leaf colors change with plant development or seasonal shifts, such as the red leaves of the Norway maple in autumn². However, the camphor tree (*Cinnamomum camphora*), a prevalent evergreen and landscape tree, is also an economically and ecologically significant aromatic tree species widely distributed in China. It is noted for its leaves that may turn red in any season.

Plant pigments impart rich colors to various organs. Studies suggest that changes in leaf color are closely linked to the synthesis and accumulation of pigments^{3–5}. Plant coloration depends primarily on the types and concentrations of three pigments, chlorophylls, anthocyanins, and carotenoids. Chlorophylls are the main components of normally green leaves. However, loss of green color often results from disruptions in the chlorophylls biosynthesis pathway, exposing other colorful pigments⁶. Carotenoids, synthesized exclusively in plants, own more than 700 members and contribute yellow, orange and red color⁷. They also synthesize other substances and protect plants⁸. Anthocyanins, water-soluble polyphenols within flavonoid group, members number over 600 and are predominantly responsible for the red colors in ornamental plant tissues⁹.

The biosynthesis pathway of anthocyanins has been extensively studied in ornamental plants, such as *Prunus persica*¹⁰, *Bauhinia variegata*¹¹, *Prunus mume*¹². Numerous studies have highlighted the roles of structural genes and transcription factors (TFs) in plants¹³. Anthocyanin synthesis is catalyzed by

several enzymes, including chalcone synthase (CHS), dihydroflavonol reductase (DFR), flavanone-3-hydroxylase (F3H), anthocyanidin synthase/leucoanthocyanidin dioxygenase (ANS/LDOX), and UDP glucose flavonoid 3-glucosyltransferase (UGT). Glycosylated anthocyanins are transported to vacuoles by specialized transporters, such as glutathione S-transferase (GST) and multidrug resistance-associated protein (MRP)^{14, 15}, to protect them from the cytoplasmic environment. Structural genes are typically regulated by TFs (e.g. bHLH, MYB and WD40)¹⁶. MYB can regulate alone or in ternary complexes with two other TFs (known as MBW). In poplar, over-expression of *PdMYB118* leads to up-regulation of most structural genes and increased anthocyanins production in the leaves¹⁷. Recent studies indicate that other TF families (e.g. MADS, WRKY and bZIP) in anthocyanin synthesis^{18–20}.

With the advent of next-generation sequencing, transcriptome sequencing (RNA-Seq) has emerged as an effective tool for identifying candidate genes involved in biological processes. The camphor tree is diploid ($2n = 24$) with 12 chromosomes. Its genome sequencing has been completed²¹, and RNA-Seq has identified 2017 differentially expressed genes (DEGs) related to the red bark mutant ('Gantong 1'), including 24 up-regulated DEGs involved in the anthocyanin biosynthesis and 6 TFs²². Luan et al. identified 121 *CcMYBs* across 12 chromosomes in the whole genome of *C. camphora*, and RNA-Seq data from seven tissues suggest that *CcMYBs* from three subgroups may be involved in the flavonoid biosynthesis pathway²³. Certain species of *Pistacia chinensis* exhibit anthocyanin accumulation in leaves, which turn red in autumn. Transcriptomic data have shown that 31 structural genes in the anthocyanin biosynthesis pathway are up-regulated, with MYB113 expression correlating with anthocyanin content under both natural and shaded conditions²⁴.

In this study, to elucidate the molecular mechanisms behind color changes in *C. camphora* leaves, pigment concentration analysis and gene expression profiling using RNA-seq were conducted to identify the primary pigment types and key genes. This study offers valuable insights into the coloration mechanisms in the *C. camphora*.

Results

Colorimetric changes during the leaf color transformation

By constant observation, we found part of *C. camphora* leaves would change their color from green to red, and this phenotype can be observed all the year around (Fig. 1A). After counted 492 WRL on 335 branches and record their locations, we found that all WRL were distributed in the last 5 leaves at the end of the branch with 44.72%, 22.97%, 22.97%, 6.91% and 2.44%, respectively (Fig. 1B). Colorimetric measurements were performed on both the adaxial (Ad) surface (Fig. 1C) and abaxial (Ab) surface (Fig. 1D) of the leaves. On the Ad surface, the L^* values were no significant difference among GL, RL and WRL. The L^* values on the Ab surface of WRL were significant lower than GL and RL. For the a^* values, both Ad and Ab surfaces shown the significant difference among GL, RL and WRL. Whether Ad or Ab surface, GL were negative means green color, while RL and WRL were positive means red color. a^* values

of WRL were higher than RL on both surfaces. These results suggested that the WRL were redder than RL. b^* values of WRL exhibited lowest level compared with GL and RL on both surface.

Major pigments in the red leaves of *C. camphora*

To determine the mainly pigments responsible for the leaf color transformation, the chlorophylls, carotenoids and anthocyanins content were measured. The results showed that color profiles were differently between GL, RL and WRL. The contents of chlorophyll a (Fig. 2A), chlorophyll b (Fig. 2B) and total chlorophyll (Fig. 2C) were significant decrease from the GL to WRL. When the leaf color completed turn to red, chlorophyll a, chlorophyll b and total chlorophyll only remain 1.79%, 9.47% and 3.98% of the GL, respectively. The contents of carotenoid were similar between RL and WRL, and significant decrease compared to GL (Fig. 2D). Compared to the GL and RL, WRL own the highest anthocyanins content, reached up to $532.13 \mu\text{g}\cdot\text{g}^{-1}$ (Fig. 2E). By the HPLC MS/MS, 27 anthocyanins were detected in GL and WRL. Among them, 11 anthocyanins were identified as differentially accumulated metabolites, including 3 cyanidins (Cyanidin-3,5-O-diglucoside, Cyanidin 3-O-sophoroside and Cyanidin 3-O-xyloside), 1 peonidin (Peonidin 3-O-galactoside), 1 pelargonidin (Pelargonidin 3-O-beta-D-glucopyranoside), 3 delphinidins [Delphinidin 3-O-sophoroside, Delphinidin 3-(6-rhamnosylgalactoside) and Delphinidin 3-rutinoside-5-glucoside] and 3 other anthocyanidins (Aurantinin, 6-Hydroxycyanidin and 5-Carboxypyranocyanidin 3-O-beta-glucopyranoside) (Fig. 2F). The WRL accumulated more anthocyanins than the GL, which are sources of red color. Among them, Cyanidin-3,5-O-diglucoside, Peonidin 3-O-galactoside, and Pelargonidin 3-O-beta-D-glucopyranoside showed the top three significance difference.

RNA library construction and sequencing

In order to characterize the difference of gene expression levels between GL and RL, 6 cDNA libraries were constructed, including 3 GL (named GL1, GL2 and GL3) and 3 RL (named RL1, RL2 and RL3), respectively. By the way, we found that the RNA of WRL was degraded lead to unable construct cDNA library. A total of 299,566,040 reads (44,934,906,000 bp) were generated in raw data. After sweep away the low-quality reads, a total of 297,256,232 reads (44,163,916,131 bp) were filtered out. On average have 92.14% of the reads were mapped to the camphor tree reference genome (Supplementary Table S1). A total of 22,948 genes were sequenced compared to reference genes, and 544 new genes were identified (Supplementary Table S2). Among these new genes, 231 unnamed, 212 belonged to *Cinnamomum micranthum* f. *kanehirae*, and other 101 distributed to 63 plants.

Contrastive analysis of transcriptome data between GL and RL

The leaf color transformation is a complex process. High transcriptome variation was observed between GL and RL, as evidenced by a large number of differentially expressed genes (DEGs). In total, 12,217 DEGs were filter out (Supplementary Table S3), including 4,065 up-regulated genes and 8,152 down-regulated genes (Fig. 3A). We selected 30 DEGs with the greatest gene expression difference, 24 DEGs were down-regulated, 5 DEGs were new genes (Fig. 3B). Ccam02G002785 was the maximum positive

value with $\log_2$ (fold change) = 14.928, while Ccam01G002624 was the maximum negative value with $\log_2$ (fold change) = -16.493. To confirm the accuracy and reliability of the RNA-seq data, 6 DEGs were randomly chosen and validation by qRT-PCR. The candidate genes expression showed large consistence between RNA-seq and qRT-PCR (Fig. 3C).

GO and KEGG analysis of the DEGs

To further elucidate the functional roles of DEGs, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis through AgriGO and GeneCodis. All DEGs were assigned to three main categories of GO classification, including biological process (BP), cell components (CC) and molecular functions (MF) (Fig. 4A). For BP, the dominant subcategories were 'cellular process' and 'metabolic process'. For CC, 'cellular anatomical entity' and 'protein-containing complex' were highly represented. In MF, 'binding' and 'catalytic activity' were the most represented.

All DEGs were further performed by KEGG pathway enrichment analysis. DEGs were mainly classified into 137 pathways. Only 23 pathways with the P-value ≤ 0.05 , including 'ribosome', 'pentose phosphate pathway', 'porphyrin metabolism', 'photosynthesis', 'carbon metabolism', 'carbon fixation in photosynthetic organisms' and 'photosynthesis-antenna proteins'. 'Ribosome' (Ko03010) contained the largest number of DEGs (247 DEGs, accounts for 8.99% of the total DEGs), and most of the DEGs were down-regulated in RL. Ribosome was the cellular factory responsible for synthesize proteins, most of DEGs were down-regulation could affect other biological process. List the top 30 most enriched pathways (Fig. 4B). 'Porphyrin metabolism' (Ko00860), 'carotenoid biosynthesis' (Ko00906) and 'anthocyanin biosynthesis' (Ko00942) were related to plant pigment chlorophylls, anthocyanins and carotenoids, respectively.

Ribosome related DEGs between GL and RL

Ribosome is composed of two subunits, which are built from RNA and protein. In cells, ribosome perform translation function and play vital role in cell division, growth and development²⁵. In this study, we found that the 'ribosome' was the richest KEGG term. Total of 247 DEGs belonged to this pathway, but only 3 DEGs (Ccam04G001485, Ccam02G003266, and MSTRG.15482) were up-regulated. 24 DEGs with $|\log_2$ (Fold change)| ≥ 3 were belong to small subunit and only PR-S23e (MSTRG.15482) is up-regulated (Fig. 5A). 61 DEGs with $|\log_2$ (Fold change)| ≥ 3 were belong to large subunit and only PR-L31 (Ccam02G003266) is up-regulated (Fig. 5B). Most of genes were down-regulated would affect the ribosome generation and protein synthesis. Thus, we measured the change of protein concentration during leaf color transformation. The results shown that protein contents were downtrend, the GL own the highest protein contention than RL and WRL (Fig. 5C).

DEGs related to chlorophyll biosynthesis and degradation

Chlorophylls biosynthesis and degradation is belonged to 'porphyrin metabolism' (Ko00860). Based on the KEGG pathway analysis, a total of 26 DEGs were identified, including 2 up-regulated DEGs and 24 down-regulated DEGs (Fig. 6). Among them, 22 DEGs related to chlorophylls biosynthesis, including *OVA3* (Ccam05G002255), *HEMA1* (Ccam01G002476 and Ccam04G001696), *GSA* (Ccam01G002922 and Ccam06G000281), *HEMB* (Ccam11G001295), *HEMC* (Ccam07G001413), *HEME1* (Ccam10G000902), *CPX1* (Ccam10G000600), *PPOX1* (Ccam10G001810), *CHLH* (Ccam10G001073), *CHLD* (Ccam02G001092), *CHLI* (Ccam04G000078), *CHLM* (Ccam02G002041), *CRD1* (Ccam01G003276), *DVR* (Ccam02G001588), *PORA* (Ccam12G000060), *CHLG* (Ccam03G003207), *CAO* (Ccam01G000545), *NYC1* (Ccam02G002556), *NOL* (Ccam03G002262) and *HCAR* (Ccam12G001422). Almost all of the genes were down-regulated, except for *NYC1* was up-regulated in RL. 4 DEGs related to chlorophyll degradation, including *CLH2* (Ccam02G002637), *RCCR* (Ccam08G000276), *SGR* (Ccam07G000443), *SGRL* (Ccam09G000375). These results indicated that chlorophylls biosynthesis was severely decreased in RL. The genes expression result was consistent with the result of chlorophylls concentration.

DEGs related to anthocyanin biosynthesis

Anthocyanin is associated with three pathways, including 'phenylpropanoid biosynthesis' (Ko00940), 'flavonoid biosynthesis' (Ko00941) and 'anthocyanin biosynthesis' (Ko00942). In this study, 31 DEGs were identified belong to four *PAL*, one *C4H*, seven *4CL*, four *CCoAOMT*, five *CHS*, one *CHI*, one *F3H*, one *FLS*, one *DFR*, one *ANS* and four *UFGT* (Fig. 7). 18 DEGs were significantly up-regulated, including three *PAL*, three *CCoAOMT*, one *C4H*, four *4CL*, three *CHS*, one *FLS*, one *ANS* and two *UFGT*. Among these up-regulated genes, *4CL* (Ccam06G000458, Ccam06G002027, Ccam08G001811 and Ccam03G001045), *ANS* (Ccam10G000921) and *UFGT* (Ccam02G000147 and Ccam02G000149) may result in increased anthocyanin synthesis in RL.

TFs analysis

TFs analysis was performed in this study, total of 1691 TFs have been identified into 58 families. 657 DEGs belongs to 51 different TF families were identified (Fig. 8, Supplementary Table S4). These DEGs are mainly in the bHLH, MYB, ERF, NAC, WRKY, C2H2 families (Fig. 8). TFs could affect the pigment biosynthesis by regulate the expression of structural genes. MYB and bHLH are the critical TFs in flavonoid and anthocyanin biosynthesis. We found 64 bHLH, 54 MYB, and 21 MYB-like genes were difference expression. Among the most 10 significantly up-regulated genes were three MYB, three LBD, one bHLH, one WRKY, one ERF, and one G2-like, while the most 10 significantly down-regulated genes were three ERF, one MYB, one bHLH, one bZIP, one LBD, one GATA, one C2H2, and one TCP.

Discussion

In plants, leaves are not only important organ for photosynthesis, transpiration and gas exchange, but also one of the most important ornamental trait. The *C. camphora* have a phenotype that leaves change in color from green to red around the year. Previous studies suggest that leaves color are predominantly determined by chlorophylls, carotenoids and anthocyanins concentration in various plants^{26–28}.

However, the factors regulating the synthesis and degradation of pigment are complex. In this study, we observed the process of leaves turning red and identified DEGs in *C. camphora* RL compared with GL.

By pigments content determination, we found that GL own the highest content level of chlorophylls and carotenoids, while WRL own the highest content level of anthocyanins. Chlorophylls degradation and anthocyanins accumulation caused leaves turn red. Evolution has been selected for high chlorophylls content in leaves related to photosynthesis and gives green color. The biosynthesis and degradation of chlorophylls play a crucial role in leaf coloration. Through a branched pathway, flavonoids are synthesized both colorless and colored pigments (including anthocyanins and proanthocyanidins). Most of anthocyanins belong to cyanidin, peonidin, pelargonidin, delphinidin, malvidin, and petunidin types²⁹. In our results, Cyanidin-3,5-O-diglucoside, Peonidin 3-O-galactoside, and Pelargonidin 3-O-beta-D-glucopyranoside showed the top three significance difference of anthocyanins. In *C. camphora* cv 'Gantong 1', the red bark contained more anthocyanin compounds than green bark of normal tree, especially Pelargonidin 3-O-glucoside (synonyms Pelargonidin 3-O-beta-D-glucopyranoside). Pelargonidin 3-O-beta-D-glucopyranoside mainly responsible for the orange-red coloration²². For *Acer pseudosieboldianum* leaves, Cyanidin-3,5-O-diglucoside content displayed significant differences between middle stage and last stage in autumn, it may be play major role for the final red color³⁰. For *Ananas comosus* var. *Bracteatus* leaves, the Cyanidin-3,5-O-diglucoside content nearly 95.6% in red samples and significantly higher than green samples³¹. By comparisons of two purple heading Chinese cabbages, Cyanidin 3,5-O-diglucoside, Pelargonidin 3-O-beta-D-glucoside, and Cyanidin O-syringic acid was directly affected color differences³².

High-throughput sequencing technologies rapid development. RNA-seq is a useful tool to screen out key genes related to special characteristics in ornamental plants, such as *C. camphora*²², *Purnus persica*³³, *Acer fabr*⁵. In our study, we identified 26 DEGs take part in chlorophyll biosynthesis and degradation. SGR is one of the most important enzyme in chlorophyll degradation and *sgr* mutation was originally described by Mendel³⁴. *SGR* expression is up-regulated during natural and dark-induced leaf senescence³⁵. The mutation of *SGR* caused the normal senescence leaves remain green for a long time through affecting the chlorophyll degradation. In rice, *SGRL* is primarily expressed in green tissues, and is significantly down-regulated in leaves undergoing natural and dark-induced senescence³⁶. The expression pattern of *SGRL* is opposite to that of *SGR1* in *Arabidopsis*³⁴, which is consistent with our RNA-seq results.

The flavonoids biosynthesis is controlled by multiple structural genes. Our RNA-Seq analysis screen out 31 DEGs related to anthocyanin biosynthesis, including *PAL*, *C4H*, *4CL*, *CCoAOMT*, *CHS*, *CHI*, *F3H*, *FLS*, *DFR*, *ANS* and *UFGT*. *4CL* is a crucial rate-limiting enzyme in flavonoids metabolism, directly participating in phenylpropane metabolism, and then synthesizing flavonoids³⁷. qRT-PCR analysis showed that the expression level of all *4CL* members were directly proportional to the degree of fruit coloring and anthocyanin accumulation³⁸. *ANS* catalyzes the colorless proanthocyanidins converted to colored anthocyanin precursors. Comparative leave transcriptome analysis of 11 *Brassica* species with purple or

green leaves, the results showed that ANS was highly up-regulated in all purple leaves³⁹. UFGT is a key enzyme can promote unstable anthocyanidins be stabilized. By transcriptomic comparison analysis of foliar senescence between *Prunus cerasifera*, UFGT were exclusively up-regulated in red leaves than green leaves⁴⁰. By compared the Chokecherry green, purple and purplish-red leaves transcriptome data, two UFGT genes (Cluster-24837.111509 and Cluster-24837.123865) with high expression during the leaf color gradually turns purple-red⁴¹. In *Cymbidium sinense*, qRT-PCR results showed that *ANS* expression in red leaf stage was 833 times higher than green leaf stage, while the expression of *UFGT* in red leaf stage was 14 times higher than green leaf stage⁴².

TFs regulating anthocyanin biosynthesis have been reported in various plants. In our study, we identified a large number DEGs belong to TFs. MYB is a large TFs superfamily with highly conserved DNA-binding domain repeats and can be divided into four sub-families on the basis of their repeats number. The structural genes encoding these enzymes is tuned by MYB or a conserved MYB-bHLH-WDR (MBW) complex. MBW comprises MYB, basic helix-loop-helix (bHLH), and WD-repeat proteins⁴³. R2R3-MYB members are critical transcriptional regulators for anthocyanin structural genes⁴⁴. AtMYB5 is homologous to TT2 (AtMYB123) in terms of function, can also form MBW complexes⁴³. 121 *CcMYBs* were identified on 12 chromosomes in the whole genome of *C. camphora*, part of *CcMYBs* were significantly associated with structural genes involved in flavonoid synthesis. *CcMYB21*, *CcMYB43*, and *CcMYB52* were significantly correlated with the *DFR* and *LDOX*²³. In *Populus deltoids*, *PdMYB118* functions have been verified as an essential transcription factor regulating anthocyanin biosynthesis by overexpressing *PdMYB118*¹⁷. Overexpression of PsMYB114L and PsMYB12L exhibited significantly accumulated more anthocyanins in transgenic Arabidopsis plants, resulting in purple-red leaves⁴⁵. More researches have shown that other TF families (like MADS, NAC, WRKY) are involved in anthocyanin synthesis. In pears, transcription factor PpWRKY44 induces anthocyanin accumulation by regulating *PpMYB10* expression. Over-expressed *PpWRKY44* in leaves and fruit peels significantly enhanced the accumulation of anthocyanin⁴⁶. These results indicating that anthocyanin synthesis and regulation mechanisms are complicated.

Materials and Methods

Plant Materials

The leaves of *C. camphora* were obtained from the campus of Huaiyin Institute of Technology (Huai'an, China). Ten trees were selected to observe and statistics the color change of leaves. Three growth uniformity trees were selected to collect the green leaves (GL), color turning red leaves (RL), and whole red leaves (WRL) as the plant materials. All samples were immediately used or frozen in liquid nitrogen and then stored at -80 °C.

Determination of color indices

The GL, RL and WRL were collected to measure the color parameters, respectively. Every color stage choose 10 leaves, and color parameters measurement were repeated three times for each leaf. A colorimeter (CR8, Shenzhen ThreeNH Technology Co., Ltd. China) was used to measure the color differences (L^* , a^* , and b^*) between the GL, RL, and WRL. L^* indicates lightness, with a value of 0 representing black and 100 representing white. a^* corresponded to red/green chromaticity, with '+' values indicating 'redness' and '-' values indicating 'greenness'. b^* corresponded to yellow/blue chromaticity, with '+' values indicating 'yellowness' and '-' indicating 'blueness'. The color of GL was used as a control.

Determination of pigment content

The same GL, RL and WRL samples were used for determination of anthocyanins, chlorophylls and carotenoids content. Total anthocyanin contents determination were followed Mei et al method³. Chlorophylls and carotenoids contents were determined followed Dong et al method⁴⁷. All pigment contents were measured in three replicates.

Anthocyanins measurement used the LC-MS/MS

Fresh samples of GL and WRL were pulverized by using tissue grinder at 50 Hz for 120 s. Anthocyanins were extracted from 50 mg leaf powder in 1 mL 60% MeOH (0.1 mol/L HCl, 0.1% Ethylenediaminetetraacetic acid disodium salt by mass fraction), vortex for 60 s. Extraction for 10 min under the conditions of ultrasonic temperature 40 °C and ultrasonic power 60%. Centrifuge for 10 min at 12,000 rpm and 4 °C, filter the supernatant by 0.22 µm membrane and transfer into the detection bottle for LC-MS detection. Liquid chromatography conditions were followed Hong et al.⁴⁸, while Mass spectrum conditions followed Zhang et al.⁴⁹. All pigment contents were measured in three replicates. Anthocyanins with a $|\text{Log}_2(\text{fold change})| \geq 2$ and p-value < 0.05 were considered differentially accumulated anthocyanins.

RNA extraction, libraries construction and sequencing

The total RNA was extracted from the GL and RL samples using the RNeasy Pure Plant Plus Kit (Polysaccharides and Polyphenolics-rich) (DP441; Tiangen Biotechnology, Beijing, China) according to the manufacturer's protocol. GL and RL own three biological replicates, respectively. RNA quality were measured by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and checked using RNase-free agarose gel electrophoresis. After purification from total RNA using the oligo (dT) magnetic beads, enriched mRNA was fragmented into short fragments using fragmentation buffer and reversly transcribed into cDNA by using NEBNext Ultra RNA library Prep Kit for Illumina (#7530, NEB, Ipswich, MA, USA). Then, the double-stranded cDNA fragments were purified, end-repaired, A base added, and ligated to the Illumina sequencing adaptors. The ligation reaction was purified with the AMPure XP Beads (1.0X). The ligated products were selected by agarose gel electrophoresis and PCR amplified. Then sequenced on the Illumina Novaseq6000 (Illumina Inc., San Diego, CA, USA) by Gene Denovo Biotechnology Co. (Guangzhou, China).

Analysis of RNA-seq data

Reads obtained from the sequencing machines includes raw reads containing adapters or low quality bases. Thus, raw reads were further filtered by fastp (version 0.18.0) to get high quality clean reads. The fastp removed reads containing adapters, containing more than 10% of unknown nucleotides (N) and low quality reads containing more than 50% of low quality (Q-value < 20) bases. Short reads alignment tool Bowtie2 (version 2.2.8) was used for mapping reads to ribosome RNA (rRNA) database. The rRNA mapped reads then will be removed. Then, the clean reads were mapped to the *C. camphora* genome with HISAT2. The *C. camphora* genome was employed as the reference genome²¹.

DEGs analysis

The mapped reads of each sample were assembled by using StringTie (v1.3.1) in a reference-based approach. For each transcription region, a FPKM (fragment per kilobase of transcript per million mapped reads) value was calculated to quantify its expression abundance and variations by RSEM software. The DEGs between GL and RL were analyzed by DESeq2 software. The genes/transcripts with the parameter of p -value < 0.05 and $|\log_2(\text{Fold change})| \geq 1$ were considered DEGs. The DEGs were subjected to enrichment analysis of GO functions and KEGG pathways.

Quantitative real-time PCR (qRT-PCR)

6 DEGs were selected to validate expression levels by qRT-PCR. The qRT-PCR assay was performed on CFX Connect™ Real-Time System (Bio-Rad, USA) with ChamQ Blue Universal SYBR qPCR Master Mix (Vazyme). The primer sequences (listed in Supplementary Table S5) were designed by Primer Premier 6.0 software. The reaction system contained 10 µL of ChamQ Blue Universal SYBR qPCR Master Mix, 0.4 µL Primer 1 (10 nM) and 0.4 µL primers 2 (10 nM), 30 ng of cDNA, and nuclease-free water to a final volume of 20 µL per reaction. *CcActin* was used as the reference gene for qRT-PCR²³. Data analysis was performed using the Bio-Rad CFX Manager 3.1 (Bio-Rad, USA). All experiments were performed with three biological replicates, and the $2^{-\Delta\Delta C_t}$ method was used to analyze the amplification results.

Protein extraction and protein content determination

0.4 g leaf samples of GL, RL, and WRL were separately ground into powder with 10% PVP by liquid nitrogen. The protein extraction was performed according to the TCA/acetone precipitation method⁵⁰. After extraction, the protein concentration was determined by the Bradford method⁵¹.

Declarations

Competing interests

The authors declare no competing interests.

Supplementary Information

Statement

The collection of plant materials complied with relevant institutional, national, and international guidelines and legislation. Authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

Author Contribution

XW conceived and designed this study. YL and TD performed the transcriptome and qRT-PCR assay. FT, LK and BP performed the color parameters and pigment concentration determination. XW and YL analyzed the data and draw the figures. XW and WZ wrote the manuscript. All authors reviewed the manuscript.

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Data Availability

All raw sequencing data described in this study have been submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA). Submission ID is SUB14435641, BioProject ID is PRJNA1110619.

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Figures

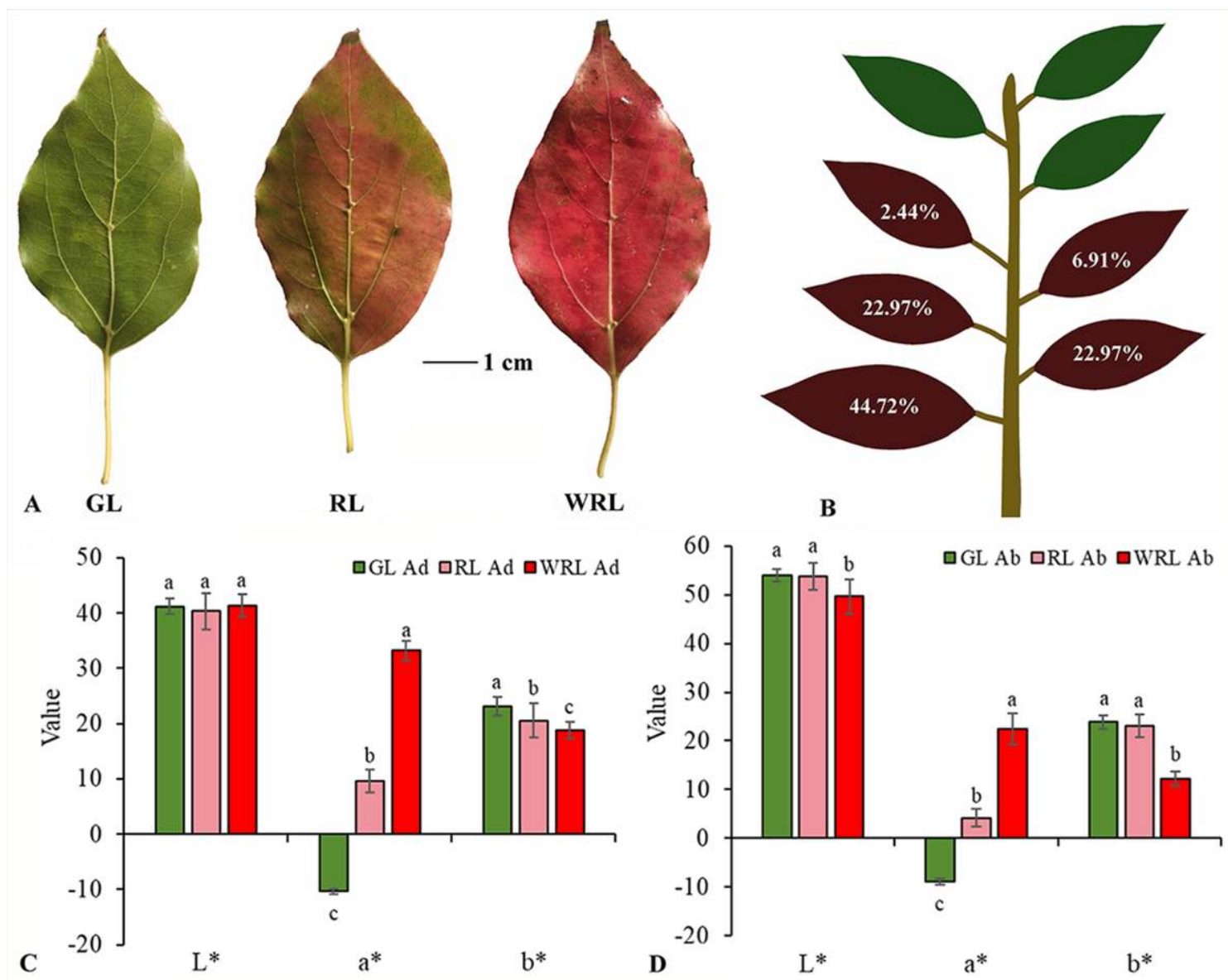


Figure 1

Observed the color transformation and detected color parameters of *C. camphora* leaves. (A), the process of leaf color transformation. GL means green leaf. RL means color turning red leaf. WRL means whole red leaf. (B), the spatial distribution of WRL. Color parameters of adaxial (C) and abaxial surfaces (D). Values sharing the same letters are not significant difference according to Duncan's Multiple Range Tests ($P = 0.05$).

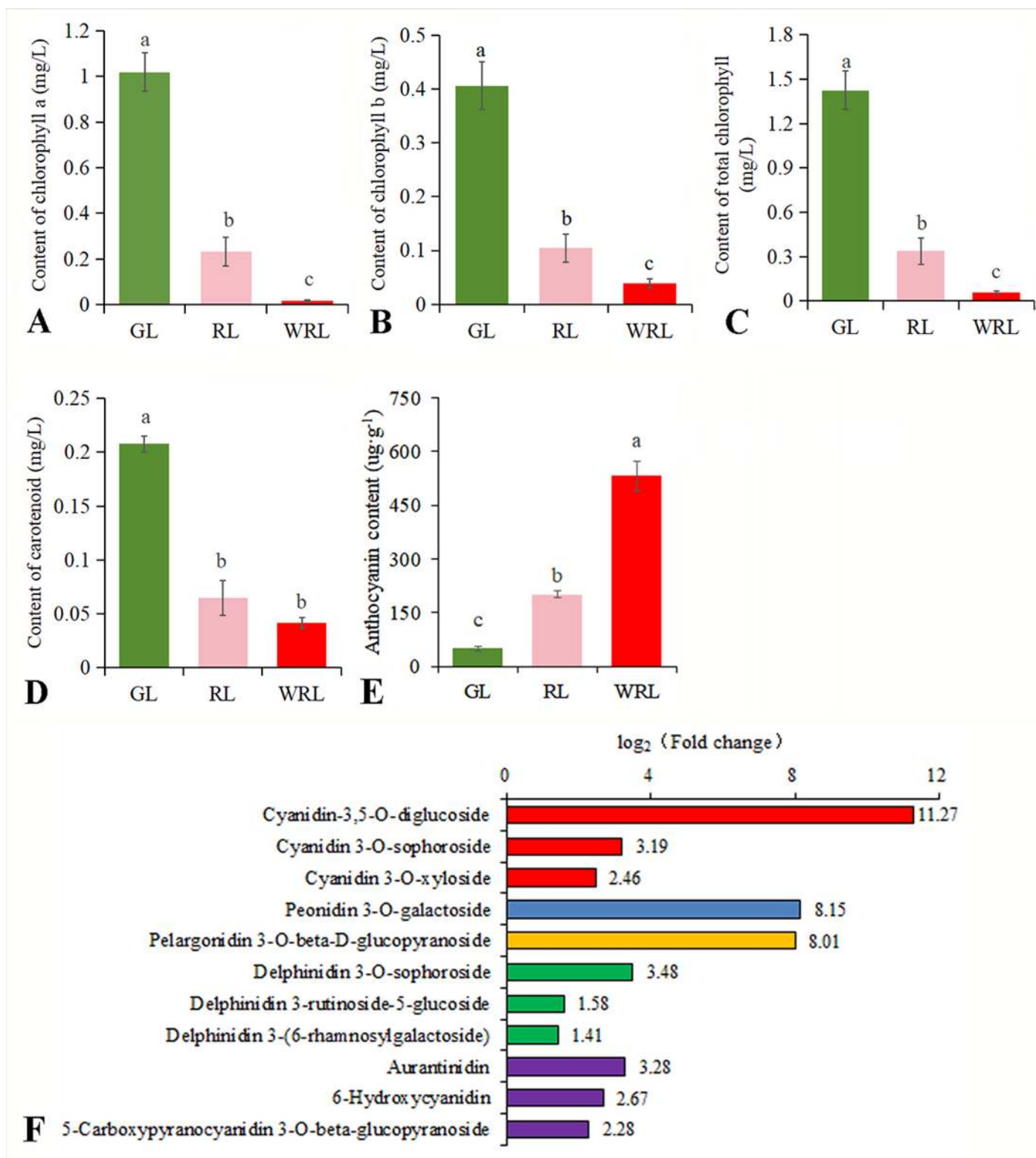


Figure 2

The content of chlorophylls, carotenoids and anthocyanins in the GL, RL and WRL. The content of chlorophyll a (A), chlorophyll b (B), total chlorophyll (C), carotenoid (D), and anthocyanin (E). (F) the difference in anthocyanins between GL and WRL.

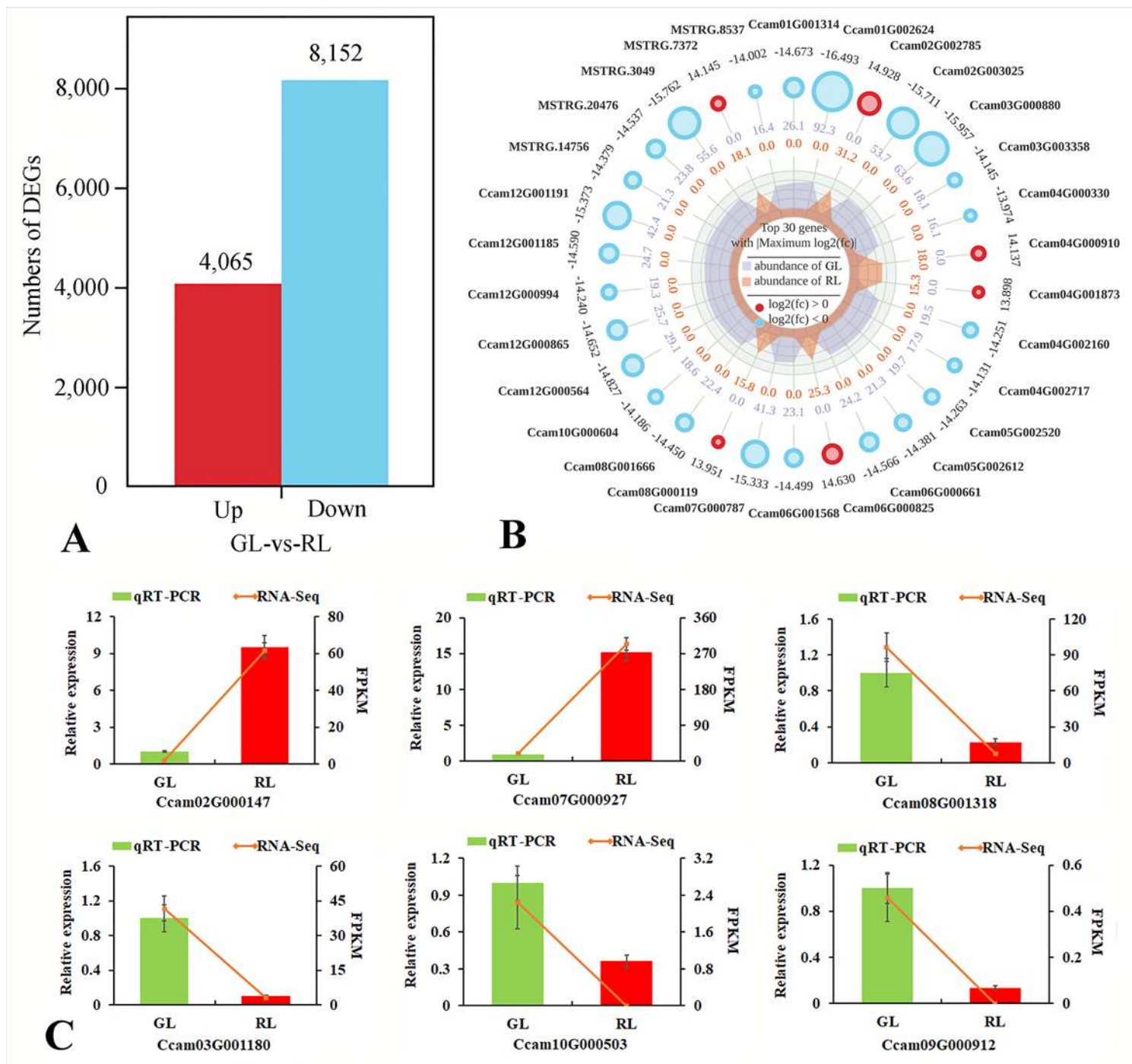


Figure 3

Analysis of the DEGs and validation by qRT-PCR. (A), histogram of DEGs identified by RNA-Seq. (B), Top 30 DEGs. (C) Gene expression of candidate genes analyzed by qRT-PCR and RNA-seq. Data are presented as the mean $\pm$ standard deviation of three biological replicates.

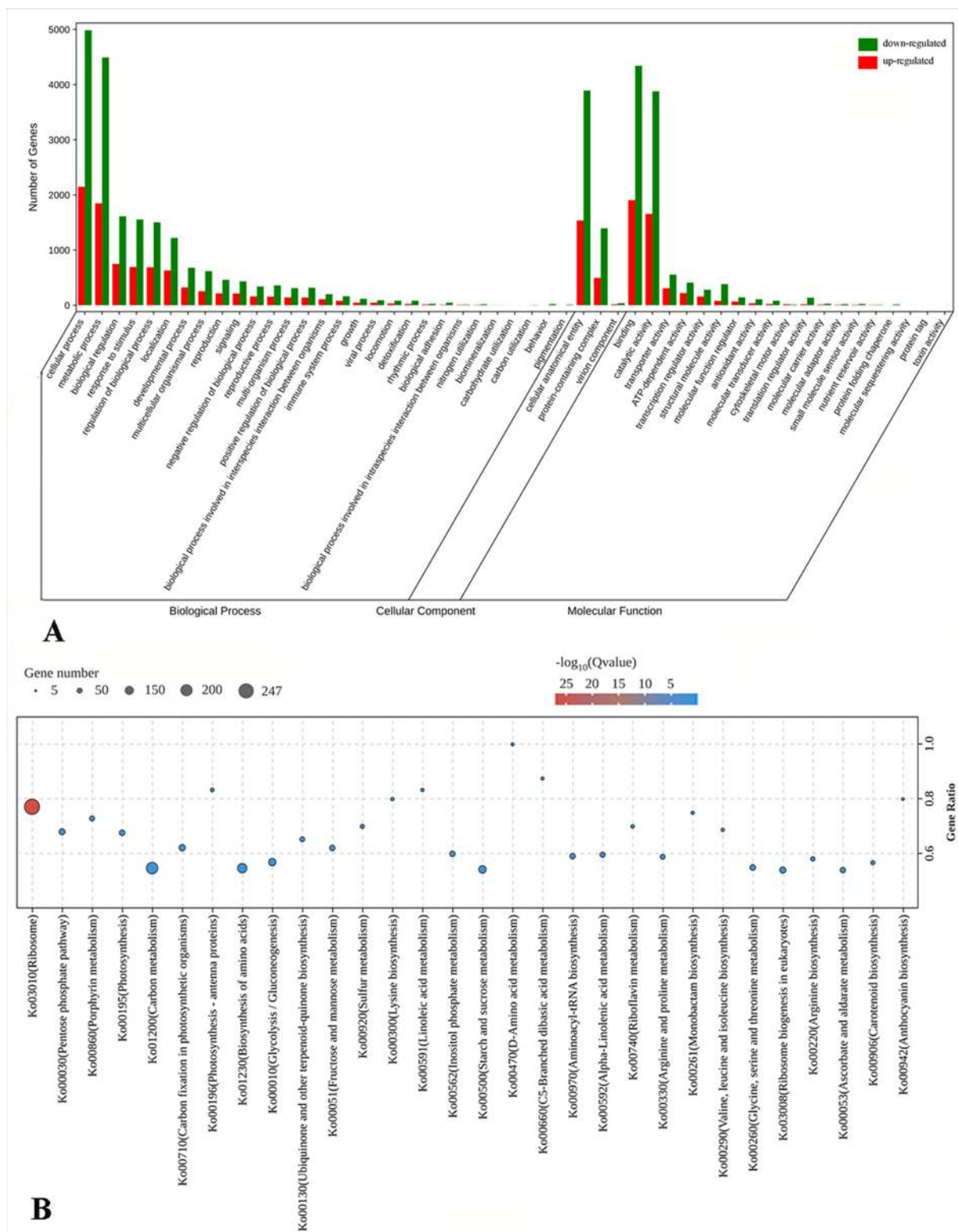


Figure 4

GO annotation (A) and KEGG pathways (B) enrichment analysis of DEGs between GL and RL.

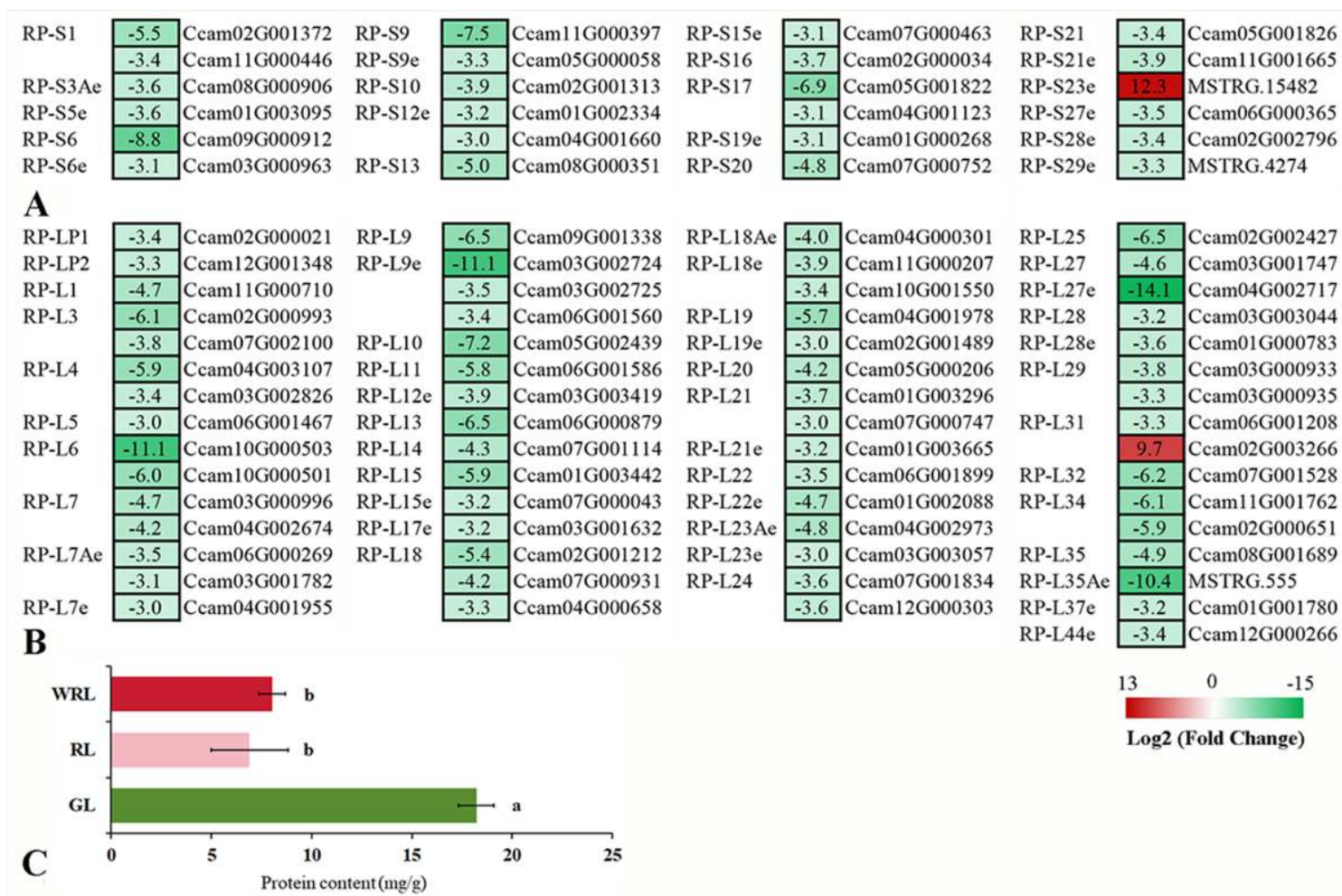


Figure 5

The DEGs related to ribosome pathway and protein content detection. (A) the DEGs ($|\log_2$ (Fold change)| ≥ 3) related to small subunit of ribosome pathway. (B) the DEGs ($|\log_2$ (Fold change)| ≥ 3) related to large subunit of ribosome pathway. (C) protein content difference between GL, RL and WRL.

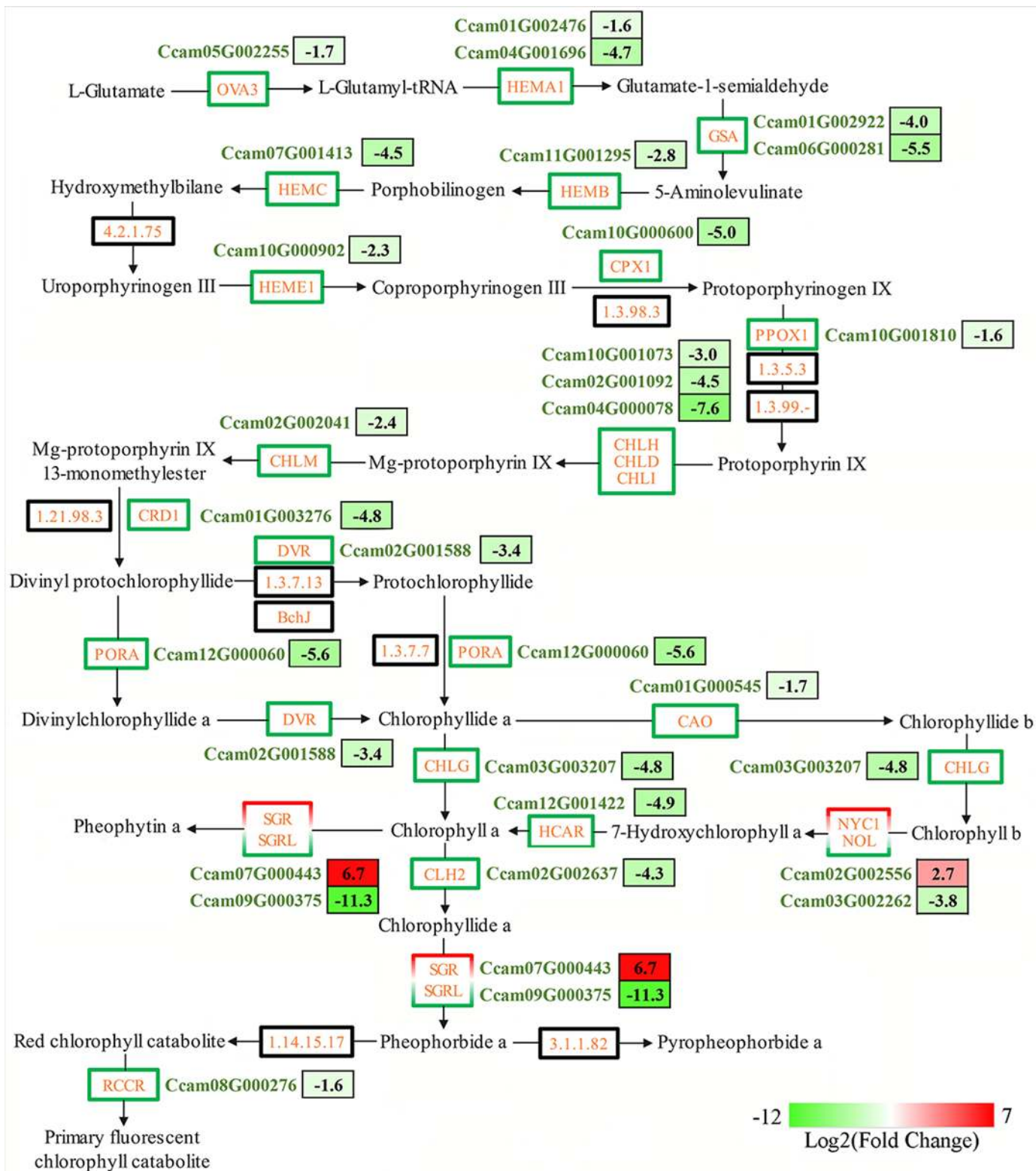


Figure 6

Genes expression profile of chlorophylls biosynthesis and degradation pathway. Different color means the expression level changes between GL and RL, Green boxes indicate gene down-regulated, red boxes indicate gene up-regulated.

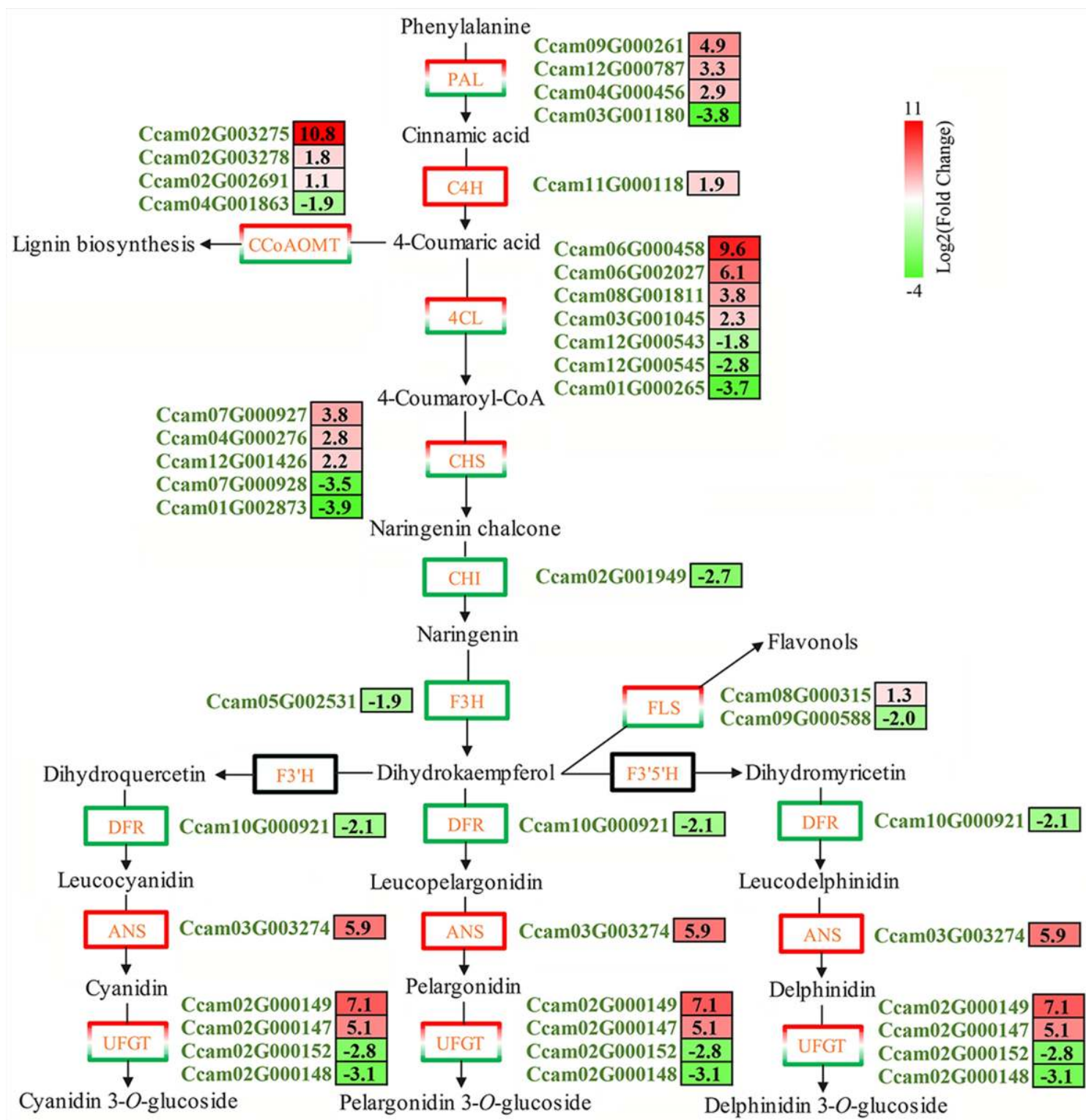


Figure 7

Expression profile of anthocyanin metabolism. Different color means the expression level changes between GL and RL, Green boxes indicate gene down-regulated, red boxes indicate gene up-regulated.

Supplementary Files

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

- [SupplementaryTableS1ThesummaryofcDNAlibraryconstructionandsequencing.xls](#)
- [SupplementaryTableS2Thenewgenesidentifiedfromsixlibraries.xlsx](#)
- [SupplementaryTableS3ThelistofDEGs.xlsx](#)
- [SupplementaryTableS4TheDEGsofTFsbetweenGLandRL.xlsx](#)
- [SupplementaryTableS5TheprimersequenceofqRTPCR.doc](#)