Biochemical and transcriptome analysis reveals pigment biosynthesis influenced chlorina leaf formation in Anoectochilus roxburghii (Wall.) Lindl

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Abstract

Anoectochilus roxburghii (Wall.) Lindl is a perennial herb of the *Orchidaceae* family; a yellow-green mutant and a yellow mutant were obtained from the wild type, thereby providing good material for the study of leaf color variation. Pigment content analysis revealed that chlorophyll, carotenoids, and anthocyanin were lower in the yellow-green and yellow mutants than in the wild type. Transcriptome analysis of the yellow mutant and wild type revealed that 78,712 unigenes were obtained, and 599 differentially expressed genes (120 upregulated and 479 downregulated) were identified. Using the Kyoto Encyclopedia of Genes and Genomes pathway analysis, candidate genes involved in the anthocyanin biosynthetic pathway (five unigenes) and the chlorophyll metabolic pathway (two unigenes) were identified. Meanwhile, the low expression of the chlorophyll and anthocyanin biosynthetic genes resulted in the absence of chlorophylls and anthocyanins in the yellow mutant. This study provides a basis for similar research in other closely related species.

Introduction

Leaf color mutations are a common feature of pigment deficiency. Variations in pigment content (Li et al. 2015), chloroplast development (Wu et al. 2007), and photosynthetic efficiency (Zhu et al. 2016) may lead to chlorotic mutants.

Chlorophyll (Chl) and anthocyanin are the main pigment classes influencing leaf color formation. While several pigments are produced in chloroplasts, Chl is the major component of normal green leaves (Li et al. 2011a; Du et al. 2008; Zhang et al. 2006). Chl metabolism is divided into three phases: Chl a synthesis, interconversion of Chl a and Chl b (Chl cycle), and Chl degradation (Hörtensteiner 2013). Large numbers of leaf color mutants are determined by the expression level of key genes involved in Chl biosynthesis and chloroplast development (Eckhardt et al. 2004; Kato et al. 2007). The function of these genes in leaf color formation has been identified through studies on leaf mutants in *Arabidopsis thaliana*, rice (*Oryza sativa*), maize (Zea *mays*), and tobacco (*Nicotiana tabacum*). Anthocyanins, a class of flavonoid compounds, mainly formed the pink, red, purple, and blue colors in a plant (Davies et al. 2012; Zhao and Tao 2015). Anthocyanin biosynthesis is one of the best-known metabolic pathways and most of the genes of this pathway have been characterized in different plant species, such as pear (Yang et al. 2013), apple (Kim et al. 2003), lettuce (Zhang et al. 2018), rose (Sui et al. 2018), and sweet potato (Wang et al. 2018).

Anoectochilus roxburghii (Wall.) Lindl is a perennial herb of the *Orchidaceae* family and is one of the most precious traditional Chinese medicines (Liu et al. 2020). We found a yellow-green mutant from *Anoectochilus roxburghii* during the process of tissue culture, and then a yellow mutant was found from the yellow-green mutant. This provides ideal genetic material for exploring the molecular mechanisms of leaf color mutation. Compared with studies in model plants with known genome sequences, little is known about the molecular mechanisms regulating Chl deficiencies in *Anoectochilus roxburghii*. In this study, the transcriptome was sequenced from the wild-type and yellow mutant leaves, and the differently

expressed genes in the two groups were identified. The main purpose of this study is to explore the molecular mechanisms regulating pigment metabolism and leaf color mutant formation in *Anoectochilus roxburghii*. This study could provide a valuable resource on leaf color formation in *Anoectochilus roxburghii* and other plant species.

Materials And Methods

Plant Materials

The wild type and leaf color mutants (green-yellow and yellow) of *Anoectochilus roxburghii* (Wall.) Lindl were used as experimental materials. The green-yellow mutant comes from the mutations of the wild type, and the yellow mutant was derived from the mutations of the green-yellow mutant (Fig. 1). All the wild plants and two mutants were rooting seedlings grown in tissue culture for approximately 3 months. The wild type and yellow mutant were collected for transcriptome analysis. The samples (three biological replicates) were flash frozen in liquid nitrogen and then stored at − 80°C for analyses.

Determination Of Chl, Carotenoid, Chl Precursors, And Anthocyanin

Approximately 0.1 g of leaves from the wild type, yellow-green mutant, and yellow mutant were cut into pieces and then immersed in a 10 mL extraction mixture (acetone:absolute ethanol = 2:1) overnight. The Chl a, Chl b, and total carotenoid contents were measured at 645, 663, and 470 nm following the procedure of Lichtenthaler (1987). Two precursors of Chl biosynthesis, Mg-Proto IX and protochlorophyllide (Pchlide), were examined. An extraction mixture [80% alkaline acetone (acetone:1M ammonia = 9:1, v/v)] was used to extract two precursors from the leaves of the wild type and two mutants. The 5-aminolevulinic acid (ALA) and Pchlide contents were measured at 575, 590, and 628 nm. Using the method described by Wu and Prior (2005), 0.1 g of leaves from the wild type and two mutants were ground in liquid nitrogen, and then a 5 mL extraction mixture (methanol:water:acetic acid = 85:15:0.5) was added to the anthocyanin extract. The anthocyanins were acquired at a wavelength of 525 nm. Cyanidin 3-O-glucoside was used as a quantification standard. All of them were determined using a UV-1800 spectrophotometer (Shanghai Mapada Instruments Co. Ltd., Shanghai, China). Three biological replicates were prepared for each sample. The data obtained were analyzed using Microsoft Excel 2016 (Microsoft China, Beijing, China).

Cdna Library Construction, Sequencing, And Transcriptome Assembly

Total RNA of the wild type and yellow mutant was extracted using a universal RNA extraction kit (Bioteke Corporation, Beijing, China). The integrity of the RNA was examined using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, MA, USA). Library construction and transcriptome sequencing were performed at the Biomarker Biotechnology Corporation (Beijing, China) using an Illumina HiSeq™ 4000 (NCBI SRA accession PRJNA594793). High-quality clean reads were obtained from the Illumina-

generated raw reads by removing low-quality reads. The de novo assembly of these reads into contigs was performed through the overlapping regions using the Trinity assembly platform (Grabherr et al. 2011) and were further assembled into transcripts using the paired-end reads. Finally, the transcripts were gathered into unigenes.

Functional Annotation

Owing to sequence similarity, BLASTx was used by searching against public databases with an E-value threshold of 10^{-5}, namely Clusters of Orthologous Groups of proteins (COG), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), euKaryotic Clusters of Orthologous Groups (KOG), protein family (Pfam), Swiss-Prot protein database, evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG), and NCBI non-redundant protein (Nr). Pfam was searched using the HMMER 3.0 program with an E-value cutoff of 10^{-10} (Finn et al. 2011). The classification of GO terms was achieved using Blast2GO software (Gotz et al. 2008).

Differentially Expressed Gene (Deg) Analysis

Gene expression was estimated using the RNA-Seq through Expectation Maximization (RSEM) software (Li and Dewey 2011b). The abundance of each gene was calculated using Fragments Per Kilobase of transcript per Million mapped reads (FPKM) (Trapnell et al. 2010). DEGs among six different libraries were evaluated using the DESeq package (Anders and Huber 2010). A false discovery rate (FDR) of < 0.01 and a fold-change value of ≥ 2 were used as thresholds to confirm the DEGs between the two samples.

Quantitative Real-time Pcr (Qrt-pcr) Analyses

qRT-PCR was performed using the ABI 7500 Real-Time PCR System (Applied Biosystems, USA). Primers were designed for identifying the relative expression levels of the candidate genes using Primer Premier 5.0 (Supplementary Table S1). Melting curve analysis was conducted to confirm PCR specificity. The actin gene was selected as an internal control. A relative quantitative computing method ($2^{-\Delta\Delta Ct}$ method) was used to calculate the expression changes in the unigene (Schmittgen et al. 2008). Three independent biological replicates and three technical replicates were analyzed.

Results

Chl, Carotenoid, and Anthocyanin Contents

The leaf color of *Anoectochilus roxburghii* (Wall.) Lindl was green at the front and red at the back. It is well known that leaf greening is determined by Chl biosynthesis and leaf reddening is the result of pigment accumulation. The results show that the Chl a and b contents were significantly lower in the two mutants than in the wild type (approximately 7% and 9% of the yellow-green mutant and 4% and 8% of

the yellow mutant) (Fig. 2). The data indicate that the carotenoid and anthocyanidin contents decreased to 10% and 10% of the levels in the yellow-green mutant and 3% and 2% in the yellow mutant (Fig. 2). The result suggests that the yellow-green and yellow mutants are due to reduced Chl, carotenoid, and anthocyanin levels, and the lower contents of all the above might result from abnormal biosynthesis in Chl, carotenoid, and anthocyanin.

de novo **Assembly and Sequence Analysis**

From the ultra-morphological and physiological description above, we proposed that the reason for the different phenotypes of the two mutants may be the different expression patterns of genes in Chl and pigment biosynthesis. To verify our hypothesis, a comparison of transcriptome sequencing was performed. Because the yellow mutant came from the green-yellow mutant, we supposed they share a similar mechanism in the formation of leaf color, and the yellow mutant may have richer genetic variations, therefore, we chose the yellow mutant as the representative to compare with the wild type to explore the mechanisms in leaf color mutation. As a result, 171.38 million high-quality clean reads were obtained from six libraries (Supplementary Table S2). After de novo assembly, 78,712 unigenes were obtained, with N50 values of 1,860 bp and a mean length of 1234 bp. Of the assembled unigenes,18,711 were longer than 1000 bp, 41.5% of the total (Table 1).

Table 1
Quality parameters of transcriptome

Length (bp)	Unigenes	
	Number	Percentage
200–300	6,630	8.42%
300–500	13,736	17.45%
500–1,000	25,564	32.48%
1,000–2,000	18,711	23.77%
2,000+	14,071	17.88%
Total number	78,712	
Total length	97,115,759	
N50 length	1,860	
Mean length	1233.81	

Functional Annotation And Deg Identification

The FPKM method was used to analyze gene expression. From the results, 67,388 and 66,738 unigenes were obtained from the library of the wild type and yellow mutants. From the FPKM calculation, 599

DEGs were detected, of which 120 were upregulated and 479 were downregulated. However, the analysis of the DEGs indicates that 50% of the DEGs remained unannotated. These unannotated DEGs may be due to untranslated regions (UTRs), noncoding RNAs (ncRNAs), or specifically expressed in *Anoectochilus roxburghii* (Wall.) Lindl (Chung et al. 2016; Kwenda et al. 2016).

To understand further information on the DEGs and related pathways of leaf color variation, the entire set of the DEGs was searched in eight databases. A total of 333 (55.59%) DEGs were mapped onto GO annotation, which was grouped into three major categories, namely, molecular function, cellular components, and biological processes. Among the three components, the metabolic process subcategory accounted for the majority of GO annotations, followed by the catalytic activity under molecular function (Fig. 3, Table 2). Next, 187 (31.22%) DEGs were matched to 25 different COG categories. Among them, general function prediction only represented the largest group, followed by posttranslational modification, protein turnover, and chaperones (Table 2, Supplementary Fig. S3)

Table 2
Summary of unigene annotations

Database	Number of annotated unigenes	Percentage of annotated unigenes	Number of DEGs	Percentage of DEGs
COG	10,621	13.49%	187	31.22%
GO	18,994	21.80%	333	55.59%
KEGG	10,692	13.58%	180	30.05%
KOG	19,765	25.11%	246	41.07%
Pfam	22,821	28.99%	418	69.78%
Swiss-Prot	20,706	26.31%	421	70.12%
eggNOG	30,939	39.31%	493	82.30%
Nr	32,898	41.80%	515	85.97%
All	33,261	42.26%	516	86.14%

To identify the biological pathways, KEGG annotation was conducted. Approximately 194 (32.39%) DEGs were assigned to 64 KEGG pathways, where six pathways, namely starch and sucrose metabolism, phenylpropanoid biosynthesis, cyanoamino acid metabolism, photosynthesis, biosynthesis of amino acids, and pentose and glucuronate interconversions, were considered significant with a cutoff FDR of < 0.01 and FC of > 2 (Fig. 4). Most DEGs of the six enriched pathways were downregulated. Chl is important for leaf color and photosynthesis, and anthocyanin is crucial for pigment metabolism. Therefore, our analysis was focused on the major genes of Chl, photosynthesis, and anthocyanin. The results show 20 DEGs in photosynthesis (seven genes), flavonoid (anthocyanin) biosynthesis (five genes), Chl biosynthesis (two genes), carbon fixation in photosynthetic organisms (one gene), and carbon

metabolism (five genes). These pathways and genes may play an important role in leaf color variation in yellow mutants.

Identification Of Genes Involved In Anthocyanin Biosynthesis

To further identify the key genes involved in the leaf color mutants, the DEGs related to pigment metabolism and Chl biosynthesis compared with the wild type and yellow mutant. The results indicate that five genes in anthocyanin biosynthesis were downregulated, comprising three chalcone synthase (CHS) genes (Unigene_21909, Unigene_29201, and Unigene_38569), one naringenin 3-dioxygenase (F3H) gene (Unigene_11620), and one flavonoid 3'5'-hydroxylase (F3'5'H) gene (Unigene_17122) (Fig. 5). The downregulation of these five genes may be due to the absence of the red color at the back of the leaves from the yellow mutant.

Identification Of The Genes Involved In Chl Metabolism

Chl content is directly related to leaf color formation. In this study, two genes in the porphyrin and Chl metabolism pathway showed significant differences, one heme oxygenase gene (Unigene_51297) was downregulated, and one oxygen-dependent protoporphyrinogen oxidase gene (Unigene_16629) was upregulated in the yellow mutant (Fig. 5). These two genes may play important roles in the abnormal leaf color of the yellow mutant.

Identification Of The Genes Involved In Photosynthesis

Chl content and photosynthesis might affect each other. Reduced Chl content might directly influence photosynthesis, and vice-versa. A total of seven DEGs that mapped to photosynthesis were identified, namely one photosystem II protein D1(psbA) gene (Unigene_08318), one photosystem II CP47 chlorophyll (psbB) gene (Unigene_09488), one photosystem II CP43 chlorophyll (psbC) gene (Unigene_37618), one photosystem I P700 chlorophyll A apoprotein A1(psaA) gene (Unigene_18753), one apocytochrome f (petA) gene (Unigene_21103), one ATP synthase CF1 subunit alpha (atpA) gene (Unigene_45495), one ATP synthase subunit beta (atpB) gene, and one ATP synthase CF1 subunit alpha (atpA) gene. All of them were significantly downregulated in the yellow mutant (Fig. 6). Meanwhile, all the genes involved in carbon fixation in photosynthetic organisms and carbon metabolism were lower in expression in the yellow mutant, including one gene that encodes ribulose bisphosphate carboxylase large chain (rbcL, Unigene_01022), one gene that encodes phosphoserine aminotransferase (PSAT, Unigene_11499), one gene that encodes D-3-phosphoglycerate dehydrogenase (PHGDH, Unigene_14400), one gene that encodes pyruvate kinase isozyme (PK, Unigene_36454), acetyl-CoA carboxylase carboxyl transferase beta (accD, Unigene_39073), and one gene that encodes serine hydroxymethyltransferase (SHMT, Unigene_41740). The above results indicate that the lower expression of these genes might have blocked photosynthesis, and further affected Chl biosynthesis in the yellow mutant.

Quantitative Real-time Pcr Analysis Of Candidate Genes

To demonstrate the reliability of the RNA-Seq data, 20 DEGs that were considered to be strongly related to leaf color mutation were analyzed using qRT-PCR. The results show that the expression of these genes via qRT-PCR was similar to those in the transcriptome data. Additionally, based on the scatter plot $\log_{10}$-fold changes (Fig. 7, Supplementary Fig. S4), a positive correlation coefficient was observed between the qRT-PCR and RNA-Seq data ($R^2 = 0.71$), indicating the reliability of the RNA-Seq data.

Discussion

In this study, we report novel chlorophyll-deficient and anthocyanin-deficient mutants that present yellow-green and yellow phenotypes in leaves. These two mutants were selected from the tissue culture of the wild type. This is a good material for studying leaf color variation. Pigment analysis showed that the Chl, carotenoid, and anthocyanin contents of the yellow-green and yellow mutants decreased significantly. It seemed that Chl and carotenoid biosyntheses were blocked and photosynthesis was inhibited in the two mutants. Recently, Wang et al. found that decreased abundances in carotenoids and Chls were the main cause of leaf color variation (Wang et al. 2014). This finding is consistent with our results. Chls and carotenoids are important for the biogenesis of the photosynthetic apparatus (Li et al. 2013; Cazzonelli et al. 2010). Meanwhile, many studies have shown that a change in anthocyanin content directly affects the color of leaves. For example, the change in color of the herbaceous peony leaves from purple to green is caused by a reduction in anthocyanins and accumulation of Chl (Tang et al. 2020). In this study, the color of the back of the leaves changed from red to yellow, mainly due to the decrease in anthocyanin content.

Chl metabolism is a highly coordinated process, which is catalyzed by numerous enzymes. Changes in the expression of the genes involved in Chl biosynthesis might result in the yellow mutant (Sakuraba et al. 2013). The heme content of plant cells has a feedback inhibition effect on Chl biosynthesis. If the synthesis from heme to phytochrome is blocked, the heme content of cells increases, and a large amount of accumulated heme feedback inhibit the synthesis of ALA, and further affect the synthesis of pchlide, thus affecting the biosynthesis of Chl and causing leaf color variation (Terry et al. 1999). In this study, the expression level of *ArHO* in the yellow mutant was significantly lower than that in the wild type, and the ALA and pchlide contents were also significantly lower in the yellow mutant, indicating that low expression of *ArHO* led to the accumulation of heme, and inhibited the synthesis of ALA and pchlide, causing leaves to turn yellow.

Anthocyanin is one of the branch products in the flavonoid pathway, that is responsible for the red, blue, and purple pigments of plant tissues (Tanaka et al. 2008). Anthocyanin synthesis is under the control of multiple structural genes and regulated by a variety of transcription factors, and the mutation of any one of these genes can cause changes in plant coloration. The CHS gene is the first key gene in anthocyanin synthesis (Tian et al. 2011). The absence of CHS blocks the synthesis of anthocyanins. Silencing the CHS genes produces the white areas of flowers in several horticultural crops, such as petunia (*Petunia hybrida*) (Saito et al. 2006; Morita et al. 2012), camellia (*Camellia japonica*) (Tateishi et al. 2010), and

dahlia (*Dahlia variabilis*) (Ohno et al. 2011). In this study, three *ArCHS* have a low expression level in the yellow mutant, which may be the main reason why the back of the leaf changed from red to yellow. In anthocyanin biosynthesis, three branches are formed under the action of F3H, F3'H, and F3'5'H. The deficiency of any one of these three genes leads to a change in flower color (Tanaka 2006). Our data show that the expression of *ArF3H* and *ArF3'5'H* in the yellow mutant was significantly lower than that in the wild type. Our results indicate that *ArF3H* and *ArF3'5'H* played a pivotal role in the anthocyanin biosynthesis of leaves in *Anoectochilus roxburghii.*

Conclusion

In this study, a yellow-green mutant and a yellow mutant were obtained from the wild type. Pigment content and transcriptome analysis revealed that chlorophyll and anthocyanin were lower in the yellow-green and yellow mutants than in the wild type, and low expression of key genes of chlorophyll biosynthesis and anthocyanin pathway is the main reason for leaf color mutant.

Declarations

Acknowledgments

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Compliance with ethical standards

Conflict of interest: The authors declare that they do not have any conflicts of interest.

Author contributions

Fan RH and Huang ML designed the research. Chen YQ , Wu JS, Zhong HQ and Lin B conducted the experiments. Ye XX analyzed the data and wrote the manuscript.

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Figures

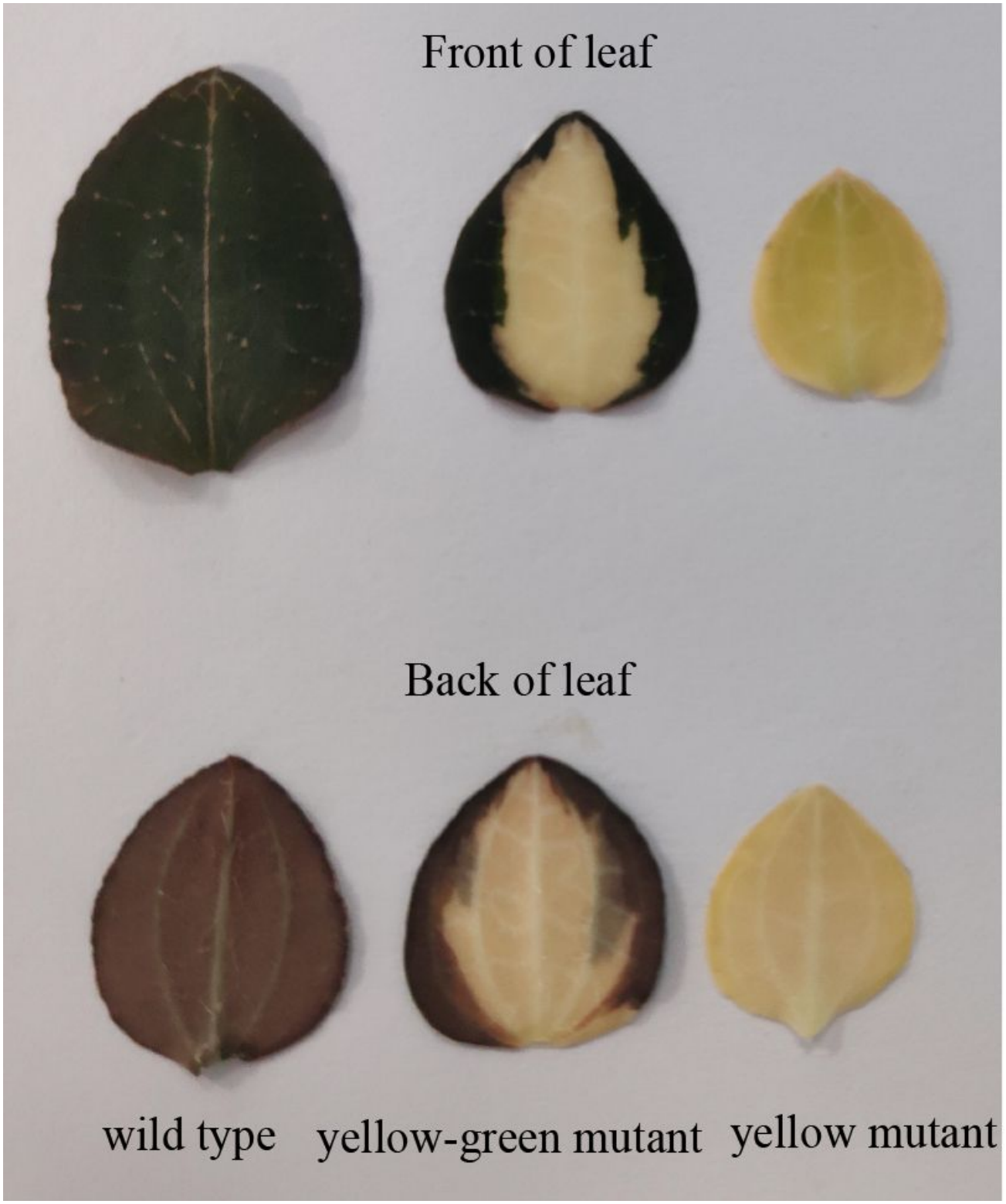

Figure 1

Phenotype of the wild type, green-yellow mutant, and yellow mutant of *Anoectochilus roxburghii*

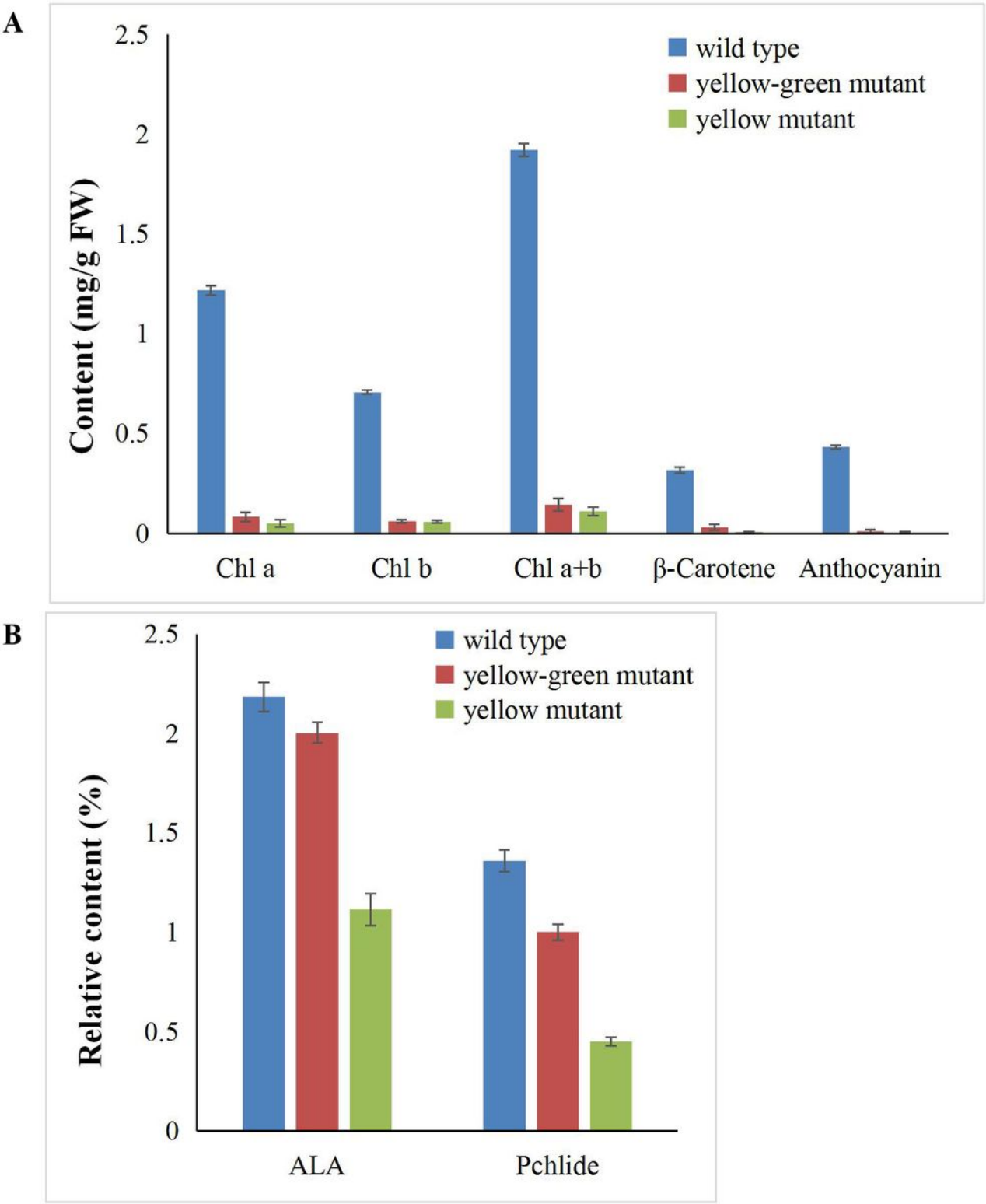

Figure 2

Chl, carotenoid, anthocyanin, ALA, and Pchlide contents of the wild type, yellow-green, and yellow mutants

A: Chl, carotenoid, and anthocyanin contents; B: ALA and Pchlide contents

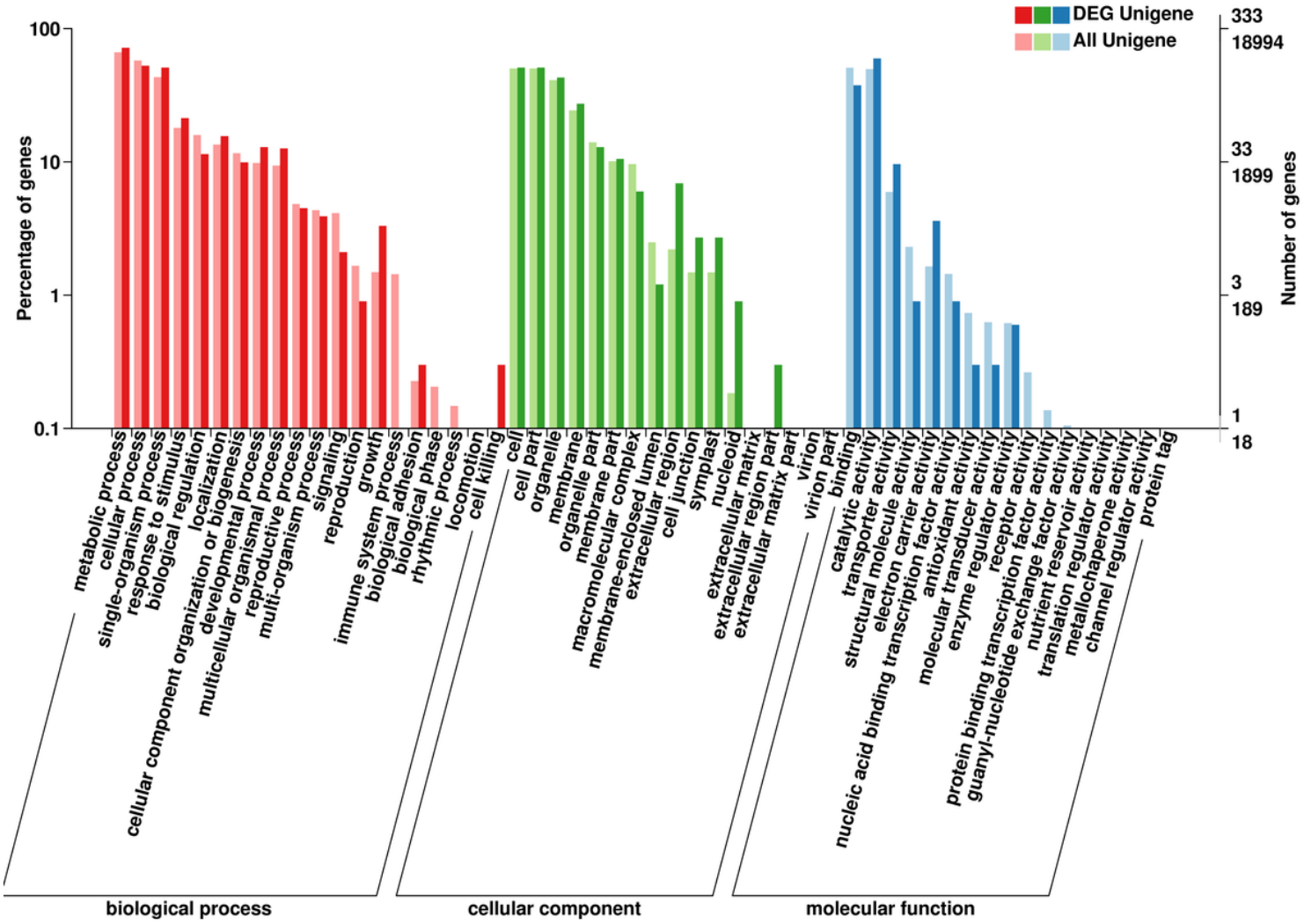

Figure 3

GO group classification in *Anoectochilus roxburghii*

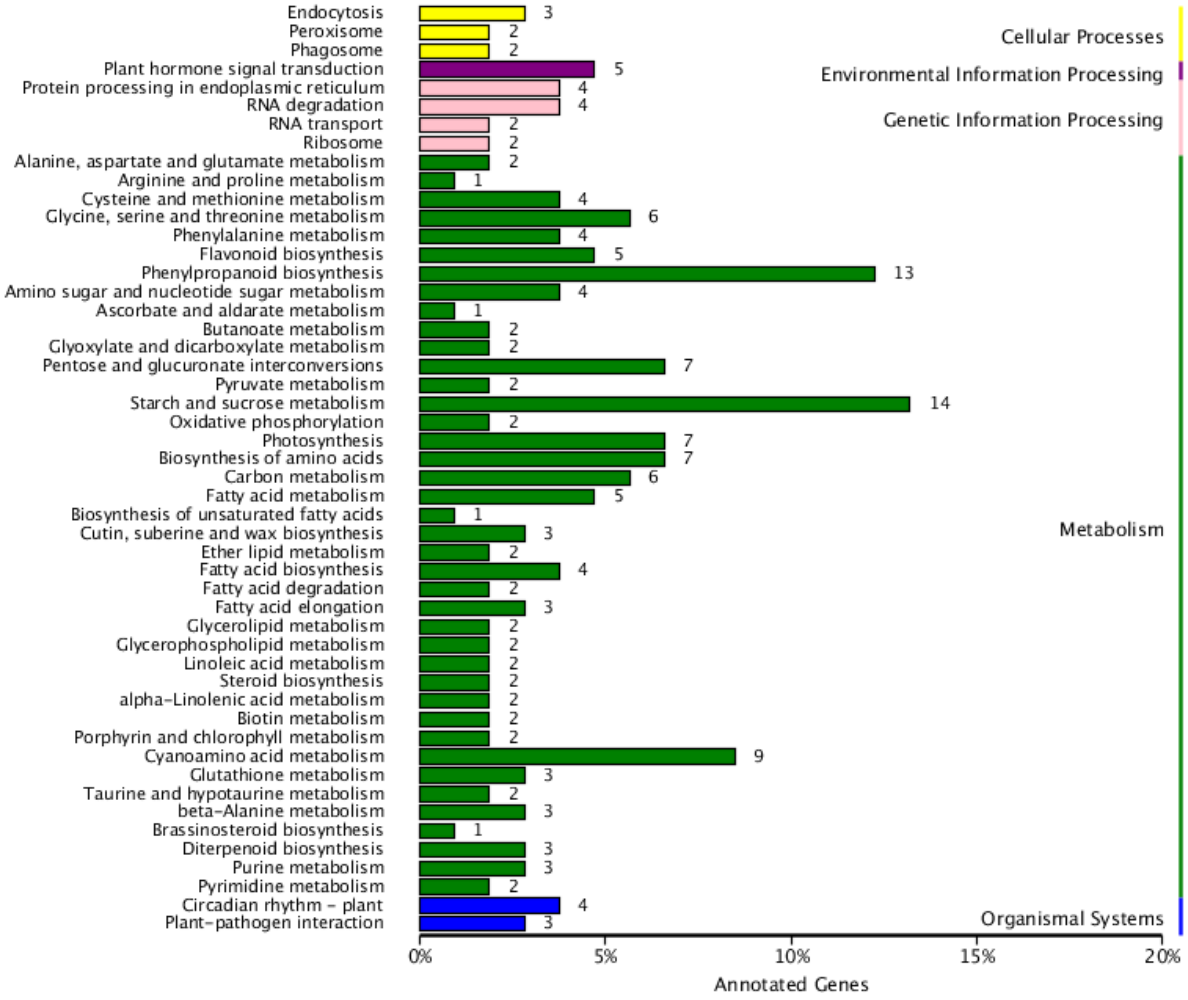

Figure 4

KEGG pathway of the DEGs in *Anoectochilus roxburghii*

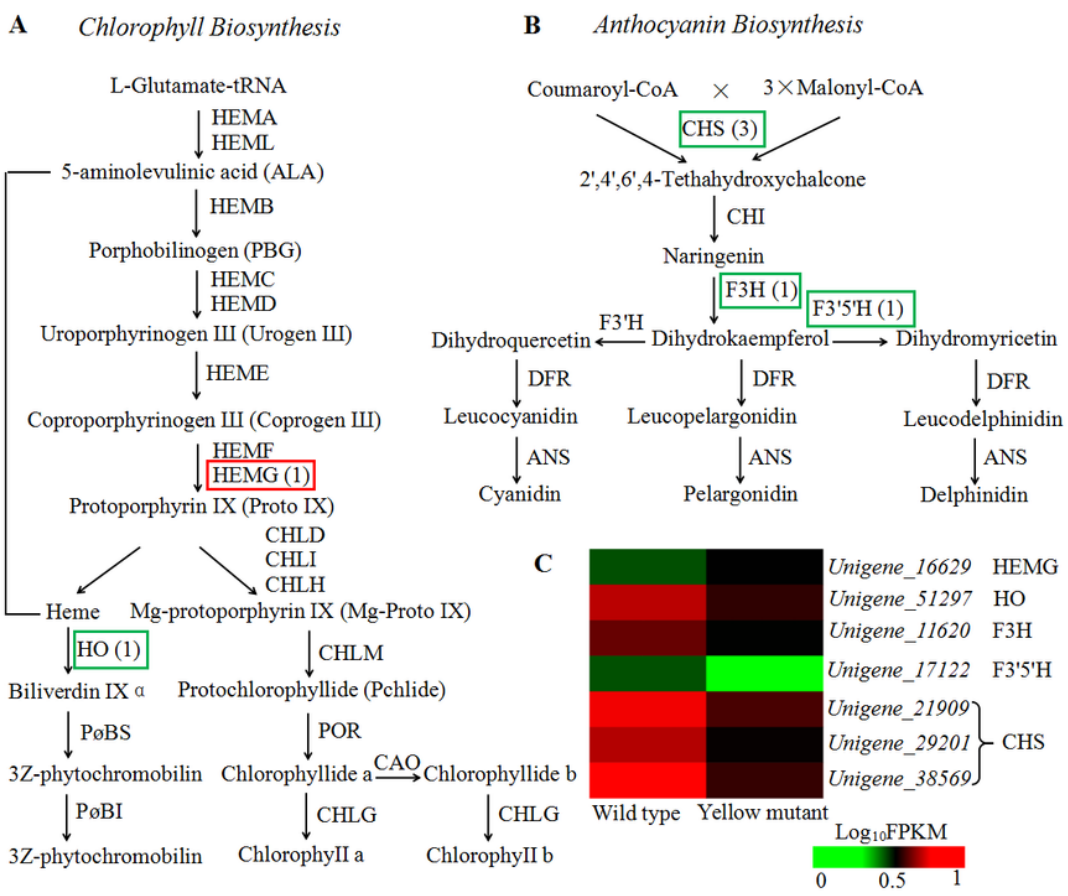

Figure 5

Expression profiles of the genes involved in the Chl and anthocyanin biosyntheses

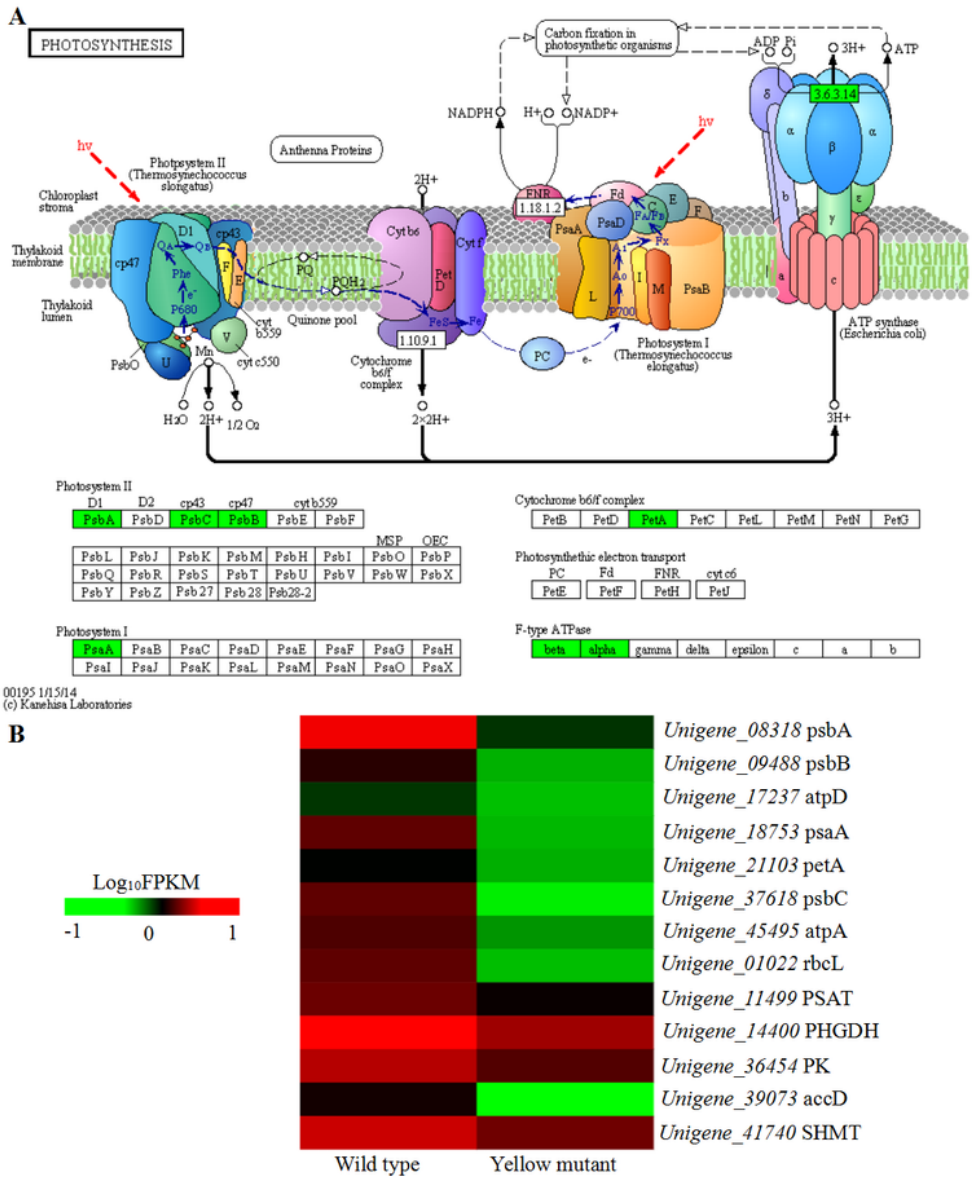

Figure 6

Expression profiles of the genes involved in the photosynthesis system

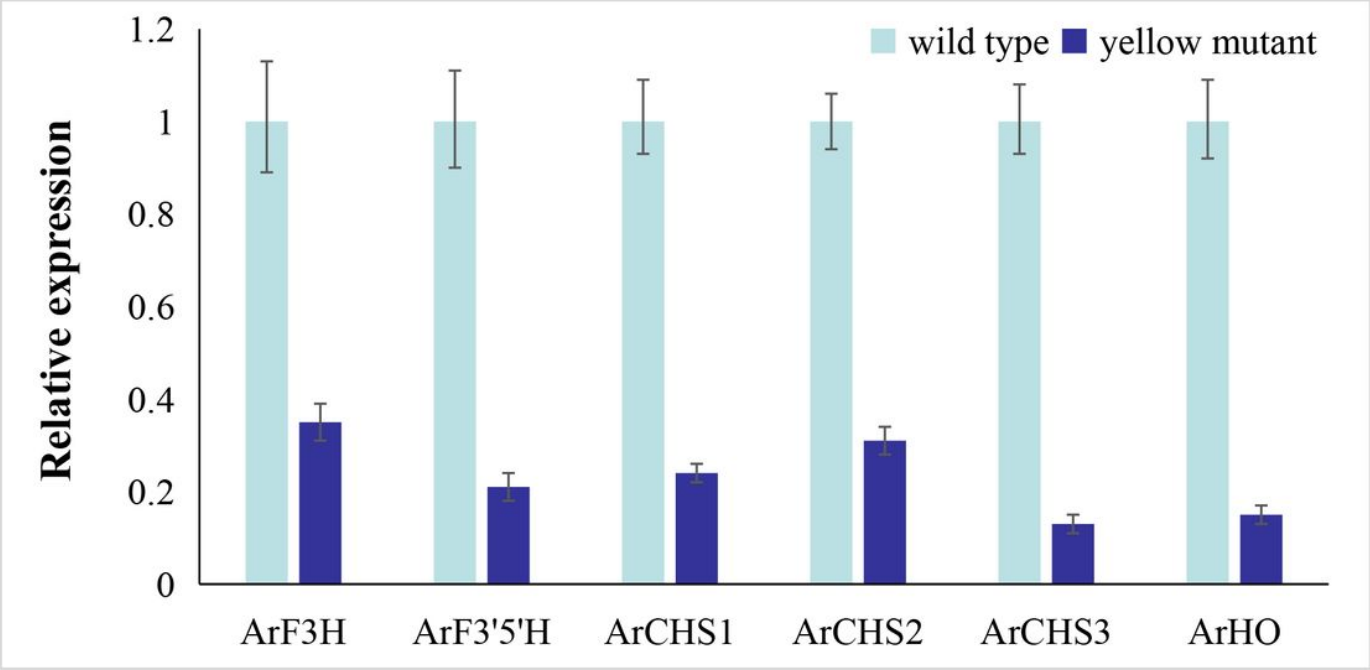

Figure 7

Expression analysis of six genes in Chl and anthocyanin biosyntheses

Supplementary Files

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

- SupportingInformation.zip