Integrated Metabolome and Transcriptome Analysis Reveals the Mechanism of Leaf Chlorosis Variation in Cyclobalanopsis gilva Postprint

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Abstract

To elucidate the mechanism underlying leaf yellowing variation in *Cyclobalanopsis gilva*, this study employed leaves from leaf color mutant and normal plants of *C. gilva* as experimental materials, and performed metabolome and transcriptome analyses using ultra-high performance liquid chromatography-tandem mass spectrometry and high-throughput RNA sequencing technology, respectively. The results demonstrated that: (1) In positive and negative ion modes, the metabolome identified 257 and 357 significantly changed metabolites (SCMs) between normal and mutant plants, respectively, among which various flavonoids including quercetin, leucocyanidin, and myricetin, as well as their glycoside derivatives (pyranofisetin A, isorhamnetin 3-glucuronide, etc.), were significantly upregulated in the mutant, while pigment contents such as chlorophyll a, chlorophyll b, and carotenoids were significantly decreased. (2) Transcriptome sequencing detected 4,146 differentially expressed genes (DEGs), with 1,711 genes upregulated and 2,435 genes downregulated. (3) KEGG enrichment analysis revealed that SCMs and DEGs were significantly enriched in pathways including photosynthesis, porphyrin and chlorophyll metabolism, and flavonoid biosynthesis. The findings indicate that leaf yellowing in the mutant may be attributed to the combined effects of blocked chlorophyll synthesis, abnormal chloroplast development, and increased flavonoid synthesis. Furthermore, MYB and bHLH family genes were significantly upregulated in the mutant, confirming that these two types of transcription factors are involved in regulating flavonoid biosynthesis. These results provide novel insights into the molecular mechanism of plant yellowing mutations and serve as a reference for mining leaf color functional genes and breeding ornamental plants.

Full Text

Combined Metabolome and Transcriptome Analyses Reveal the Mechanism of Leaf Yellowing Mutation in *Cyclobalanopsis gilva*

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Abstract: To elucidate the mechanism underlying leaf yellowing mutation in *Cyclobalanopsis gilva*, we conducted integrated metabolomic and transcriptomic analyses using leaves from both yellowing mutant and normal plants. Ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS) and high-throughput RNA sequencing were employed to profile metabolic and transcriptional changes. The results revealed: (1) A total of 257 and 357 significantly changed metabolites (SCMs) were identified in positive and negative ion modes, respectively. Multiple flavonoids and their glycoside derivatives, including quercetin, leucocyanidin, myricetin, pyranodelphinin A, and isorhamnetin 3-glucuronide, were significantly upregulated in the mutant, while chlorophyll a, chlorophyll b, and carotenoid pigments were markedly reduced. (2) Transcriptome sequencing identified 4,146 differentially expressed genes (DEGs), with 1,711 upregulated and 2,435 downregulated. (3) KEGG enrichment analysis demonstrated that both SCMs and DEGs were significantly enriched in photosynthesis, porphyrin and chlorophyll metabolism, and flavonoid biosynthesis pathways. These findings indicate that the yellow leaf phenotype likely results from combined effects of impaired chlorophyll synthesis, abnormal chloroplast development, and enhanced flavonoid biosynthesis. Notably, MYB and bHLH family genes were significantly upregulated in the mutant, confirming their involvement in regulating flavonoid biosynthesis. This study provides novel insights into the molecular mechanisms of plant etiolation and offers valuable references for functional gene mining and breeding of ornamental plants with distinctive leaf coloration.

Keywords: *Cyclobalanopsis gilva*, leaf-color mutant, etiolation, metabolome, transcriptome

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Introduction

Leaf color mutation refers to phenotypic changes in foliage pigmentation during plant growth and development. First categorized by Gustafsson (1942) into

five types—striped, spotted, pale green, yellowing, and albino—this classification was later expanded to include yellow-green, purple, and lesion-mimic variants (Manjaya and Nandanwar, 2007). Yellowing represents a significant mutation type that has been extensively utilized in studies of photosynthetic physiology (Zhang et al., 2019), chloroplast ultrastructure (Li et al., 2023), and chlorophyll biosynthesis (Zhu et al., 2014). In horticulture, yellowing mutants have important breeding applications, with many popular "golden-leaf" cultivars derived from such mutations, including *Sophora japonica* 'Aurea', *Ulmus pumila* 'Jinye', and *Acer palmatum* 'Aurea'.

Current research on leaf yellowing has primarily focused on model plants and major crops such as *Arabidopsis thaliana*, rice (*Oryza sativa*), and cucumber (Li et al., 2012; Maekawa et al., 2015; Xiong et al., 2023). Among ornamental species, reports remain limited to a few taxa including *Koelreuteria bipinnata* var. *integrifoliola* (Lyu et al., 2017), *Ginkgo biloba* (Li et al., 2019), and hybrid paper mulberry (*Broussonetia kazinoki* × *B. papyrifera*) (Wang et al., 2022).

High-throughput sequencing technologies have become powerful tools for elucidating molecular mechanisms underlying biological phenomena (Xiong et al., 2023). Integrative multi-omics approaches provide comprehensive insights into both static and dynamic changes in organisms, offering deeper understanding of complex traits (Lu et al., 2020). For instance, Lyu et al. (2017) identified nine chlorophyll metabolism genes and 14 carotenoid biosynthesis genes in the golden-leaf cultivar 'Jinyan' of *K. bipinnata* var. *integrifoliola*. Wang et al. (2022) employed multi-omics to demonstrate that the yellow leaf phenotype in hybrid paper mulberry is associated with altered photosynthetic pigment content, chloroplast structural defects, and functional impairment. Yamashita et al. (2021) showed that etiolation in the tea cultivar 'Koganemidori' resulted from deficiencies in chloroplast development and chlorophyll synthesis genes, while elevated amino acid levels stemmed from widespread protein degradation caused by upregulated metabolic genes. Luo et al. (2022) integrated transcriptomic and metabolomic data to reveal that sugarcane leaf chlorosis involves reduced chlorophyll synthesis, decreased photosynthetic gene expression, dysfunctional metal ion regulation, and altered secondary metabolism. Collectively, these studies demonstrate that leaf yellowing is closely associated with mutations or suppressed expression of chlorophyll metabolism genes, combined with altered metabolism of other pigments such as anthocyanins and carotenoids (Lin et al., 2022).

Cyclobalanopsis gilva (Fagaceae) is an evergreen tree species distributed in mountainous regions of southern China at elevations of 300–1,500 m (Editorial Committee of China Flora, 1998). Valued for its hard, fine-textured timber, it is used for high-quality lumber, mixed afforestation in low mountain regions, understory planting, and urban landscaping (Ouyang et al., 2021; Qin et al., 2023). In 2016, our research team discovered a spontaneous yellow-leaf mutant in a seedling population at the Xixia nursery base in Haishu District, Ningbo. The mutant exhibited yellow mature leaves, golden-yellow young foliage, and bright

yellow branches. Through grafting propagation initiated in late 2018, we established multiple individuals that have stably maintained these distinctive traits for four consecutive years. This study employs integrated metabolomic and transcriptomic analyses combined with physiological and biochemical measurements to address two key questions: (1) What are the physiological mechanisms underlying the golden-yellow leaf phenotype in the *C. gilva* mutant? (2) What transcriptional regulatory mechanisms govern leaf yellowing? The findings will provide valuable references for breeding and genetic improvement of golden-leaf ornamental plants.

Materials and Methods

1.1 Plant Materials The study utilized yellow-leaf mutant (YL) and normal green-leaf (NYL) plants of *Cyclobalanopsis gilva*, both cultivated at the Forest and Specialty Crop Base of Ningbo Agricultural Technology Promotion Station (121°42 24 E, 29°48 46 N). Leaf samples were collected in August 2021 [Figure 1: see original paper], immediately flash-frozen in liquid nitrogen, and stored at -80°C until analysis.

1.2 Metabolomic Analysis 1.2.1 Metabolite Extraction

Fifty milligrams of leaf tissue were transferred to centrifuge tubes containing 400 µL of methanol:acetonitrile (1:1 v/v) extraction solution with internal standards. Samples were homogenized using a cryogenic tissue grinder (Wonbio-96c, Shanghai Wanbai), incubated at 4°C, and centrifuged at 13,000 × g for 15 minutes. The supernatant was collected for LC-MS analysis. Six biological replicates were prepared for each group (YL and NYL), with 20 µL aliquots from each sample pooled for quality control analysis.

1.2.2 Metabolite Detection

Metabolites were analyzed using a Thermo Fisher Scientific UHPLC system coupled to a Q Exactive HF-X mass spectrometer. Chromatographic separation was performed on an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 µm; Waters, USA) at 40°C with a flow rate of 0.4 $mL \cdot min^{-1}$ and injection volume of 3 µL. Mobile phases consisted of (A) 95% water + 5% acetonitrile (0.1% formic acid) and (B) 47.5% acetonitrile + 47.5% isopropanol + 5% water (0.1% formic acid). Gradient elution profiles were optimized for positive and negative ion modes. Mass spectrometry employed electrospray ionization (ESI) with spray voltages of +3,500 V and -3,500 V, heated capillary temperature of 425°C, sheath gas flow of 50 Arb, auxiliary gas flow of 13 Arb, and scan range of 70–1,050 m/z. Data were acquired using sequential MS scans at resolutions of 60,000 (MS^1) and 7,500 (MS^2) with collision energies of 20, 40, and 60 eV.

1.2.3 Data Analysis

Raw data were processed using Progenesis v2.2 software (Waters, USA) for peak alignment and integration. Metabolites were identified by matching against HMDB, METLIN, and other public databases. Statistical analyses and heatmap generation were performed using R packages. Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were conducted to assess sample clustering and identify significant metabolites. Significantly changed metabolites (SCMs) were defined by variable importance in projection (VIP) > 1, $P \leq 0.05$, and fold change (FC) > 1.10 or < 0.91. KEGG pathway enrichment analysis was performed to identify affected metabolic pathways.

1.3 Transcriptomic Analysis 1.3.1 RNA Sequencing and Library Construction

Total RNA was extracted from fresh YL and NYL leaf samples following the method of Lu et al. (2020). Libraries were constructed using the TruSeq™ RNA Sample Prep Kit (Illumina). Polyadenylated mRNA was isolated using oligo(dT) magnetic beads, fragmented, and reverse-transcribed to synthesize first-strand cDNA using SMARTScribe™ Reverse Transcriptase. After RNase H degradation of RNA templates, second-strand cDNA was synthesized. Double-stranded cDNA was end-repaired, A-tailed, and ligated to sequencing adapters. Following PCR enrichment and AMPure XP bead purification, libraries were quantified using TBS-380 (Picogreen) and pooled for sequencing. Cluster generation was performed on a cBot platform, and paired-end sequencing (2×150 bp) was completed on an Illumina NovaSeq 6000 platform.

1.3.2 Data Processing

Image data were converted to raw reads via CASAVA base calling. After quality control filtering, high-quality clean reads were assembled de novo using Trinity v2.8.5 (Grabherr et al., 2011). Initial assemblies were optimized using TransRate v1.0.3 (Smith-Unna et al., 2016) and CD-hit v4.5.7 (Li and Godzik, 2006), with final transcriptome completeness assessed using BUSCO v3.0.2 (Simão et al., 2015). Transcripts were annotated against six major databases (NR, Swiss-Prot, Pfam, eggNOG, GO, and KEGG). Gene expression levels were quantified as transcripts per million (TPM) using RSEM v1.3.1 (Li and Dewey, 2011). Differentially expressed genes (DEGs) were identified using DESeq2 v1.24.0 (Michael et al., 2014) with thresholds of FC ≥ 2 and adjusted P-value < 0.05. GO and KEGG pathway enrichment analyses were performed using Goatools v0.6.5 (Klopfenstein et al., 2018) with adjusted P-value < 0.05 to identify key genes involved in leaf color variation.

1.3.3 Correlation Analysis Between Metabolites and Genes

Pearson correlation analysis between SCMs and DEGs was performed using Prism 8.0 (GraphPad, USA). Molecular interactions were identified using correlation coefficient $|r| \geq 0.8$ and $P < 0.05$ as significance thresholds.

1.3.4 qRT-PCR Validation

Eight DEGs were selected for quantitative real-time PCR validation to confirm transcriptome data reliability. Primers were designed using Primer Premier 5.0 . The *CACs* gene (Accession: ID6728500) served as the internal reference (Marum et al., 2012). Three biological replicates were performed, and expression levels were Log_2-transformed for comparison with transcriptome data.

1.4 Chlorophyll Content Determination Fresh leaves from YL and NYL plants were weighed, ground, and filtered following the method of Zhang et al. (2021). Chlorophyll content was measured spectrophotometrically with three biological replicates.

1.5 Photosynthetic Parameter Measurement Photosynthetic parameters including net photosynthetic rate, stomatal conductance, intercellular CO_2 concentration, and transpiration rate were measured using a CI-340 portable photosynthesis system (CID, USA) on fully expanded leaves of YL and NYL plants. Three biological replicates were conducted.

Results

2.1 Metabolomic Analysis 2.1.1 Multivariate Statistical Analysis of Metabolome

UHPLC-Q Exactive HF-X data from YL and NYL samples were subjected to multivariate statistical analysis. PCA revealed that in positive ion mode, the first two principal components (PC1 and PC2) explained 40.60% and 19.10% of total variance, respectively, while in negative ion mode they explained 37.30% and 24.10% [FIGURE:2A, B]. OPLS-DA models showed good predictive power with R^2 values exceeding Q^2 values and Y-intercepts below zero in both ion modes [FIGURE:2C, D], indicating robust sample separation.

A total of 614 SCMs were identified between YL and NYL groups: 257 in positive mode (148 upregulated, 109 downregulated) and 357 in negative mode (147 upregulated, 210 downregulated) [FIGURE:2E, F].

2.1.2 Analysis of Major SCMs

Among the 614 SCMs, the top 30 metabolites by relative abundance were characterized . Notably, nine flavonoids were upregulated in YL, with pyranodelphinin A showing the highest fold change (2.28-fold), followed by quercetin 3-O-(6' ' -acetyl-glucoside) (1.55-fold). Five nucleosides and derivatives showed differential accumulation, with two pyrimidine nucleosides upregulated and three purine nucleosides downregulated. Among nine lipid metabolites, five were upregulated, primarily prenol lipids. Two organic acids showed opposite trends, with one significantly upregulated and the other downregulated.

2.1.3 KEGG Pathway Analysis of SCMs

KEGG enrichment analysis of SCMs revealed 66 significantly enriched pathways, including glycerophospholipid metabolism, cofactor biosynthesis, flavonoid biosynthesis, ABC transporters, isoflavonoid biosynthesis, and amino sugar and nucleotide sugar metabolism [Figure 3: see original paper]. Flavonoid biosynthesis pathways showed the highest enrichment, with 12 metabolites in general flavonoid biosynthesis, 7 in isoflavonoid biosynthesis, and 4 in flavone and flavonol biosynthesis, suggesting a strong association between flavonoid accumulation and the yellow leaf phenotype.

2.2 Transcriptomic Analysis 2.2.1 Sequencing Quality Assessment

RNA sequencing of NYL and YL samples yielded 44.26 Gb of clean data after quality control . Clean read counts ranged from 43,864,122 to 56,594,944 per sample, with Q20 values between 97.61–97.79%, Q30 values between 93.03–93.49%, and GC content of 44.49–44.99%, meeting requirements for downstream analysis.

2.2.2 Differential Gene Expression Statistics

A total of 46,391 and 48,018 expressed genes were identified in YL and NYL, respectively, with 16,699 and 15,072 uniquely expressed in each group [Figure 4A: see original paper]. Using NYL as control, 4,146 DEGs were detected in YL, including 1,711 upregulated and 2,435 downregulated genes [Figure 4B: see original paper].

2.2.3 GO Functional Analysis of DEGs

GO enrichment analysis of 3,612 DEGs ($P < 0.05$) revealed significant enrichment in 12 biological processes, 14 cellular components, and 2 molecular functions [Figure 5: see original paper]. The most enriched biological processes included "generation of precursor metabolites and energy," "photosynthesis,""pigment biosynthetic process,""pigment metabolic process,"and "porphyrin-containing compound biosynthetic process," predominantly involving downregulated genes. Cellular component analysis showed highest enrichment in "plastid" and "chloroplast," followed by "thylakoid membrane," "photosynthetic membrane," and "chloroplast thylakoid membrane." Molecular function enrichment was limited to "tetrapyrrole binding" and "chlorophyll binding," both with predominantly downregulated genes (>90% for chlorophyll binding). These results indicate that leaf yellowing in YL is closely associated with disrupted photosynthesis and chlorophyll metabolism.

2.2.4 KEGG Pathway Enrichment Analysis of DEGs

KEGG analysis annotated 744 DEGs to known pathways, with 192 genes significantly enriched in 12 pathways including plant-pathogen interaction, photosynthesis, and glyoxylate/dicarboxylate metabolism . Five pathways were photosynthesis-related: photosynthesis-antenna proteins (enrichment factor =

0.6667, 10 LHC genes), photosynthesis (23 genes, primarily reaction-center complex components), carbon fixation in photosynthetic organisms (16 genes including TIM and GAPA), porphyrin and chlorophyll metabolism (12 genes), and glyoxylate/dicarboxylate metabolism (22 genes). Additional DEGs participated in flavonoid biosynthesis, anthocyanin biosynthesis, amino acid metabolism, and phenylpropanoid biosynthesis.

2.2.5 Identification and Analysis of Differential Transcription Factors

Transcription factors (TFs) regulate gene expression by binding to functional gene regulatory regions. Among 4,146 DEGs, 39 TF genes belonging to 15 families were identified [Figure 6: see original paper]. MYB family showed the highest representation (8 DEGs), followed by AP2/ERF (5 DEGs) and bHLH (4 DEGs). WRKY, bZIP, and SBP families each contributed 3 DEGs. While WRKY, GRAS, AP2/ERF, and C2H2 families were predominantly downregulated, most other families showed upregulation in YL.

2.2.6 RT-qPCR Validation of DEGs

Quantitative PCR validation of eight selected DEGs confirmed expression trends consistent with transcriptome data, verifying the reliability of sequencing results [Figure 7: see original paper].

2.3 Integrated Analysis 2.3.1 Correlation Analysis of Pigment Metabolism-Related DEGs and SCMs

Leaf color is determined primarily by chlorophyll, carotenoids, and anthocyanins. Physiological measurements revealed that YL leaves contained significantly reduced chlorophyll a (0.35 $mg \cdot g^{-1}$), chlorophyll b (0.31 $mg \cdot g^{-1}$), total chlorophyll (0.66 $mg \cdot g^{-1}$), and carotenoids (0.05 $mg \cdot g^{-1}$) compared to NYL (2.38, 1.49, 3.87, and 0.44 $mg \cdot g^{-1}$, respectively). The chlorophyll a/b ratio was also significantly lower in YL [Figure 8: see original paper], suggesting potential environmental stress (Sun et al., 2022). This altered ratio modifies light absorption characteristics and consequently affects leaf coloration (Li et al., 2022).

In the chlorophyll biosynthesis pathway, nine enzyme-encoding DEGs were downregulated in YL, including glutamyl-tRNA reductase (HemA), uroporphyrinogen decarboxylase (HemE), protoporphyrinogen oxidase (HemF), and protochlorophyllide oxidoreductase (POR) [Figure 9A: see original paper]. Notably, *HemE* expression decreased by 80%, while two *POR* genes were downregulated by >66%. Genes encoding HemA, HemB (δ-aminolevulinic acid dehydratase), DVR (divinyl protochlorophyllide a 8-vinyl-reductase), and ChlI (Mg-chelatase subunit ChlI-1) all showed ~50% reduced expression.

Carotenoid content in YL (0.056 ± 0.009 $mg \cdot g^{-1}$) was significantly lower than in NYL (0.442 ± 0.083 $mg \cdot g^{-1}$). In the carotenoid biosynthesis pathway [Figure 9B: see original paper], phytoene synthase (PSY), the first committed enzyme, showed 80% reduced expression, likely impacting downstream flux. Lycopene

-cyclase (LCYE), a key enzyme in lutein biosynthesis, was also downregulated by ~80%, affecting carotenoid composition and leaf color.

Flavonoid biosynthesis begins with p-coumaroyl-CoA derived from the phenylpropanoid pathway. While 4-coumarate-CoA ligase (*4CL*) was upregulated in YL [Figure 9C: see original paper], p-coumaroyl-CoA levels remained unchanged. However, early flavonoid pathway genes chalcone synthase (*CHS*) and chalcone isomerase (*CHI*) were significantly upregulated, leading to increased naringenin chalcone and naringenin accumulation (FC = 1.05). Subsequent pathway genes also showed elevated expression: flavonol synthase (*FLS*) was upregulated 6.98-fold, flavonoid 3' -monooxygenase (*F3' H*) 5.77-fold, and dihydroflavonol 4-reductase (*DFR*) 4.44-fold. These expression changes strongly correlated ($|r| \geq 0.8$) with accumulation of quercetin, leucocyanidin, myricetin, and other flavonoids in YL [FIGURE:9D, FIGURE:10].

2.3.2 Correlation Analysis of Photosynthesis-Related DEGs and SCMs

In the photosynthetic apparatus, 28 DEGs encoding core proteins of PS I, PS II, and light-harvesting complexes (LHCs) showed altered expression [Figure 11: see original paper]. Eight PS I reaction center genes were downregulated in YL. Among ten PS II reaction center DEGs, only *psbA* (encoding the D1 protein) was upregulated, while five oxygen-evolving enhancer proteins and four other PS II components were downregulated. Additionally, ten genes encoding chlorophyll a/b-binding proteins (LHCA1, LHCA2-1, LHCA2-2, LHCA4, LHCB1-1, LHCB1-2, LHCB2, LHCB4, LHCB6, LHCB7) were downregulated. These results suggest that reduced expression of photosynthesis-related genes impaired chloroplast development, consistent with decreased chlorophyll content and reduced photosynthetic rates in YL .

Discussion

3.1 Impaired Chlorophyll Synthesis and Abnormal Chloroplast Development Underlie Leaf Yellowing Leaf color is a complex trait involving multiple biological processes (Wang et al., 2022). In higher plants, leaf coloration is primarily determined by chlorophyll metabolism, with yellowing mutants typically arising from mutations that block chlorophyll synthesis or accelerate its degradation (Zhu et al., 2020). In YL, nine enzyme-encoding genes involved in chlorophyll biosynthesis were downregulated, spanning most steps from L-glutamyl-tRNA to chlorophyllide. HemA catalyzes the first rate-limiting step of chlorophyll synthesis by reducing L-glutamyl-tRNA (Zhao et al., 2014), while HemB participates in the conversion of δ-aminolevulinate to porphobilinogen, another critical step (Yang et al., 2015). Both genes were downregulated by ~50% in YL, likely reducing precursor availability and causing downstream metabolic blockage. Mutations in later-stage genes typically produce striped or spotted phenotypes (Sakuraba et al., 2015); for example, *HemE* mutations

cause lesion mimics in maize (Hu et al., 1998), while *HemF* mutations induce yellow necrosis (Williams et al., 2006).

Additionally, downregulation of *ChlI* may contribute to YL etiolation. ChlI encodes a subunit of Mg-chelatase (Mgch), the first enzyme in the magnesium branch of chlorophyll synthesis and a key rate-limiting step (Luo et al., 2015). Previous studies show that *ChlI* and *ChlD* expression is influenced by substrate concentrations (Zhang et al., 2006). The 50% reduction in *ChlI* expression in YL likely reflects decreased protoporphyrin IX levels, which may further suppress downstream reactions and ultimately reduce chlorophyll content. The significant decrease in carotenoids also contributed to the yellow phenotype, although the chlorophyll-to-carotenoid ratio remained unchanged (8.21 in YL vs. 8.31 in NYL), suggesting this ratio has limited influence on the golden-leaf appearance.

Abnormal chloroplast structure can also cause leaf yellowing (Jiang, 2021). In YL, 33 photosynthesis-related DEGs were altered, including 28 encoding PS I, PS II, and LHC components. Downregulation of *Psa* and *Psb* family genes may impair PS I and PS II protein function. Studies confirm that reduced PS II complex expression leads to defective granal stacking (Xiong et al., 2023). LHC proteins bind chlorophyll for light harvesting and photoprotection; their decreased expression causes abnormal granal architecture and yellowing (Kim et al., 2009). In rice yellow-leaf mutants, chloroplast development and PS II complex genes are regulated by chlorophyll synthesis intermediates (Wu et al., 2007). In *Arabidopsis*, *LHCB* expression is feedback-regulated by chlorophyll synthesis enzymes including Mgch (Mochizuki et al., 2001). We hypothesize that blocked chlorophyll synthesis in YL alters intermediate metabolite and enzyme levels, suppressing expression of RCC and LHC genes, impairing thylakoid membrane formation, and causing chloroplast developmental defects that manifest as leaf yellowing. Chlorophyll deficiency and abnormal chloroplast development also reduce light-harvesting capacity, negatively impacting photosynthetic performance.

3.2 Flavonoid Biosynthesis Provides the Metabolic Basis for YL Yellowing Untargeted metabolomics detected 614 SCMs in YL, predominantly flavonoids, amino acids, and amino sugars/nucleotide sugars. Flavonoids constituted the largest group (23 SCMs), including leucocyanidin, myricetin, quercetin, and their glycosides. Pyranodelphinin A showed the greatest upregulation (2.28-fold). Originally identified in black currant seeds (Lu et al., 2000), its biosynthetic pathway remains uncharacterized. Accumulation of nitrogen-containing flavonoid SCMs suggests metabolic reprogramming of carbon and nitrogen pathways in YL.

While DEGs involved in chlorophyll synthesis, carbon fixation, and photosynthesis were downregulated (e.g., *HemA*, *TIM*, *GAPA*, *LHC*), potentially inhibiting photosynthesis and downstream carbon metabolism (Song et al., 1998), metabolomic data revealed no significant changes in glycolysis or TCA cycle

intermediates. Surprisingly, pyruvate kinase expression increased 3.14-fold and malate synthase 20.42-fold in YL, suggesting enhanced rather than reduced glycolysis and TCA cycle activity. Similar observations in a *Vitis vinifera* yellow mutant were attributed to stronger carbon and nitrogen mobilization in etiolated leaves (Chen, 2011), ensuring adequate carbon skeleton supply for flavonoid synthesis. Additionally, reduced nitrogen consumption by the blocked chlorophyll synthesis pathway may have contributed to nitrogen accumulation, further promoting flavonoid biosynthesis and providing the metabolic foundation for the yellow leaf phenotype (Jiang, 2021).

3.3 Transcription Factors Participate in YL Leaf Yellowing Transcription factors play crucial roles in plant development and stress responses (Sun et al., 2022). In YL, the most enriched TF families among DEGs were MYB, AP2/ERF, and bHLH. MYB TFs are known to mediate transcription of key flavonoid biosynthesis enzymes (An et al., 2017), while bHLH TFs respond to environmental stresses and co-regulate flavonoid biosynthesis with MYB proteins (Liu et al., 2018; Wang et al., 2018). Upregulation of *CHS*, *CHI*, *F3H*, and *FLS* in YL likely reflects regulation by MYB and bHLH TFs.

Four AP2/ERF family DEGs showed significant expression changes. This family primarily responds to abiotic stresses such as high light intensity and temperature (Wu et al., 2015). Enrichment of stress-related TFs including WRKY and HSF suggests YL experiences environmental stress, likely due to high light or water deficit. Chlorophyll-deficient mutants are more susceptible to photoinhibition. Jiang (2021) demonstrated that high light induces MYB upregulation during etiolation. Additionally, YL' s transpiration rate was 1.42-fold higher than NYL , potentially causing water stress (Zhang et al., 2021). We propose that high light or water deficit stress induces upregulation of MYB, bHLH, and other TFs, promoting flavonoid biosynthesis and accumulation in YL.

Conclusion

This integrated metabolomic and transcriptomic study elucidates the mechanism of leaf yellowing in *Cyclobalanopsis gilva*. The golden-yellow phenotype results from combined effects of impaired chlorophyll synthesis, abnormal chloroplast development, and enhanced flavonoid biosynthesis. Significant upregulation of MYB and bHLH family genes confirms their regulatory role in flavonoid biosynthesis. These findings advance our understanding of yellow-leaf trait formation and provide theoretical guidance for breeding golden-leaf ornamental plants.

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Note: Figure translations are in progress. See original paper for figures.