

Transcriptomic Analyses Reveal Leaf Colour Changes and L-theanine Accumulation in Variegated Tea

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Abstract

Background

Camellia sinensis 'Yanlinghuayecha' (YHC) is a variegated mutant developed recently in China. To dissect the physiological and molecular mechanisms of leaf variegation, we compared the leaf pigmentation, cellular ultrastructure, amino acid content, and transcriptome between the albino (A), mosaic (M), and green (G) sectors.

Results

The contents of photosynthetic pigments were significantly lower in sector A and higher in sector G than in sector M. Chloroplasts with well-organized thylakoids were found only in the mesophyll cells of the G sector but not in those of the A sector. The A sector had a significantly higher content of total and free amino acids. In particular, the levels of theanine, glutamate, and alanine in the A sector were higher than those in the G sector. Transcriptomics analysis showed that a total of 44,908 unique transcripts were identified. Comparing the differentially expressed genes (DEGs) in the three sectors, we conducted an in-depth study on chloroplast biogenesis, chlorophyll biosynthesis, and theanine synthesis pathways. The expression of *CsPPOX* in "porphyrin and chlorophyll metabolism" was significantly downregulated in the A sector. *CsLHCB6* in "Photosynthesis - antenna proteins" and *CsSCY1* in "Protein processing in endoplasmic reticulum", both of which were associated with chloroplast biogenesis, were significantly downregulated in the A sector. The expression of *CsTS1* was notably upregulated in the A sector.

Conclusion

Taken together, variegation alters the gene activities involved in chloroplast biogenesis, and our results suggest that leaf colour change in the A sector incorporates three aspects compared with that in the G sector: (1) Decreased *CsPPOX* expression slows the rate of chlorophyll synthesis, resulting in a decrease in chlorophyll content; (2) downregulated expression of *CsLHCB6* and *CsSCY1* inhibits chloroplast biogenesis, decreasing thylakoid morphogenesis and grana stacking; and (3) the metabolic flow of glutamate changes, possibly from chlorophyll biosynthesis to theanine biosynthesis. The accumulation of precursor synthetic substances and the high expression of *CsTS1* generates a high theanine content. These analyses provide valuable insights into variegation in tea plants with regard to leaf colour change and L-theanine accumulation.

Introduction

Leaf variegation typically shows green and yellow sectors, and this attractive trait often enhances the commercial value of ornamental plants[1, 2]. Variegations can be caused by mutations in nuclear, plastid, or mitochondrial genes[3]. The immutan (im) variegation mutation and yellow variegation (var2) of *Arabidopsis*[4, 5], the rice zebra leaf 16 (zl16) mutation[6], and other gene mutations cause foliar

variegation. These mutants are ideal materials for revealing the potential mechanisms of chlorophyll biosynthesis, chloroplast structure and function, chloroplast development, and photosynthesis.

The tea plant (*Camellia sinensis* (L.) O. Kuntze.) is one of the most important economic crops in China. The role of leaf colour in determining the quality of tea has largely been reported[7]. Plants with special leaf colours (white and yellow) among tea plantations represent important fundamental materials for tea breeding[8]. Morphological analysis of albino leaves showed that the membrane system of their chloroplasts was severely disrupted, even including cavitation of the chloroplasts, compared with that of green-leaved cultivars. Chemical analysis of albino leaves showed that the leaves contain a high concentration of total amino acids, especially theanine, but lower levels of pigment, total polyphenol, and caffeine than green-leaved cultivars[9-11].

Variegation is responsible for one of three types of albino tea plants[12]. Many studies have attempted to reveal the principle of leaf colour conversion from albino to green. The leaf colour changes in albino tea are mainly affected by chloroplast biogenesis and pigment biosynthesis. Numerous genes and signal integrators participate in chloroplast biogenesis[13], suppression of the etioplast-chloroplast transition and damage to grana in the chloroplast[10]; repression of genes encoding LHC[14] and expression of genes during protein processing can cause the leaf colour to change in tea[15]. Leaf colour changes are also largely influenced by flavonoid and carotenoid biosynthesis[16], the phenylpropanoid biosynthesis pathway[17], and the shikimic acid pathway[18]. The secondary metabolites of albino tea plants have been studied extensively for their health effects and impacts on tea quality[19]. DNA methylation may be responsible for changes in the contents of the major secondary metabolites in Yujinxiang[20]. Chloroplast deletion in albino leaves likely destroys the balance of carbon and nitrogen metabolism, leading to a high accumulation of free amino acids and a low concentration of polyphenols in albino leaves[9]. L-theanine accumulation in albinism-induced yellow tea leaves was due to weak L-theanine catabolism[21], and PAL might be a core regulator that decreases catechin biosynthesis in albino tea plants[22].

The variegated leaves of tea plants may be formed by physiological and pathological processes. 'Yinghongjiuhao' undergoes physiological changes, forming branches with variegated leaves. The branches with variegated leaves display abnormal chloroplasts compared with the normal branches, and the decrease in photosynthetic protein abundance might be associated with the changes in leaf colour[23]. Variegation may also be caused by viral infection. With metagenomic analysis based on next-generation methods, seven kinds of plant viruses were identified in 26 tea plant samples with typical discolouration symptoms[24].

Although numerous studies have been conducted on albino tea cultivars, the majority of previous studies have focused on leaves of different varieties or in different periods of conversion from albino to green. There are few reports of variegated leaves in *Camellia sinensis*, and research on the albino and green parts of variegated tea plants has not been reported. This study explores the chemical and genetic mechanisms of leaf colour in YHC. We applied integrated approaches to examine pigmentation, chloroplast ultrastructure, and secondary metabolites in this mutant. This study demonstrated that the

genes encoding *CsLHCB6* and *CsSCY1* were closely linked to aberrant chloroplast development, and the increase in theanine levels was due to the high expression of *CsTS1* and accumulation of precursors for theanine synthesis in the albino YHC.

Results

2.1 Changes in phenotypic and photosynthetic pigments

The leaves of YHC exhibited yellowish or white margins and a green interior (Figure 1A, B). We compared the contents of photosynthesis-related pigments such as chlorophyll-a (Chl a), chlorophyll-b (Chl b), and carotenoids (Carot) in the A, G, and M leaves. The results revealed that all of the Chl a, Chl b, and Carot contents were significantly lower ($p < 0.05$) in A and significantly higher ($p < 0.05$) in G than in M. The concentration of Chl b in G was 25.64 as much as that in A (Figure 1C). The photosynthesis-related pigments in A were close to zero.

2.2 Changes in cell ultrastructure

Transmission electron microscopy revealed that G contained well-developed chloroplasts with stacked grana (Figure 2CF). M had fewer thylakoids and grana stacking and some vacuoles than G and A (Figure 2BE). Plastids rarely contained stacked grana but contained many vacuoles in A (Figure 2AD). However, the number of chloroplasts in cells rarely changed (Figure 2).

2.3 Changes in amino acids concentrations/contents

The levels of total amino acids in M were all substantially higher than those in G and substantially lower than those in A. In addition, the content of free amino acids in M was generally lower than in A and higher than G. The content of theanine in A was 2.47% and dramatically (1.25-fold) higher than in G. The contents of aspartic acid, glutamate, histidine, and arginine in A were 0.32%, 0.30%, 0.48%, and 0.66%, respectively 2.13-, 1.88-, 2.82-, 1.78-fold higher, respectively, than those in G. The alanine content of A was 0.04% and was twice the content of G (Table 1).

Table 1 Contents of amino acid in YHC. A, albino sector. M, mosaic sector. G, green sector. Data are presented as the mean of three replicates (\pm standard deviation). Data marked with different letters indicate statistically significant differences among samples ($p \leq 0.05$). DW, dry weight.

Amino acid	A (% , DW)	M (% , DW)	G (% , DW)
Aspartic acid	0.32±0.07a	0.24±0.05ab	0.15±0.01b
Serine	0.12±0.01a	0.09±0.04ab	0.05±0.01b
Glutamate	0.30±0.05a	0.21±0.09ab	0.16±0.01b
Histidine	0.48±0.05a	0.27±0.03b	0.17±0.00c
Arginine	0.66±0.08a	0.53±0.05b	0.37±0.03c
Threonine	0.03±0.00a	0.04±0.01a	0.03±0.00a
Alanine	0.04±0.01a	0.03±0.00b	0.02±0.00b
Proline	0.08±0.01a	0.04±0.03b	0.04±0.00b
Theanine	2.47±0.25a	2.06±0.08b	1.98±0.03b
Valine	0.21±0.05a	0.05±0.01b	0.02±0.01b
Methionine	0.01±0.00a	0.01±0.00a	0.01±0.00a
Lysine	0.04±0.01a	0.01±0.01b	0.01±0.00b
Isoleucine	0.02±0.00a	0.01±0.01b	0.01±0.00b
Phenylalanine	0.01±0.00a	0.01±0.00a	0.01±0.00a
Total amino acids	6.01±0.03a	5.68±0.05b	4.88±0.36c

2.4 GO and KEGG enrichment analyses

The Q20 of the raw data surpassed 97.79%, indicating the presence of high-quality reads worthy of further analysis. A total of 44908 unique transcripts were identified (Figure S1). Compared to G plants, A plants showed 2760 significantly regulated genes, which included 1241 upregulated genes and 1519 downregulated transcripts (Table S1). GO analysis suggested that differentially expressed genes (DEGs) in A compared with G were mostly involved in three categories: “nucleic acid binding transcription factor activity”, “transcription factor activity, sequence-specific DNA binding” and “hydrolase activity, acting on glycosyl bonds” (Figure S2). DEGs in A compared with M and in G compared with M were importantly involved in the “membrane protein complex” and “thylakoid”. Mapping to the KEGG database revealed significantly enriched pathways in A compared with G: “arginine and proline metabolism” (ath00330) (Figure S2), in A compared with M: “photosynthesis” (ath00195) and in G compared with M: “photosynthesis - antenna proteins” (ath00196). Some DEGs in the “protein processing in endoplasmic reticulum” (ath04141) pathway were also involved. DEGs were also discovered in other pathways, such as “alanine, aspartate and glutamate metabolism” (ath00250), “biosynthesis of amino acids” (ath01230), and “porphyrin and chlorophyll metabolism” (ath00860), which are known to be involved in the responses

of albino plants. Functional categories of DEGs from GO and KEGG analyses are listed in the supplementary material.

2.5 DEGs involved in chlorophyll and chloroplast biogenesis

The decrease in photosynthetic pigments can be explained by the changes in the activities of genes involved in chlorophyll biosynthesis (Figure 3A). *CsPORA* showed a clear upregulation in A compared with G. The transcript levels of *CsHEMA1*, *CsHEMG2*, and *CsGUN5* increased in A compared with G, but the level of *CsPPOX* decreased. In addition, DEGs believed to function in chloroplast biogenesis were also investigated. Especially for proteins in the endoplasmic reticulum and photosynthetic antenna (Figure 3B), an increase was detected in the expression of *CsRMA3*, and *CsLHB1B2*, but expression of *CsSCY1* and *CsLHCB6* was lower in A than in G.

2.6 DEGs involved in theanine biosynthesis

Theanine synthase (*CsTS1*) was highly expressed and in A compared with G. Some DEGs were downregulated in A compared with G, e.g., glutamate synthetase (*CsGS2*), glutamate dehydrogenase 2 (*CsGDH2*), and glutamine synthetase (*CsGSR_1*). Alanine decarboxylase (*CsAlaDC*) was not differentially expressed in A, M or G (Figure 4). Analysing the correlation between DEGs and the free amino acid content associated with the glutamate pathway, proline dehydrogenase (*CsERD*), *CsTS1*, delta 1-pyrroline-5-carboxylate synthase (*CsP5CS2*) and *CsAlaDC* were positively correlated with the content. *CsGS2*, alanine transaminase (*CsALAAT2*), and proline iminopeptidase (*CsPIP*) were negatively correlated with the content.

2.7 Transcription factor correlation with theanine synthesis

The expression levels of the *CsTS1* gene and MYB transcription factors (TFs), including TEA027578, TEA000509, TEA009081, and TEA004608, were significantly consistent. Seven WRKY TFs but only one bHLH transcription factor were expressed consistently with *CsTS1*. The correlation between *CsTS1* and *CsAlaDC* and TFs showed similar trends. Most of the correlations between *CsTS1*, *CsAlaDC* and *CsALAAT2*, *CsGS2*, *CsGSR_1* and *CsGDH* and TFs showed opposite trends. More DEGs associated with MYB TFs were correlated with theanine synthesis than with WRKYS and bHLHs (Figure 5A). MYB genes in five data-rich plants, including *Arabidopsis thaliana*, *Theobroma cacao*, *Prunus persica*, *Actinidia rufa*, and *Vitis vinifera*, used to conduct evolutionary comparative analysis with the known tea plant genome as a reference, TEA007928 and TEA009081, had high homology and consistency. These two genes were shown to group together with the *Camellia sinensis* gene *CsMYB36-like* in a cluster. TEA012145 was shown to group together with the *Prunus persica* gene *MYB5*, and TEA019409 was shown to group together with the *Actinidia chinensis* gene *MYB123* in a cluster (Figure 5B).

2.8 QRT-PCR verification

Among A, G, and M, the number of DEGs between A and G was the largest. Therefore, the 16 candidate genes in A and G were selected from the DEGs for qRT-PCR analysis. These DEGs were related to the

following metabolic pathways: “porphyrin and chlorophyll metabolism”, “photosynthesis - antenna proteins”, “alanine, aspartate and glutamate metabolism”, “arginine and proline metabolism” and “MAPK signalling pathway – plant”. The expression patterns of the selected genes obtained from qRT-PCR validation were similar to the expression trends of the Illumina sequencing results, except that the *LHCB4* gene showed similar expression (Figure 6).

Discussion

3.1 *CsPPOX* may inhibit chlorophyll biosynthesis in albino sector

The low expression of *CsPPOX* inhibits chlorophyll synthesis, resulting in low chlorophyll contents in albino sector. It is well documented that the leaves of variegated plants have fewer photosynthetic pigments in the albino sector, and YHC is no exception[25]. The inhibition of chlorophyll synthesis or an increase in the chlorophyll degradation rate may cause chlorophyll deficiency[26]. We determined the expression levels of key enzyme genes related to chlorophyll biosynthesis and degradation. It was found that chlorophyll deficiency was caused by a decrease in the rate of chlorophyll synthesis because 5 DEGs were found to be involved in chlorophyll synthesis, and DEGs were not found to be involved in chlortophyll degradation. *CsPPOX* catalyses the last step in the branched tetrapyrrole biosynthetic pathway, which is a common reaction for chlorophyll and haem biosynthetic pathways[27]. Inhibition of *PPOX* can lead to the observed bleaching of photosynthetically active parts of tobacco[28]. *Arabidopsis* mutations can inactivate PPOX, and offspring display yellow-green leaves, or even white and nonviable leaves[29]. We speculate that the low expression of *CsPPOX* is the main reason for the decrease in the rate of chlorophyll biosynthesis, which can be inferred by detecting the accumulation of intermediate products in the process of chlorophyll synthesis.

3.2 Low expression of *CsLHCB6* and *CsSCY1* may inhibit chloroplast biogenesis

Chloroplast biogenesis is a sophisticated process that includes chloroplast gene expression, chloroplast protein processing, signal transduction, synthesis, assembly, and maintenance of the thylakoid protein complex at the molecular level[30-33]. The low contents of Chl a and Chl b molecules that are required for stabilization of the proteins within the photosynthetic membrane probably causes the breakdown of thylakoid membranes[34]. Any developmental defect of the chloroplast can also negatively regulate the stability of photosynthetic pigments, thus changing the content and proportion of photosynthetic pigments, ultimately leading to leaf variegation[35]. *SCY1* is a multispanning membrane protein anchored in thylakoid membranes[36]. Plastids contain two different Sec systems with distinct functions. *SCY1*, as a loss-of-function mutation in components of the thylakoid-localized Sec system in *Arabidopsis*, can lead to albino and even lethal seedlings[37]. *LHCB6* (*CP24*) was detected as a component of light-harvesting complex II (LHCII) assemblies[38]. The formation and stacking of grana prove to be important for chloroplast biogenesis. The *Theangulata 10* (*anu10*) mutant lacks grana due to reduced levels of trimeric LHCII, leading to a pale leaf phenotype[39]. A missing assembly of the trimeric LHCII results in no granum formation in the seedling lethal *gdc1* mutant (grana-deficient chloroplast 1)[40]. The repressed expression

of genes encoding LHCII is correlated with albinism in tea[14]. Without the light-harvesting complex *LHCB6*, PSII cannot properly organize PSII complexes in the membrane[41], which affects the structure and function of the granum membranes of chloroplasts[42]. It is suggested that lower expression of *CsSCY1* and *CsLHCB6* in albino sector separately inhibited thylakoid and grana membranes of chloroplasts, leading to the albino tea.

3.2 *CsTS1* regulates theanine contents in albino sector

Theanine is a special secondary metabolite of tea plants, and its metabolism is closely related to nitrogen metabolism[43]. The degradation of chloroplasts causes substantial protein degradation, and protein degradation increases nitrogen sources and activates nitrogen metabolism. Total amino acids are significantly improved by nitrogen levels[9]. Glutamate is a common precursor substance for the biosynthesis of chlorophyll and theanine[44], and the production of less chlorophyll results in the consumption of less glutamate and glutamate accumulation in cells. Theanine is biosynthesized by glutamine and ethylamine under the catalysis of theanine synthase[45]. The expression of the *CsTS1*[46] gene and the content of theanine in tea increased simultaneously in albino sector. The downregulation of *CsGS2*, *CsGSR-1*, and *CsALAAT* in albinism reduces the breakdown of glutamate and alanine, ensuring that there are enough precursor substances to synthesize theanine. *CsA/aDC* is not differentially expressed in variegated plants, but the expression of this gene is highly correlated with the theanine content and the expression of *CsTS1*[47].

3.3 Transcription factors are positively correlated with theanine synthesis

With the great effort that has recently been made to functionally dissect theanine enzyme genes, study of the regulation of theanine enzyme genes via TF genes may become an important field[48]. The transcriptional regulation of theanine biosynthesis has attracted interest. Many studies have reported the roles of TF genes in the transcriptional regulation of plant-specialized metabolites, such as flavonoids[49] and anthocyanins[50]. However, the regulation of theanine enzyme genes by TF genes remains rare because theanine has no reference pathway in model plants[48]. Twenty *CsMYB* genes associated with theanine biosynthesis were screened, of which *CsMYB73* can specifically bind to the promoter region of *CsGDH2* to regulate theanine synthesis[51, 52]. Starting from the transcriptome data, we performed an exhaustive coexpression analysis between TF genes, including MYB, WRKY, and bHLH, and theanine enzyme genes in tea plant. MYB is one of the most prominent TF families involved in the transcriptional regulation of plant-specialized metabolites[53]. Based on correlation expression analysis, eight MYB genes may be related to theanine biosynthesis. *CsMYB36* was screened in our transcriptome data and publicly available tea transcriptome data[48]. *MYB36* can affect yellow-green peel colouration in cucumber fruit[54], and whether *CsMYB36* regulates the expression of genes related to theanine synthesis remains to be further studied. *CsMYB5* might form complexes with bHLH and WD40 proteins[51]. MYB5 can affect strawberry fruit ripening by regulating anthocyanin synthesis, which affects amino acids[55]. The possible regulation of theanine enzyme genes by the eight transcription factor genes is still unknown and needs to be verified.

Conclusions

Leaf variegation is rare in tea. Knowledge of the secondary metabolites and molecular basis of tea variegation provides valuable information that is relevant to research and commercial tea production. This paper provided a mechanistic understanding of variegation at multiple biological levels from transcriptomic, molecular to cellular levels, suggesting that the expression of *CsPPOX*, *CsLHC6*, and *CsSCY1* is closely linked to variegation in tea plants. The dynamic expression levels of *CsTS1* were highly correlated with the theanine contents observed with albinism, and sufficient precursor substances for theanine synthesis is one of the reasons for the high theanine content.

Materials And Methods

5.1 Plant materials

The tea plants (*Camellia sinensis* L. O. Kuntze cv. 'Yanlinghuayecha') were cultivated at the Tea Research Institute of Hunan Province, China. On April 4, 2019, tea leaves with one bud and two leaves were randomly plucked, washed using ultrapure water and wiped dry. Then, the green sector (G), albino sector (A), and mosaic sector (M) were cut with scissors, fixed with liquid nitrogen immediately and brought back to the lab for storage in a -80 °C refrigerator.

5.2 Measurement of chlorophyll abundance

An aliquot of the ground samples (0.02 g) was mixed with 95% ethanol and incubated in darkness for 12 hours. The chlorophyll extract was filtered and analysed with a NanoDrop 2000 ultraviolet spectrophotometer (Thermo Fisher Scientific, USA). The ultraviolet absorption wavelengths of chlorophyll a and b and total carotenoids were recorded as follows: absorption peaks of 665 nm for chlorophyll a, 649 nm for chlorophyll b, and 470 nm for total carotenoids. The abundances of chlorophyll a, chlorophyll b, and total carotenoids were calculated.

5.3 Transmission electron microscopy (TEM)

Small pieces of fresh leaf tissue (1.0 mm × 1.0 mm) were obtained from green, albino and mosaic sectors of each individual and then cut and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C for 12 hours. After removing OsO₄, the tissues were rinsed in 0.1 M sodium phosphate buffer (pH 7.4) 3 times for 15 min each. These samples were then fixed in pure acetone for 20 minutes following ethanol-series dehydration. These materials were then embedded in Spurr's resin (DER = 6.0) (Spurr, 1969) and polymerized at 70 °C for 12 h. These samples were then cut into semithin sections (60-80 nm) with an ultramicrotome (Leica UC7, Leica Microsystems, Wetzlar, Germany), and the tissues were removed onto 150 mesh cuprum grids with a formvar film. For further transmission electron microscopy (TEM) examination, sections were stained with 2% uranium acetate saturated alcohol solution for 8 min, rinsed in 70% ethanol 3 times, and then rinsed in ultrapure water 3 times. Then, 2.6% lead citrate was added to avoid CO₂ staining for 8 min, and then samples were rinsed with ultra-pure

water 3 times. After the sections were dried by filter paper, the cuprum grids were put into the grid board and dried overnight at room temperature. Then, the cells were examined using TEM (HT7800, Hitachi, Tokyo, Japan).

5.4 Determination of the free amino acid content

The free amino acid content was determined by using an HPLC (Agilent Technology, San Diego, CA, USA) with an ASB C18 analytical column (250 mm × 4.6 mm, 5 µm) according to the following setup: injection volume, 2.0 µL; column temperature, 30 °C; mobile phase A, 97% sodium acetate (0.1 M) and 3% acetonitrile, pH 6.5; solvent B, 80% acetic acid and 20% water; mobile phase flow rate, 1.0 mL/min; detection wavelength, 254 nm. Gradient conditions were such that 93% of solution A was maintained for 5 min; this was then changed to 62% solution A and 38% solution B until 25 min, then to 100% solution B from 30 to 40 min, both in a linear manner; finally, solution B decreased to 7% for 5 min and this was held for 10 min. Individual amino acids were identified and quantified by comparing their retention times and peaks with those of authentic standards. Their contents were expressed as percent dry weight of tea samples. Standard samples of free amino acids were purchased from Sigma-Aldrich Chemical Reagent Co., Ltd. (SigmaAldrich, St. Louis, MO, USA). All chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, People's Republic of China). The total amount of free amino acids was determined by weighing 3 g of the sample and 450 ml of boiling distilled water into a 500 ml Erlenmeyer flask, immediately heating it in water for 45 minutes, and shaking it several times during this period. Then, the hot solution was filtered, and the filtrate was diluted to 500 ml. After the solution was cooled, 1 ml of the solution, 0.5 ml of phosphate buffer solution, and 0.5 mL of ninhydrin were absorbed in a 25 ml test tube and heated at 100 °C for 15 minutes. After cooling the solution, the volume was brought up to 25 ml with distilled water and a spectrophotometer (NanoDrop 2000 spectrophotometer) was used to measure the absorbance at 570 nm.

5.5 RNA extraction, cDNA library preparation, and sequencing

Extraction, cDNA library preparation, and sequencing of libraries were performed using the NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations. The RNA concentration was measured using a Qubit® RNAAssay Kit in a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA), and RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyser 2100 system (Agilent Technologies, CA, USA).

A total of 3 µg of mixed RNA from each sample was used for cDNA library construction. Sequencing libraries were prepared using the NEBNext®Ultra™ RNA Library Prep Kit for Illumina® (NEB, MA, USA) following the manufacturer's instructions. Briefly, the mRNA was purified using Oligo (dT). The purified mRNA was fragmented using NEBNext First Strand Synthesis Reaction Buffer. The first-strand cDNA was then synthesized using random hexamer primers, and then the second strand was synthesized using RNaseH, DNA Polymerase I, and dNTPs. After adenylation of the 3' end and purification of the cDNA library, polymerase chain reaction (PCR) was performed using Phusion HighFidelity DNA polymerase. Finally, the PCR products were purified using the AMPure XP system (Beckman Coulter, Indianapolis, IN,

USA), and the library quality was assessed using the Agilent 2100 Bioanalyser. After clustering with TruSeq PE Cluster Kit v3-cBot-HS (Illumina), the generated cDNA library was sequenced on the Illumina HiSeqTM 2500 platform (Biomarker Biotech, Beijing, China), and paired-end reads were generated. Three biological replicates were conducted for each sample.

5.6 Quality control and transcriptome analysis

The clean data were produced by removing low-quality reads, adapters, and reads with poly-A. The clean reads were aligned to the tea plant genome[45] by HISAT2 v2.0.5. FeatureCounts v1.5.0-p3 was used to count the read numbers mapped to each gene. Then, the FPKM value of each gene was calculated based on the length of the gene and read counts mapped to this gene. Differential expression analysis of three groups (three biological replicates per condition) was performed using the DESeq2 R package (1.16.1). Genes with an adjusted P-value <0.05 found by DESeq2 were considered differentially expressed. Gene Ontology (GO)[56] enrichment analysis of DEGs was implemented by the cluster profile R package, in which gene length bias was corrected. GO terms with a corrected p-value less than 0.05 were considered significantly enriched by DEGs. We used the cluster profile R package to test the statistical enrichment of DEGs in KEGG pathways[57] (<https://www.genome.jp/kegg/>). New gene prediction was performed with StringTie differential expression analysis of fragments[58].

5.7 Phylogenetic analysis

To better understand the possible functions of the above-identified thea-related TF genes, we focused on the MYB, bHLH, WRKY, and WD40 genes involved in theanine biosynthesis. Phylogenetic analysis was conducted based on the protein sequences of the screened MYB genes in tea plants and their counterpart MYB genes in five plant species, including *Arabidopsis thaliana* (<https://www.arabidopsis.org/>), *Actinidia chinensis*, *Vitis vinifera*, *Prunus persica*, and *Theobroma cacao* (<https://www.ncbi.nlm.nih.gov/>). Multisequence alignments were performed using ClustalX32 (version 1.8). A phylogenetic tree was then constructed using MEGA33 (version 6.0)[59] through the neighbour-joining method and tested using the bootstrap method with 1000 replicates.

5.8 qRT-PCR validation

16 DEGs were validated by real-time quantitative reverse transcription PCR (qRT-PCR). Total RNA was reverse transcribed into cDNA using a PrimeScript RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan). Quantitative PCR amplification was conducted on the ABI 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). A 20 µL portion of the PCR system and TB Green Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan) were used for PCRs. The PCR amplification conditions were as follows: 90 s of degeneration at 95 °C, followed by 30 s of degeneration at 95 °C, 60 s of annealing at 55 °C, for a total of 40 reaction cycles, and finally 1 min of extension at 60 °C. Dissociation curves were collected at temperatures from 60 to 95 °C. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The sequences of differential gene primers were listed (Table S2). The relative expression levels of the genes were normalized by the 2- $\Delta\Delta CT$ method.

5.9 Statistical analysis

Analysis of variance was performed with SPSS software (v22.0, SPSS Inc., Chicago, IL) to determine the least significant differences between different treatments ($p < 0.05$), and Duncan's multiple range test was used to compare the averages. The mean and standard deviation (SD) were calculated based on three independent biological replicates. GraphPad Prism (v8.0.1)[60] was used to process the data and generate the figures. TBtools[61] and pseudoQC[62] were used to analyse correlations.

Abbreviations

A: albino sector; Chl a, chlorophyll a. Chl b, chlorophyll b. Ch, chloroplast; Carot, β -carotene. DEG: differentially expressed genes; DW, dry weight. FW, fresh weight. GO: Gene Ontology; Gr: grana; G: green sector; HPLC: high-performance liquid chromatography; KEGG: Kyoto Encyclopedia of Genes and Genomes; LHCII: light-harvesting complex II; M: mosaic sector; TEM: transmission electron microscopy; TF: transcription factor; Th: thylakoids; CsTS1: theanine synthase; Vc, vacuole. YHC: Yanlinghuayecha

Declarations

Authors' contributions

Nianci Xie conceived and designed the experiments with input from Kunbo Wang and Chengwen Shen. Pinqian Zhou and Xizhi Gao provided help in metabolite determination. Shuanghong Tian and Cui Lu provided assistance with the sample collection. Nianci Xie conducted experiments, analysed data, and drafted the manuscript. Chengwen Shen and Chenyu Zhang critically reviewed and edited the manuscript.

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Availability of data and materials

Raw sequencing data files are available in the NCBI SRA database with project accession NO. PRJNA695417. TreeBASE link for phylogenetic relationships among the 8 MYB genes in tea plants and

their counterpart genes in five other model plants.

(<http://purl.org/phylo/treebase/phylows/study/TB2:S27711>).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interest.

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Figures

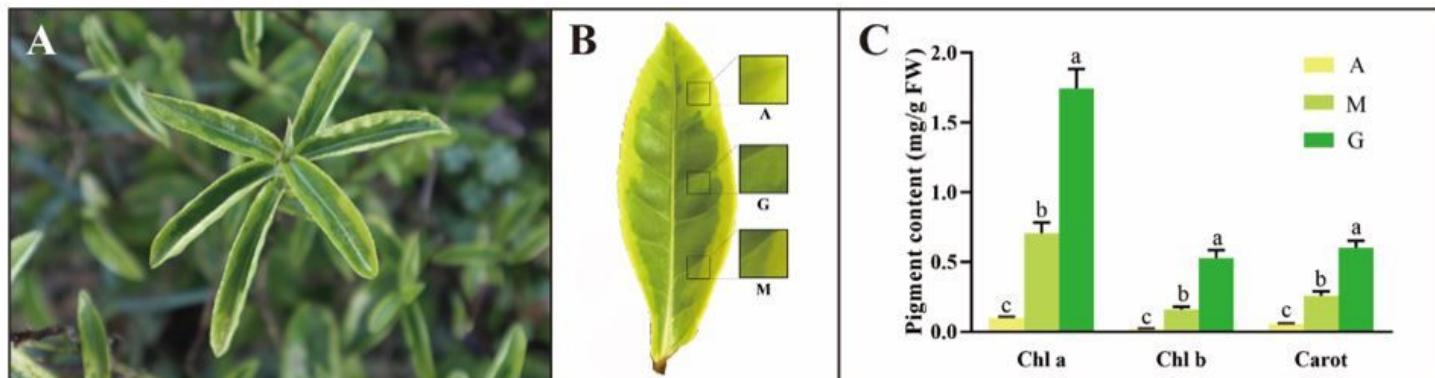


Figure 1

Phenotypic characteristics and pigment contents of YHC leaves. (A, B) Phenotypic characteristics of YHC; C: photosynthetic pigments of YHC; A, albino sector. M, mosaic sector. G, green sector. FW, fresh weight. Chl a, chlorophyll a. Chl b, chlorophyll b. Carot, Carotenoids. Data are presented as the means of three replicates (\pm standard deviation). The values in columns labelled with different letters are significantly different (p -value < 0.05).

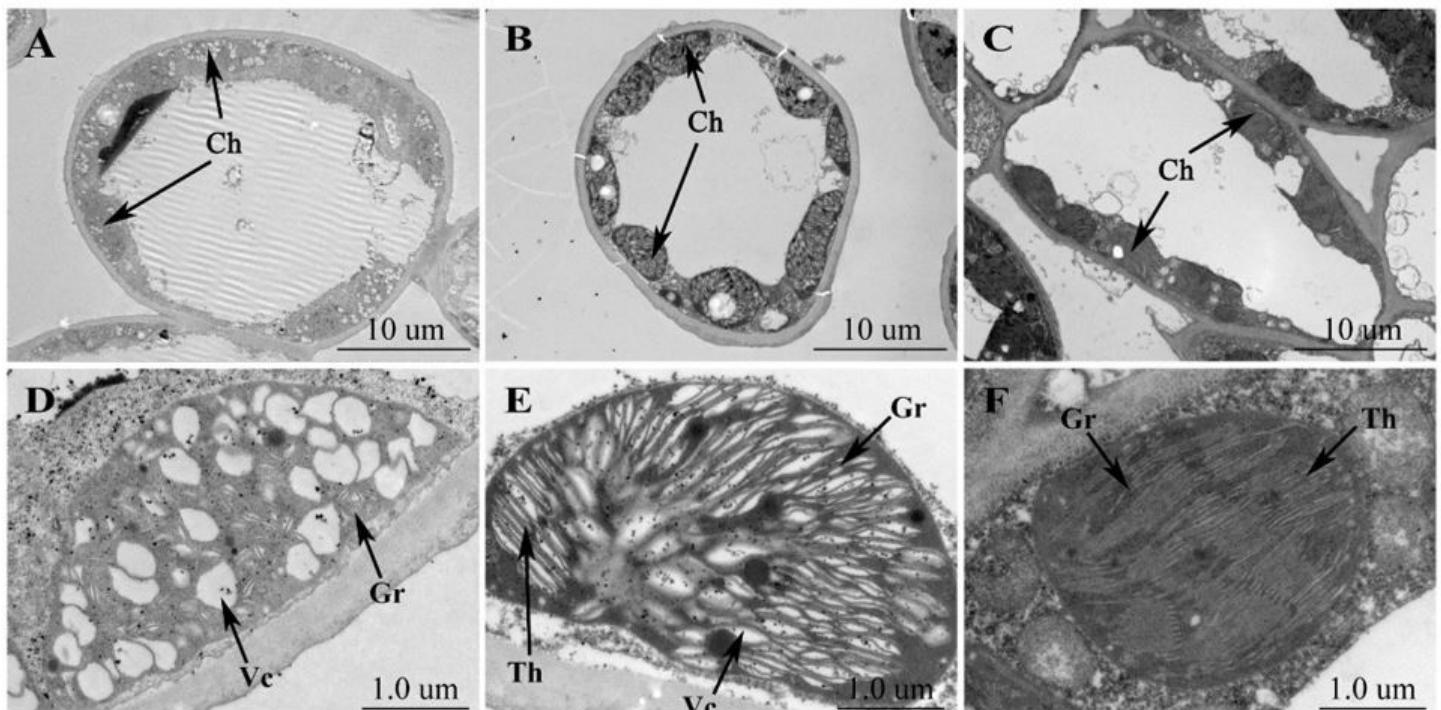


Figure 2

Chloroplast ultrastructure of different sectors of Yanlinghuayechia. Chloroplast ultrastructure of albino sector. (A, D) Chloroplast ultrastructure of mosaic sector. (B, E) Chloroplast ultrastructure of green sector (C, F). Ch, chloroplast; Th, thylakoid; Gr, grana; Vc, vacuole.

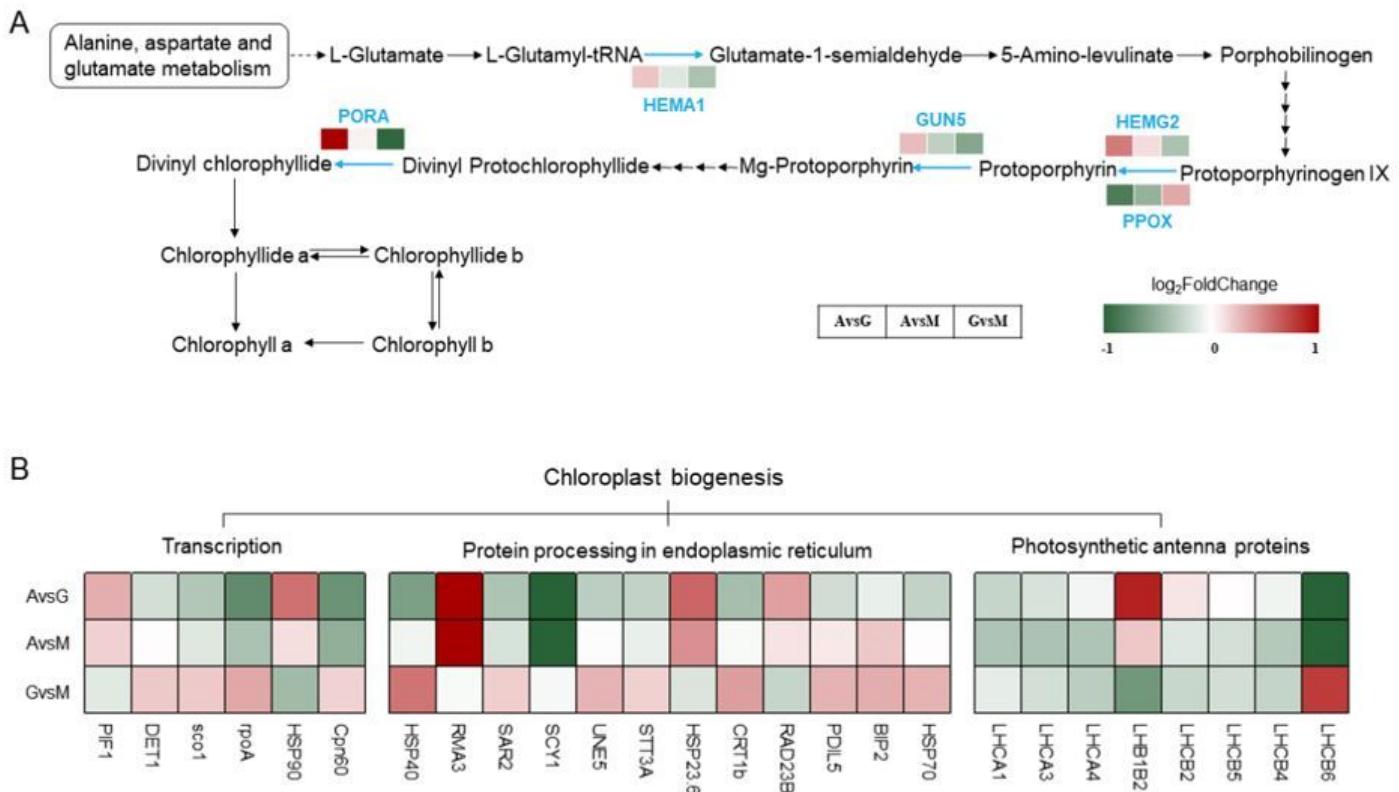


Figure 3

DEGs associated with chlorophyll biosynthesis and chloroplast biogenesis. (A) DEGs encoding enzymes related to chlorophyll biosynthesis. (B) DEGs encoding enzymes related to chloroplast biogenesis. A, albino sectors. M, mosaic sectors. G, green sectors.

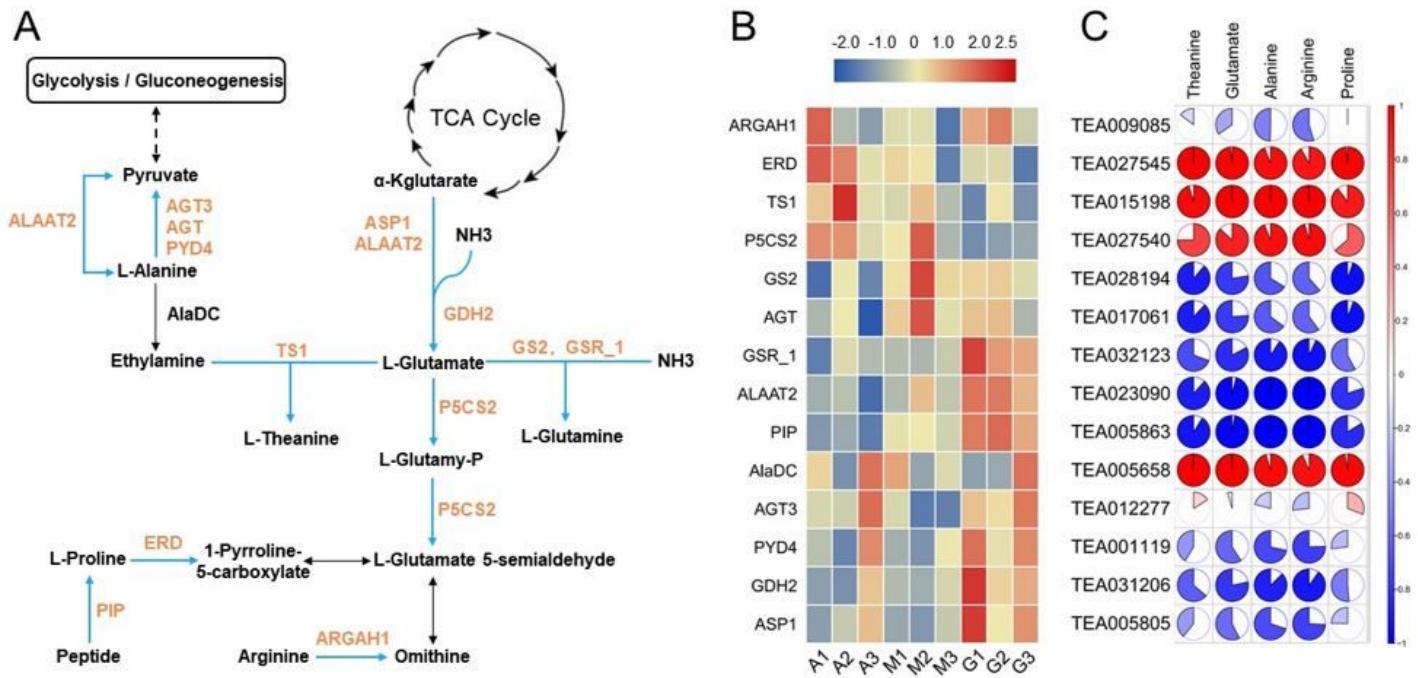
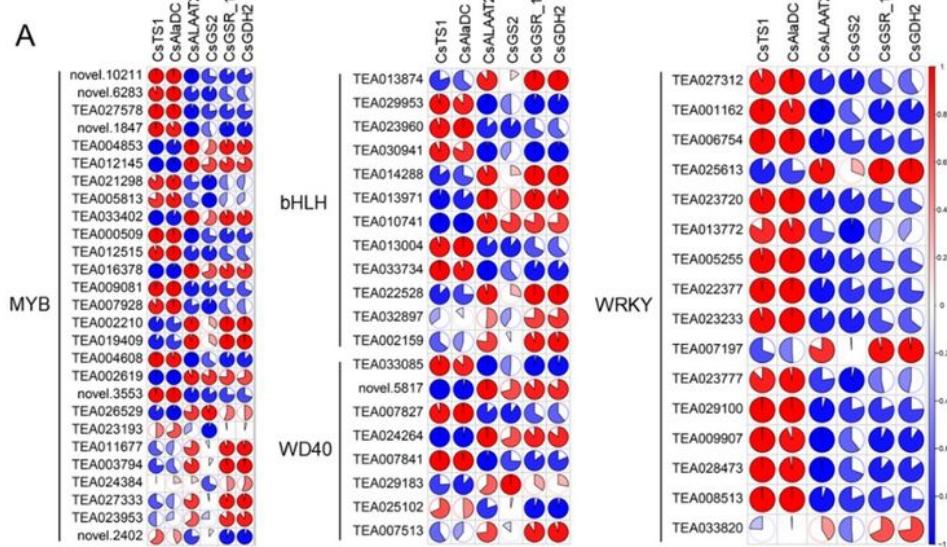


Figure 4

DEGs associated with theanine biosynthesis. (A) DEGs encoding enzymes related to synthesis. (B) Expression levels of DEGs related to glutamate amino acids. (C) Correlation analysis between substances related to theanine biosynthesis and DEGs. A, albino sector. M, mosaic sector. G, green sector.



B

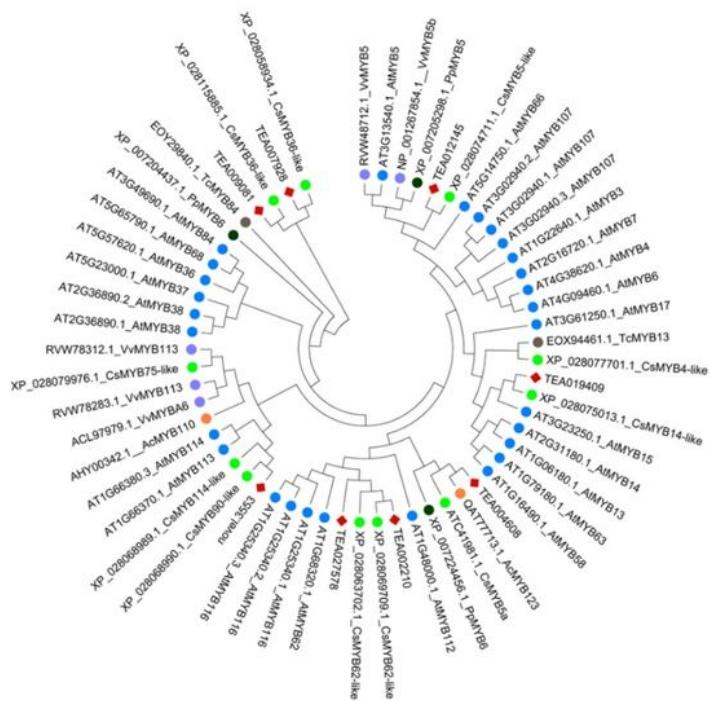


Figure 5

DEGs associated with transcription factors. (A) DEGs encoding transcription factors. (B) Phylogenetic relationships among the 8 MYB genes in tea plants and their counterpart genes in five other model plants. Eight MYB genes were labelled with a red diamond. The MYB counterpart genes in these plants were labelled with circles of different colours as follows: green, *Camellia sinensis*; blue, *Arabidopsis thaliana*; orange, *Actinidia chinensis*; purple, *Vitis vinifera*; dark green, *Prunus persica*; brown, *Theobroma cacao*.

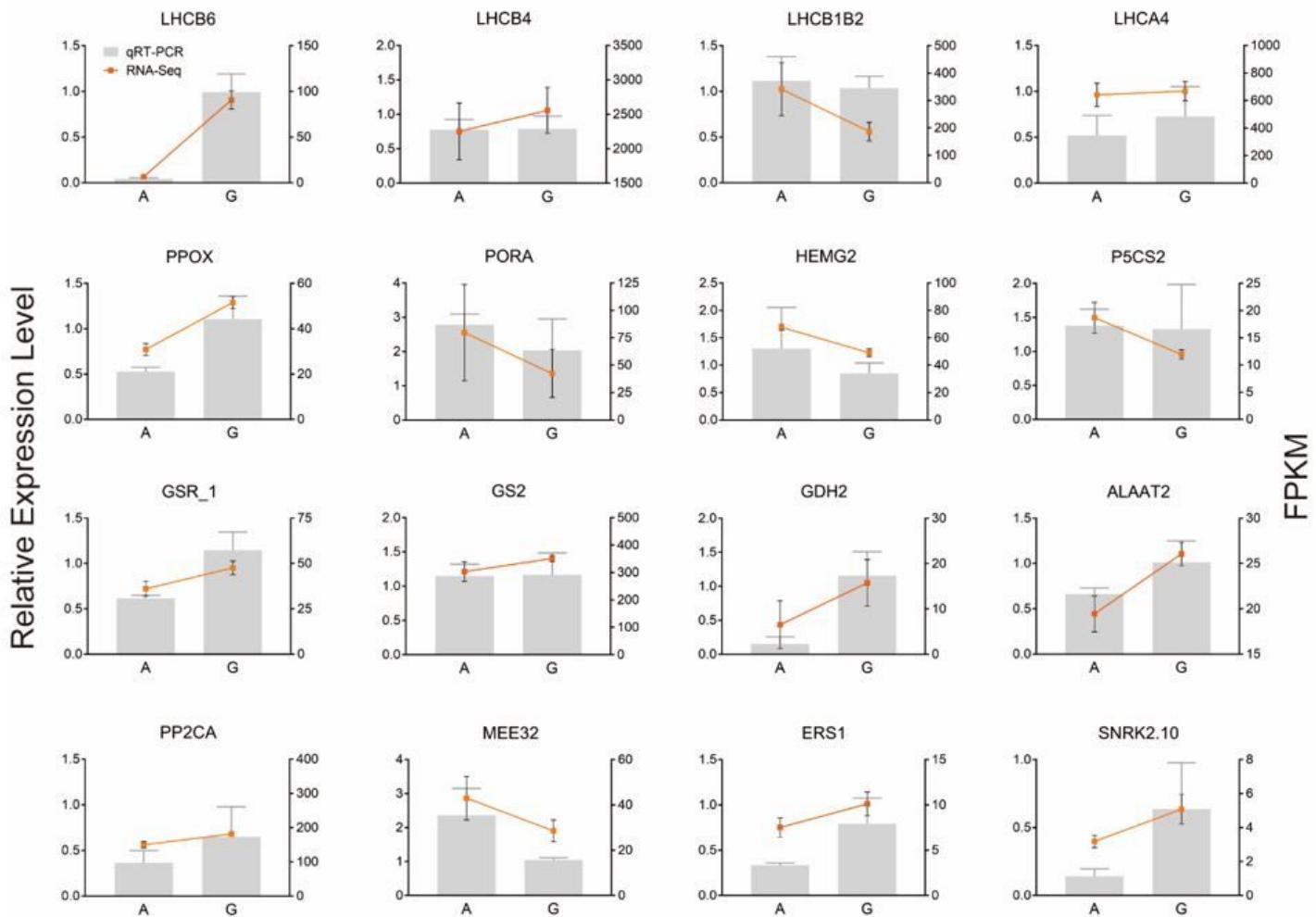


Figure 6

QRT-PCR validation of 16 DEGs. The data are presented as means with standard deviations. FPKM: Fragments per kilobase of exon model per million mapped fragments. A, albino sector. G, green sector.

Supplementary Files

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