



OPEN Full-length transcriptome sheds light into the molecular mechanism of tea leaf yellowing induced by red light

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Light, as an energy source for plant photosynthesis, can not only affect the growth and development of plants, but also affect their leaf color. This study used white (WL), red (RL), and blue light (BL) to treat tea cuttings, aiming to investigate the effect of light quality on the color of tea leaves. The results showed that tea leaves turned yellow under red light, the SPAD and *Fv/Fm* values were significantly lower than WL and BL. Full-length transcriptome was analyzed, photosynthesis and chlorophyll biosynthesis related genes such as *PsbS*, *Psb28*, *HemL*, and *POR* had the lowest expression levels under RL. Carotenoid biosynthesis related genes *ZEP*, *ABA2*, and *CRTISO* had the higher expression levels under RL. This study revealed the molecular mechanism of RL induced leaf yellowing in tea plants, providing new insights for the application of light quality in tea plants.

Keywords *Camellia sinensis*(L.) O. Kuntze, Tea cutting, Leaf color, Light quality, Full-length transcriptome

In recent years, yellow variant varieties of tea plant have been cultivated, such as “Zhonghuang 1” and “Huangjinya”, which have relatively low catechin content and high amino acid content^{1,2}, and have gained people’s love. However, the yellow variant varieties of tea plant are very rare. Therefore, it is crucial to cultivate new yellowing tea varieties.

Light quality is considered an environmental factor that affects the color of plant leaves, flowers, and fruits³. For example, red light can maintain the green color of lettuce, while blue light can make its leaves turn yellow⁴. However, other study had found that red light reduces the chlorophyll content of *Brassica oleracea*⁵. This indicates that the impact of light on different species of plants is different. So, what are the effects of different light qualities on the color of tea leaves? There are currently no relevant reports. In addition, short cutting is a commonly used breeding method for tea plant, so this study used short cuttings as the research material. This is crucial for us to cultivate new yellowing tea plant varieties and breed existing yellowing varieties.

PsbS is a key protein for managing excess energy in plants. Górecka et al. found that *PsbS* is a regulator of chloroplast retrograde signaling and plays a novel and important role in light memory and light domestication⁵. Previous studies have shown that red light can down-regulate the expression of *PsbS* gene in ‘Huangjinya’ tea plants⁶. These indicated that the *PsbS* gene may play an important role in the regulation of tea leaf color by light quality.

Light emitting diodes (LEDs) are the main application light source in plant factories. It is more environmentally friendly and more economical than traditional lighting, while having a safer way of handling and disposal^{7–9}. In addition, LEDs are cold light sources that do not cause the environment to become hot, and their performance does not deteriorate over time of use¹⁰. At the same time, LEDs have their specific spectral range, and LEDs with corresponding spectrum can be customized according to the needs of plants. Therefore, LEDs were chosen as the light source in this study.

Our previous research found that tea plant cuttings treated with red light had lower SPAD values and yellowing of leaves, but our research focuses on the formation and growth of adventitious roots¹¹. The aim of this study is to reveal the intrinsic molecular mechanism by which light quality affects the leaf color of tea

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plant cuttings, and to provide new ideas for cultivating new yellowing varieties and breeding excellent existing yellowing varieties.

Results

Phenotypic differences of tea cuttings under different light quality

After two months of irradiation, the leaves color of tea cuttings was observed. Under RL, the mature and tender leaves turned yellow (Fig. 1A). Meanwhile, SPAD and N content were measured, the results showed that their trend of change was BL > WL > RL (Fig. 1B). In addition, red light can significantly promote the growth of new shoots in tea plant cuttings (Figure S1).

Full-length RNA-seq and functional annotation

In order to reveal the molecular mechanism of light quality regulating stem elongation of cuttings, we performed Oxford Nanopore Technologies (ONT) RNA-seq on leaf samples. There was a total of 9 cDNA libraries from three treatments (three biological replicates per treatment). After removing low-quality reads, a total of 43.1 million clean reads were obtained, with an average of over 4.78 million reads per library. Among them, more than 94% could be mapped to the reference genome sequence (Table S2).

After mapping to the reference genome *Camellia sinensis* 'Shuchazao', we identified 59,523 genes in the transcriptome. In addition, a total of 28,744 open reading frames (ORFs) were identified. As shown in Fig. 2A, only 2.77% of the coding sequences (CDS) encode peptides with a length of > 800 amino acids (aa), while 81.08% of CDS encode smaller peptides with a length between 0 and 400aa. Finally, 46,713 of the 53,574 non-redundant transcripts could be annotated to different databases. Specifically, 12,876, 37,183, 29,657, 25,590, 29,901, 30,117, 36,447 and 46,497 new isoforms were annotated to COG, GO, KEGG, KOG, Pfam, Swiss-Prot, eggNOG and NR databases, respectively (Fig. 2B).

Identification and functional annotation of DEGs

Three pairwise comparisons were established, including WL vs. RL, WL vs. BL, and BL vs. RL, to study the number of DEGs in cuttings under different light quality treatments. Compared with WL, 304 DEGs were identified under RL, of which 125 were significantly up-regulated and 179 were significantly down-regulated; 415 DEGs were identified under RL, of which 217 were significantly up-regulated and 198 were significantly down-regulated. Compared with BL, 844 DEGs were identified under RL, of which 325 were significantly up-regulated and 519 were significantly down-regulated (Figure S2). The above results indicated that RL vs. BL showed greater transcriptional differences.

A total of 1,244 DEGs were identified in WL vs. RL and BL vs. RL, of which 135 DEGs were common (Fig. 3A). KEGG analysis showed that several DEGs are involved in the "photosynthesis (ko00195)", "anthocyanin biosynthesis (ko00942)" and "phenylpropanoid biosynthesis (ko00940)" pathways (Fig. 3B). COG enrichment analysis showed that DEGs are mainly involved in "carbohydrate transport and metabolism", "secondary metabolites biosynthesis, transport and catabolism", "posttranslational modifications, protein turnover, chaperones", and "signal translational mechanisms" (Fig. 3C).

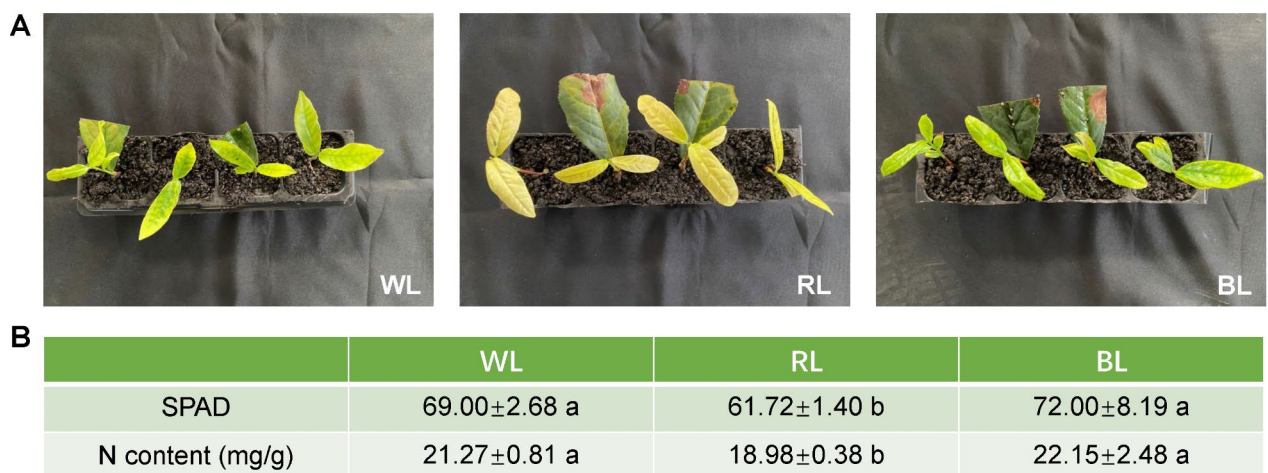


Fig. 1. (A) Phenotypic differences in tea cuttings under white, red, and blue light. (B) The SPAD and N content values of mature leaves of tea cuttings under white, red, and blue light. Lowercase letters represent significant differences ($P < 0.05$).

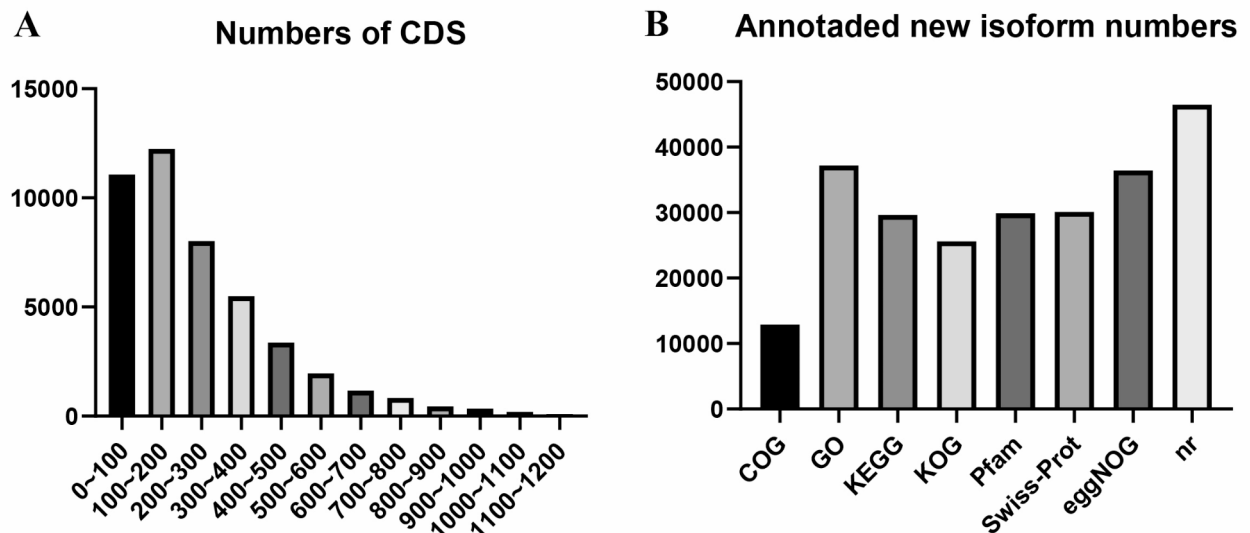


Fig. 2. (A) Plots showing the distribution of the lengths of predicted aa sequences. (B) Functional annotation of new isoforms in the indicated database.

Effect of white, red blue light on photosynthesis of tea cuttings

The *Fv/Fm* (maximal photochemical efficiency of PSII in the dark) of the cuttings was measured under different light quality treatments. The results showed that *Fv/Fm* values were as follows: BL (0.783) > WL (0.665) > RL (0.500) (Fig. 4A).

To further identify the genes related to photosynthesis under different light quality treatments, the “photosynthesis (ko00195)” pathway was analyzed. The results showed that, compared with WL and BL, 2 photosystem II 22 kDa protein genes *PsbS*, 1 photosystem II 13 kDa protein gene *Psb28* and 1 ferredoxin gene *PetF* were down-regulated in RL, except for 1 F-type ATPase gene *atpA*, and these genes were more significantly changed in RL vs. BL. This is consistent with the *Fv/Fm* result (Fig. 4B; Table 1).

Effect of white, red, and blue light on chlorophyll metabolism pathway

To reveal the molecular mechanism of light quality affecting color of tea leaves, the “porphyrin and chlorophyll metabolism (ko00860)” was analyzed. The result showed that some genes were involved in this pathway, including two glutamate-1-semialdehyde 2,1-aminomutase genes *HemL* (CSS0011648 and CSS0042059), one magnesium chelatase subunit H gene *ChlH* (CSS0016317), one magnesium-protoporphyrin IX monomethyl ester (oxidative) cyclase gene *ChlE* (CSS0026074), and one protochlorophyllide reductase gene *POR* (CSS0041527). Compared with WL and BL, these genes were down-regulated under RL (Fig. 5, Table S3).

Effect of white, red, and blue light on carotenoids metabolism pathway

Carotenoids also have an impact on the color of tea leaves, therefore the “carotenoid biosynthesis (ko00906)” pathway was analyzed. The result showed that some gene were involved in this pathway, including two xanthoxin dehydrogenase genes *ABA2* (CSS0031301 and CSS0027466), one zeaxanthin epoxidase gene *ZEP* (CSS0041624), and one prolycopene isomerase gene *CRTISO* (CSS0006960). Compared with WL and BL, these genes were down-regulated under RL (Fig. 6, Table S3).

qRT-PCR analysis

To verify the full-length RNA-Seq data, seven genes were randomly selected for qRT-PCR analysis. Expression profiles between white and red light, and between white and blue light were compared by qRT-PCR. The results showed that the expression trends of genes were consistent with the results of full-length RNA-seq analysis, which proved that the ONT RNA-Seq data were reliable (Fig. 7).

Discussion

This study used different light qualities to irradiate tea plant cuttings and found that red light can promote yellowing of tea plant cuttings leaves and promote the growth of new shoots. This indicates that red light may play an important role in promoting the growth and development of excellent yellowing tea varieties and increasing their yield. Light quality, as a key environmental factor for plant growth and development, has also been widely studied in other plant species. For example, red light can maintain the green color of lettuce⁴ and reduce the chlorophyll content of *Brassica oleracea*³. This indicates that different plants have different responses to light quality. We speculated that this was closely related to the biological characteristics and growth environment of plants. In addition, our previous research has found that blue light can significantly promote the formation and

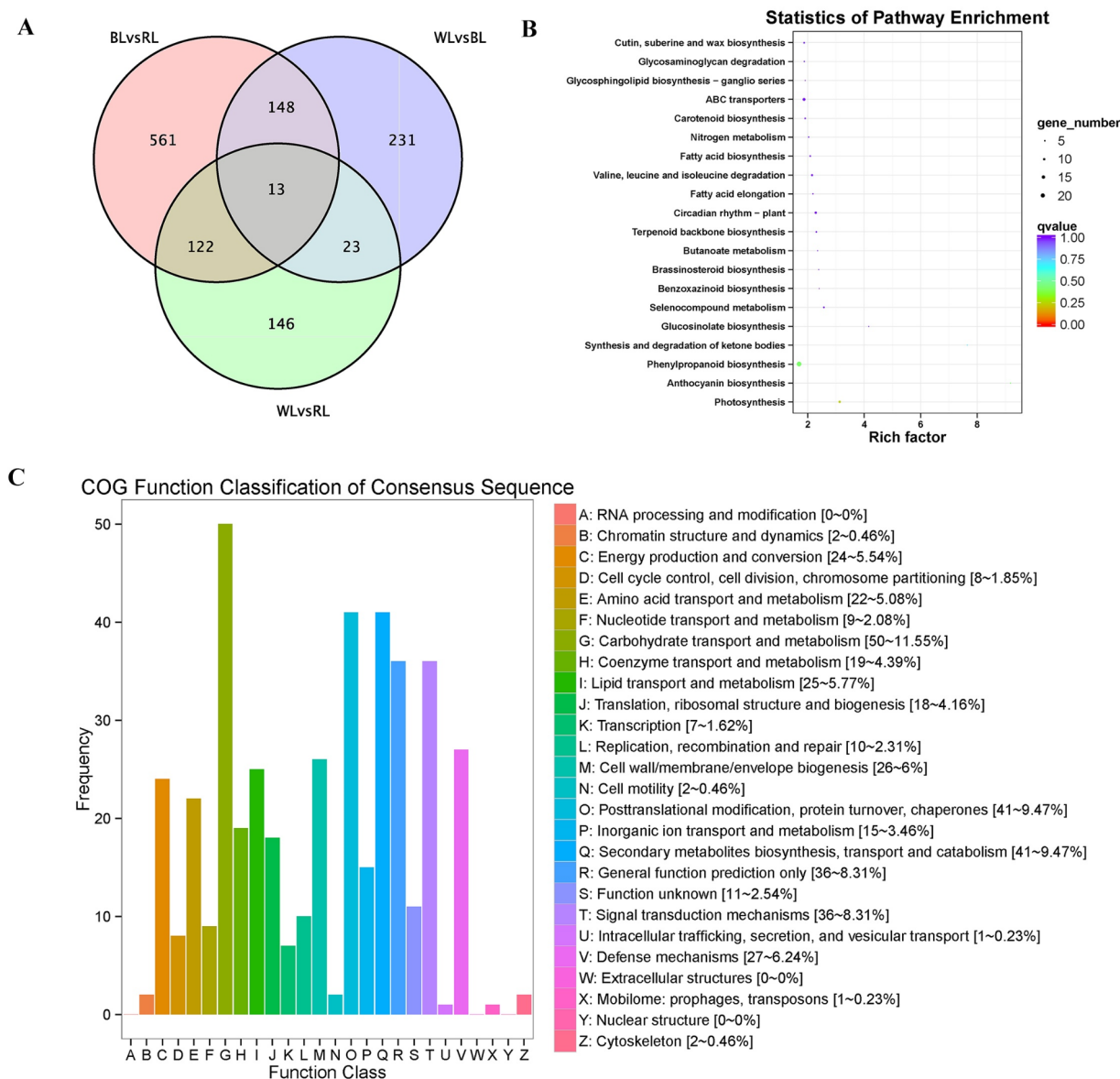


Fig. 3. (A) VENN diagram between WL vs. RL, WL vs. BL, and BL vs. RL. (B) KEGG annotation of DEGs in WL vs. RL, WL vs. BL, and BL vs. RL. (C) COG annotation of DEGs in WL vs. RL, WL vs. BL, and BL vs. RL.

growth of adventitious roots in tea short cuttings¹¹. Therefore, we speculated that mixed red and blue light can promote the growth and development of tea plant cuttings to a greater extent, but this requires further research to prove.

Red light reduced the photosynthetic capacity of tea cuttings

Light quality regulation directly determines plant photomorphogenesis and indirectly affects photosynthetic efficiency, plant growth and development. *Fv/Fm* is the maximum photochemical quantum yield of PSII (optimal/maximal photochemical efficiency of PSII in the dark), which reflects the endowment light energy conversion efficiency of PSII reaction center (intrinsic PSII efficiency) or the maximum PSII photochemical efficiency (optimal/maximal PSII efficiency). In this study, the *Fv/Fm* ratio under RL was significantly smaller than that of BL (Fig. 4A), which was similar to the results of Trojak et al.¹², indicating that RL may have reduced the photosynthetic function of cuttings. Photosynthesis in plants depends on the functional coordination of photosynthetic system (PS I) and photosystem II (PS II)¹³. *Psa* and *Psb* are components of PS I and PS II, respectively, and are considered to be key elements of photosynthesis¹⁴. In addition, when exposed to photooxidative stress, PSI and PSII are disrupted with a significant decrease in *Psa* and *Psb* expression¹⁵. In this study, both *PsbS* and *Psb28* were significantly down-regulated under RL compared with BL (Fig. 4B; Table 1), similar to the results of Tian et al.¹⁶. These results suggested that RL might inhibit the normal development of

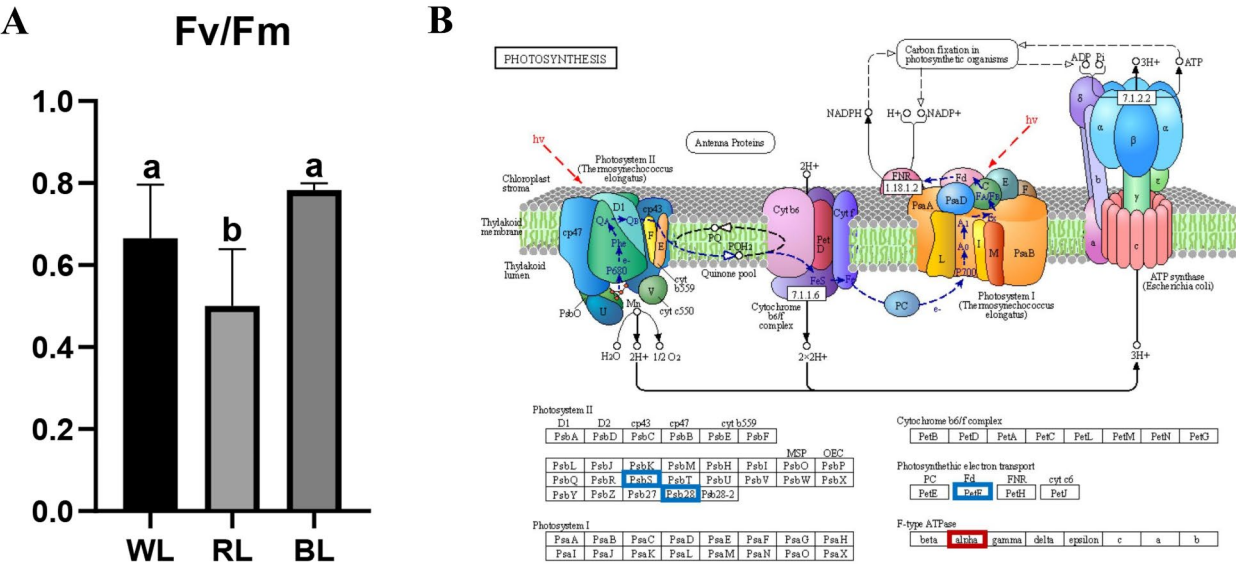


Fig. 4. (A) *Fv/Fm* values of cuttings ($P < 0.05$). (B) Expression of photosynthesis-related genes under different light qualities. Red circle represents up-regulation, blue circle represents down-regulation.

Gene id	NR annotation	Gene name	WL vs. RL log ₂ FC	BL vs. RL log ₂ FC
CSS0012566	photosystem II reaction center PSB28 protein, chloroplastic [<i>Camellia sinensis</i>]	<i>Psb28</i>	-0.409870929	-1.0013745
CSS0029272	hypothetical protein HYC85_007934 [<i>Camellia sinensis</i>]	<i>PsbS</i>	-1.161720486	-1.634562669
CSS0004563	ferredoxin, root R-B1-like [<i>Camellia sinensis</i>]	<i>PetF</i>	-0.690871992	-1.675938597
CSS0039743	hypothetical protein HYC85_007934 [<i>Camellia sinensis</i>]	<i>PsbS</i>	-0.684968901	-1.440072271
ONT.26,449	hypothetical protein HYC85_012512 [<i>Camellia sinensis</i>]	<i>atpA</i>	0.415278163	1.44643514

Table 1. Annotation and trend of photosynthesis-related genes.

the photosystem in cuttings, resulting in reduced light trapping capacity and photosynthetic efficiency. And this inhibition was eliminated by up-regulating the expression of the above genes after the plants were exposed to BL. In order to limit light damage, photosynthetic organisms have developed mechanisms to dissipate excess energy, and the involvement of PsbS protein has been clearly demonstrated in higher plants. In addition, Li et al. identified PsbS as a crucial protein for regulating photosynthetic light harvesting through analysis of *Arabidopsis thaliana* mutant isolates that cannot dissipate excessive absorption of light energy¹⁷. Therefore, we speculated that red light, as low-energy light, may be the reason for the down-regulation of *PsbS* gene. Meanwhile, studies have also found that the *PsbS* gene, like other members of the *LHC* family, is considered a pigment binding protein because proteins isolated from plants contain chlorophyll and xanthophyll¹⁸. So, we speculated that the down-regulation of *PsbS* gene under red light is closely related to the yellowing of tea leaves.

Red light caused tea leaf yellowing by down-regulating chlorophyll synthesis related genes

SPAD value is a parameter that measures the relative content of plant chlorophyll and represents its greenness. In this study, the SPAD value was the smallest under RL, indicating that the decrease in RL lowered the chlorophyll content of tea leaves. Previous studies have shown that the *Osh1H* mutant in rice has underdeveloped thylakoid membranes and low chlorophyll content¹⁹. Meanwhile, the silencing of the *ChlH* gene leads to a decrease in chlorophyll content in *Forsythia*, proving that *ChlH* was a key gene related to yellow leaf coloring in *Forsythia*²⁰. In addition, studies have shown that the expression level of *ChlE* in the yellow leaf mutant of *Paphiopedilum* is very low, and it has been proven to be a key gene responsible for leaf yellow²¹. This indicated that *ChlH* and *ChlE* are both key genes in plant chlorophyll synthesis. *POR* encodes the protochlorophyllide oxidoreductase and plays an important role in the light dependent step of chlorophyll biosynthesis, which is crucial for plant photosynthesis^{22–26}. Meanwhile, the high expression of *CsPOR* leads to an increase in chlorophyll content in tea plants²⁷. In the study of tea yellowing variants, *POR* was significantly up-regulated under RL, *ChlE* and *ChlH* showed no differential expression⁶. However, in this study, *POR*, *ChlE*, and *ChlH* were down-regulated under RL (Fig. 5). This indicated that tea varieties with different leaf colors have different responses to the same light quality. The above results indicated that RL might reduce the synthesis and accumulation of chlorophyll by down-regulating the expression of *POR*, *ChlE*, and *ChlH*, leading to yellowing of tea leaves.

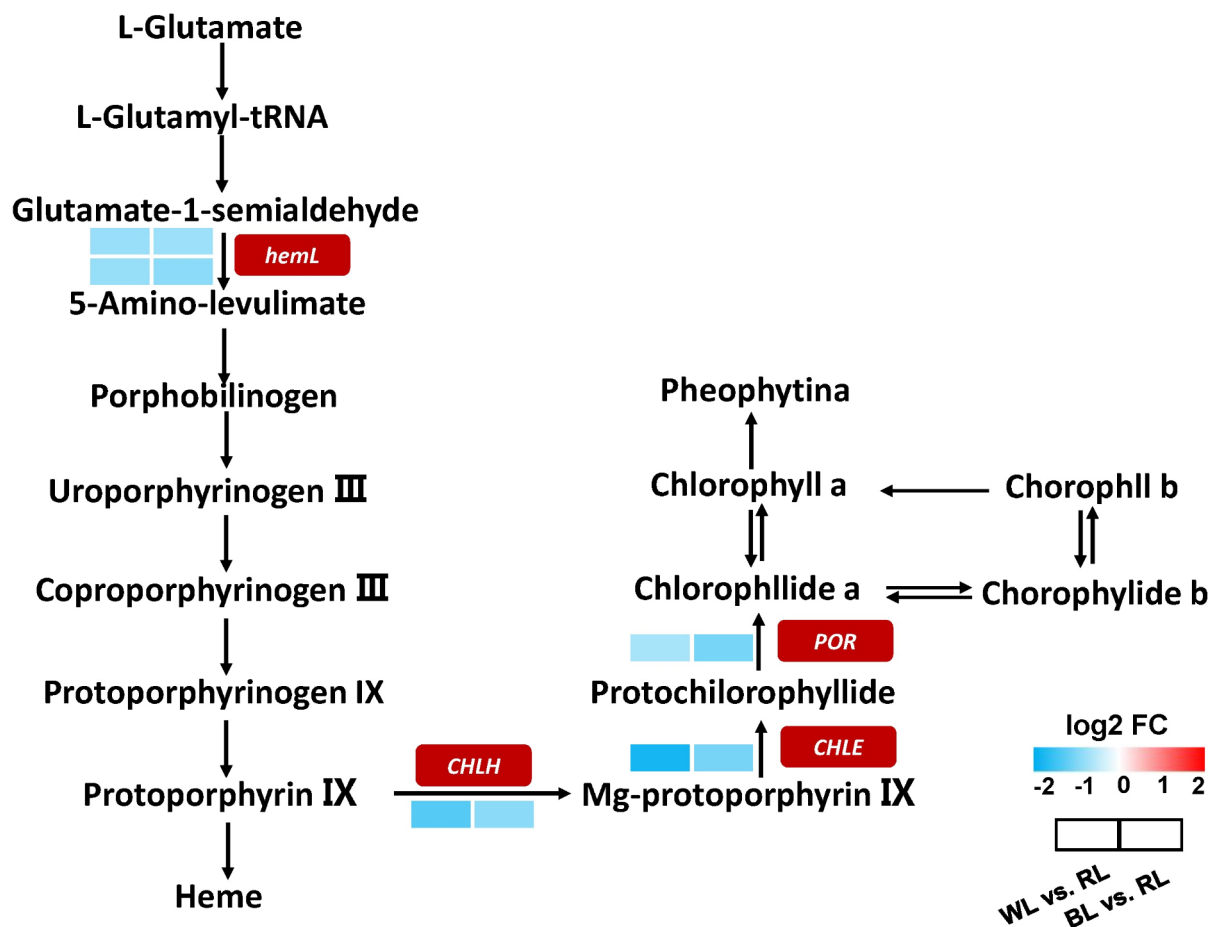


Fig. 5. Chlorophyll biosynthesis pathway and related gene expression.

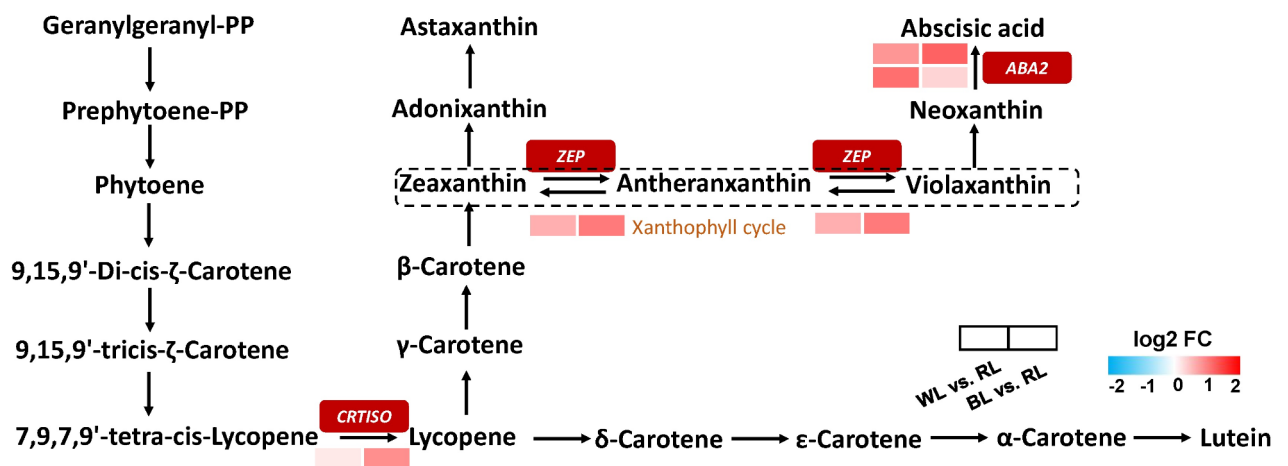


Fig. 6. Carotenoids biosynthesis pathway and related gene expression.

Red light caused tea leaf yellowing by up-regulating carotenoids synthesis related genes

The color of tea leaves is not only related to chlorophyll content, but also to the content of carotenoids. Previous studies have shown that the yellowing of plant leaves is due to a decrease in chlorophyll content, an increase in the proportion of yellow pigments such as carotenoids, or an increase in the content of yellow pigments caused

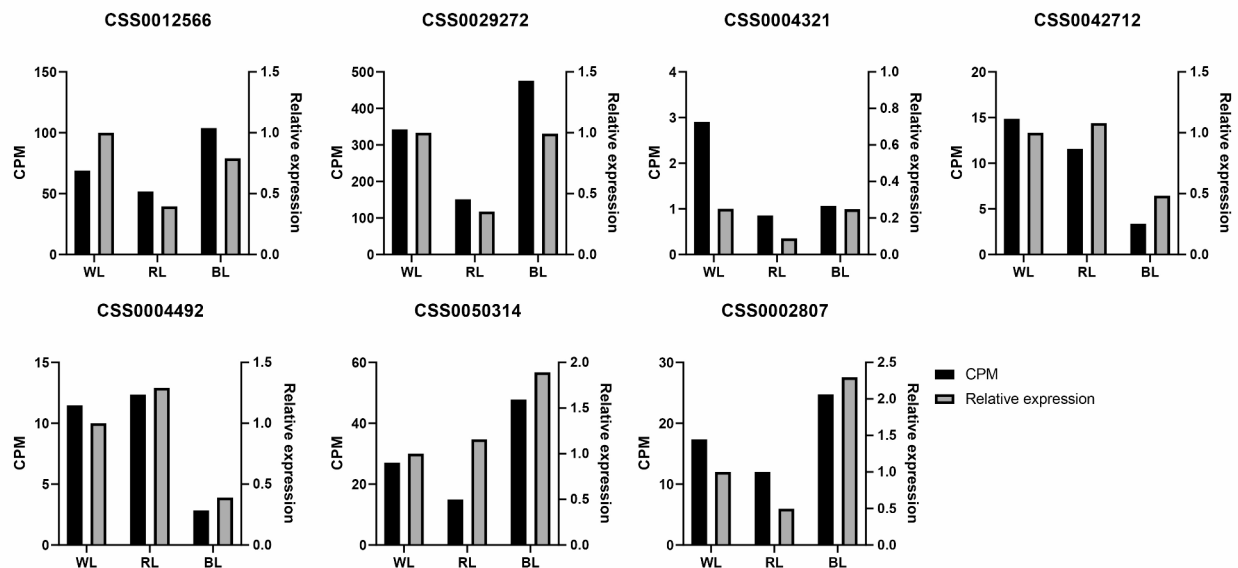


Fig. 7. Validation of seven genes by qRT-PCR. The left Y-axis represents counts per million (CPM) values for Oxford Nanopore Technologies (ONT) RNA-Seq, and the right Y-axis represents relative qRT-PCR expression levels. In the figure, the bars represent ONT RNA-Seq, and the lines represent qRT-PCR.

by the synthesis of carotenoids^{28,29}. Excessive accumulation of carotenoids often leads to the emergence of yellow leaf mutants in plants³⁰. Studies have shown that the content of carotenoids in tomato leaves is positively correlated with the levels of *CRTISO-L1* and *CRTISO-L2* transcripts³¹. At the same time, the loss of *BnaCRTISO* gene function leads to a down-regulation of key genes in the carotenoid pathway of *Brassica napus* L³². These indicate that the *CRTISO* gene is a key gene in plant carotenoid synthesis. In addition, overexpression of *NoZEP1* or *NoZEP2* increased the carotenoid content in *Nannochloropsis oceanica*³³. This indicated that *ZEP* is a key gene in carotenoid biosynthesis^{34,35}. Meanwhile, *ZEP* has been shown to play a key role in the yellowing of *Ginkgo biloba* leaves³⁶. The yellowing of tea leaves had also been proven to be highly correlated with carotenoid content³⁷. In this study, *CRTISO* and *ZEP* were up-regulated under RL (Fig. 6). The above results indicated that RL might promote the accumulation of carotenoids by up-regulating the expression of *CRTISO* and *ZEP*, leading to yellowing of tea leaves.

In rice, the ABA content decreases in the *aba2* mutant³⁸. Meanwhile, studies have shown that the *ABA2* overexpression in plants leads to an increase in ABA content in mature seeds³⁹. These results indicated a close relationship between the expression of *ABA2* gene and ABA synthesis. Studies have shown that the ABA biosynthesis in *Arabidopsis* leaves can promote the chlorophyll degradation⁴⁰. And Gao et al. found that high light intensity causes reactive oxygen species stress, triggering the ABA biosynthesis. The interaction between ABA and transcription factors limits the chlorophyll accumulation⁴¹. This indicated that endogenous ABA can reduce the chlorophyll content. In this study, the *ABA2* gene was significantly up-regulated under red light (Fig. 6). Based on the above, we speculated that red light may promote the expression of *ABA2* gene, increase the ABA content of leaves, and lead to leaf yellowing.

Conclusion

Light quality has a significant impact on the color of tea leaves. RL could cause yellowing of tea leaves, reducing *Fv/Fm* and SAPD values. Meanwhile, RL inhibited the expression of genes related to photosynthesis and chlorophyll synthesis, promoting the expression of genes related to carotenoid synthesis. In actual production, RL can be added to promote yellowing of tea leaves.

Materials and methods

Light treatment and sampling

Short cuttings of *Camellia sinensis* “Jiukengzao” were placed into artificial climate chamber of the Rizhao Tea Research Institute (Rizhao, Shandong, China). Using white (WL), red (660 nm, RL) and blue (430 nm, BL) LEDs with $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ to irradiate the short cuttings. All LED tubes were purchased from Shenzhen Hongyang Lighting Co., Ltd. (Shenzhen, China). Other environmental conditions: photoperiod (16 h day/8 h night); temperature (28 °C day/20 °C night); air humidity is $85 \pm 5\%$. After two months of irradiation, the leaves were sampled (At 22:00 that day, after 16 h of light exposure.). All samples were wrapped in tin foil, immediately frozen in liquid nitrogen, and then stored in an ultra-low temperature refrigerator at -80 °C for use in full-length transcriptome sequencing. Under each light treatment, there are three biological replicates. The tea plant samples come from our own experimental base (Rizhao Tea Research Institute, Rizhao, Shandong, China) and do not require permission.

Phenotype and photosynthetic data determination

The FP110-LM/D instrument (PSI, Czech Republic) was used to measure the *Fv/Fm* value of mature leaves under different light quality. Meanwhile, using the TYS-4 N plant nutrient meter (Zhejiang Topo Yunnong Technology Co., Ltd., China) to measure the soil and plant analyzer development value (SPAD) and Nitrogen content (N content) of mature leaves under different light quality. Ten cuttings from each treatment were randomly selected to measure the new shoots' length using a ruler with a scale of 0.1cm.

RNA extraction and full-length transcriptome sequencing

Using RNAPrep Pure Plant Kit (Tiangen, Beijing, China) to isolate RNA from leaves under different light quality, every treatment had three biological replicates. After evaluating quality of RNA quality, 9 cDNA libraries were conducted by cDNA-PCR Sequencing Kit (SQK-LSK110+EXP-PCB096) protocol (provided by Oxford Nanopore Technologies (ONT)). Biomarker Technologies Co., Ltd. (Beijing, China) conducted full-length transcriptome analysis.

Data Processing and full-length transcriptome identification

First, filter raw read (minimum average read quality score=6 and minimum read length=350 bp). Then, primers at both ends of the reads were searched to identify full-length non-chemiric (FLNC) transcripts. FLNC transcript clusters were obtained after mapping to the reference genome of *Camellia sinensis* "Shuchazao" (CSS_Chrlv, <http://eplant.njau.edu.cn/tea/download.html>) using mimimap2, and consistent isoforms were obtained after polishing using pinfish (<https://github.com/nanoporetech/pinfish>) within each cluster. Mapped reads were further collapsed by cDNA_Cupcake package (min-coverage=85% and min-identity=90%). 5' difference was not considered when collapsing redundant transcripts. This produced full-length transcripts.

Identification of differentially expressed genes

Gene expression levels were calculated as CPM (Counts per million) values. DESeq R package (1.18.0) was used to identify differentially expressed genes (DEGs), and the DEGs were defined as $|\log_2 \text{foldchange}| > 1$ and $P\text{-value} < 0.05$.

Annotation of gene function

All genes were annotated by these databases: Kyoto Encyclopedia of Genes and Genomes (KEGG), Gene Ontology (GO), Homology Clusters of Proteins (KOG/COG/eggNOG), NCBI Non-redundant Protein Sequences (NR), Protein families (Pfam), and A manually annotated and reviewed protein sequence database (Swiss -Prot).

Quantitative real-time PCR analysis

Randomly selected seven genes for expression level verification to verify the accuracy of full-length transcriptome data. Using Beacon Designer 8 to design primers, and primer sequences are listed in Table S1. The instrument used is the analytikjena-qTOWER2.2 fluorescence quantitative PCR instrument (Analytik Jena, Germany). The SYBR Green QPCR Mix (DF Biotech., ChengDu, China) was used to conduct Quantitative real-time PCR (qRT-PCR). Three biological replicates were analyzed. The glyceraldehyde 3-phosphate dehydrogenase (*CsGAPDH*) gene was regarded as an internal reference gene, the relative expression was quantified by using the $2^{-\Delta\Delta C_t}$ method to quantify the relative expression of candidate genes.

Statistical analysis

The SPSS 20.0 software (SPSS Inc., Chicago, USA) was used to analyze data, through one-way analysis of variance (ANOVA) and Duncan's multiple intervals. The software GraphPad Prime 8.0 (GraphPad Software, Inc.) and Adobe Photoshop CC 2019 were used to create pictures.

Data availability

Sequence data that support the findings of this study have been deposited in the NCBI SRA with the primary accession code PRJNA853565.

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Author contributions

YZ. S. conducted the experiments, analyzed the data, and wrote the manuscript. X.H., and H. W. directed and reviewed the manuscript; SB. D. and DP.S. collected the samples. Y.W. and ZT. D. put forward hypotheses and designed experiments. LT.S., JZ.S. and K.F. reviewed the manuscript. All authors contributed to the article and approved the submission.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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