Article

Comparative Transcriptome Analyses of Gene Response to Different Light Conditions of *Camellia oleifera* Leaf Using Illumina and Single-Molecule Real-Time-Based RNA-Sequencing

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Abstract: *Camellia oleifera* Abel. is a critical oil tree species. *Camellia* oil, which is extracted from the seeds, is widely regarded as a premium cooking oil, with the content of oleic acid being over 80%. Light is thought to be one of the largest essential natural components in the regulation of plant developmental processes, and different light qualities can considerably influence plant physiological and phenotypic traits. In this research, we examined the growth and physiological responses of *C. oleifera* “MIN 43” cultivar plantlets to three different wavelengths of light, containing white, red, and blue light, and we utilized the combination of the PacBio single-molecule real-time (SMRT) and Illumina HiSeq RNA sequencing to obtain the mRNA expression profiles. The results showed that plantlets growing under blue light conditions displayed superior growth performance, including stimulated enhancement of the leaf area, increased leaf number, increased chlorophyll synthesis, and improved photosynthesis. Furthermore, SMAT sequencing created 429,955 reads of inserts, where 406,722 of them were full-length non-chimeric reads, and 131,357 non-redundant isoforms were produced. Abundant differentially expressed genes were found in leaves under different light qualities by RNA-sequencing. Gene expression profiles of actin, dynein, tubulin, defectively organized tributaries 3 (DOT3), and ADP ribosylation factor 5 (ARF5) were associated with the greatest leaf performance occurring under blue light conditions. Moreover, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis identified hundreds of pathways involved in different light conditions. The pathways of the plant circadian rhythm and hormone signal transduction were associated with different light quality responses in *C. oleifera*. Phytochrome B (PHYB), constitutively photomorphogenic 1 (COP1), long hypocotyl 5 (HY5), auxin/indole-3-acetic acid (AUX/IAA), Gretchen Hagen 3 (GH3), and small auxin-up RNA (SAUR), which were differentially expressed genes involved in these two pathways, play a vital role in responses to different wavelengths of light in *C. oleifera*. In addition, blue light significantly promotes flavonoid biosynthesis via changing expression of related genes.

Keywords: *Camellia oleifera*; PacBio SMRT; RNA-seq; light quality

1. Introduction

Light is thought to be the largest essential natural component in the regulation of plant morphology. Plant physiological and phenotypic traits can be altered greatly by differing light quality [1,2]. Most recently, research has focused on the fields regarding the regulation of plants through various wavelengths of light. Fukuda et al. explored the responses of *Petunia hybrida* to three different qualities of light and discovered that red light hindered shoot prolongation while blue light notably heightened it [3]. A large portion of blue light can significantly withhold plant stem elongation [4–6]. However, the research on lettuce indicated that both blue and red light could improve biomass above ground [7]. Prior studies revealed that light-emitting diodes are greatly suitable for plant growth under controllable backgrounds such as plant culture systems [8]. Different light qualities altered plant metabolism processes, such as synthesis of pigments, phenolic compounds, and antioxidants. Furthermore, rising blue light fundamentally increased the content of chlorophyll compared with the control in tomatoes [9]. The previous study demonstrated that the rate of photosynthesis increased when illuminated with a high proportion of blue light in *Fagus sylvatica* [10]. Another experiment revealed that blue light promoted the content of total antioxidant phenolics in lettuce plants [11].

Variation in plant phenotypic traits caused by the different wavelengths of light is typically accompanied by altering gene expression. The RNA-seq approach has been employed to analyze how blue light enhances grape development at the molecular level in vitro [12]. Previous studies reported that gibberellin biosynthesis was promoted when illuminated with red light, while blue light triggered defense responses by improving the contents of secondary metabolites in Norway spruce [13].

Camellia oleifera Abel., classified into the family of Theaceae, is a unique oil tree species in China [14]. *Camellia* oil is rich in unsaturated fatty acids, with an oleic acid content reaching over 80% and is regarded as premium cooking oil [15–17]. The cutting technique is a critical breeding technique for the propagation of *C. oleifera*. Inducing *C. oleifera* cutting development via precisely controlling the illumination supply is a requirement for fostering robust plantlets as well as achieving valuable varieties. In recent years, the RNA-seq approach has been employed in the study of *C. oleifera*, mainly focusing on self-incompatibility [18] and lipid metabolism [19]. Unfortunately, the presently obtainable RNA-seq data for the *C. oleifera* leaf induced by various wavelengths of light is lacking.

Second-generation sequencing technology has been used to detect transcript isoforms, as the technology presents high throughput potential and supplies excellent reads. Nonetheless, its restrictions, principally short-read length, mean that sequence assembly is necessitated, thus likely introducing errors [20,21]. Single-molecule real-time (SMRT) sequencing, known for long-read sequencing, could precisely create full length transcripts and be applied to species without genome sequences [22–24]. Extensive utilization of this method is inhibited due to the high-error rate and low-throughput [20].

To address these issues, SMRT-seq and Illumina-seq data were generated from the *C. oleifera* cultivar “Min 43” and then exploited to screen gene expression profiles and characterize candidate genes engaged in the adaptation of *C. oleifera* growing under various wavelengths of light. In this research, the growth and physiological traits of *C. oleifera* and the differentially expressed genes (DEGs) of interest were considered to establish the potential molecular mechanisms by which *C. oleifera* responds to various light qualities. Overall, differentially expressed genes were obtained in all three different light settings, and numerous candidate genes were identified. The result of this research will provide insights into how to use various wavelengths of artificial light to regulate *C. oleifera* growth.

2. Materials and Methods

2.1. Materials and Experimental Treatments

One-year-old *C. oleifera* plantlet cuttings of the cultivar “Min 43” were used in this study. The experiment was conducted at the Fujian *Camellia oleifera* engineering research center, Fuzhou, China. The plantlets were transferred into pots filled with a medium composition of vermiculite, yellow sand, and yellow soil in a ratio of 2:1:1. After 2 days of pre-culture under white light conditions,

plantlets were positioned in the cutting incubator for 90 days with white, red, and blue light sources. All light sources used in this experiment were LED lights (Fujian Talos Technology Co., Ltd., Fuzhou, Fujian, China). The wavelengths of all LED lights (460 nm for the peak wavelength of blue light and 660 nm for the peak wavelength of red light) were tested with a light meter (LI-250A, LI-COR Biosciences Co., Lincoln, NE, USA). The photosynthetic photon flux density was retained at around $50 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ for all three light conditions, and the incubator environment was maintained at a twelve hour photoperiod with 75% relative humidity at $25 \pm 2^\circ\text{C}$. Each treatment had three replicates.

2.2. *C. oleifera* Plantlet Growth and Physiological Parameter Measurements

After 90 days of culture under different light sources, fifteen plantlets of different treatments were at random gathered to measure growth and physiological parameters. Plant height and stem diameter were determined with a meter ruler and a vernier caliper. The numbers of leaves were recorded. Leaf area was determined by a leaf area meter (Yaxin-1041, Beijing Yaxinlily Science and Technology Co., Ltd., Beijing, China). Photosynthetic indexes including net photosynthesis rate, stomatal conductance, intercellular CO_2 , and transpiration rate were quantified utilizing an intelligent portable photosynthesis system (LCpro-SD, ADC BioScientific Ltd., Hoddesdon, UK) between 8:30 and 11:30 a.m. The assimilation chamber settings of the LCpro-SD device were relative humidity of 75%, CO_2 concentration of 400 $\mu\text{mol/mol}$, and temperature of 25°C . Chlorophyll (Chl a and Chl b) and total carotenoid content were determined by spectrophotometry consuming 100 mg FW of leaves powdered with 8 mL of acetone 80% (v/v) for extraction. Once the whole extraction was complete, the mix was purified, and precooled acetone was added up to 10 mL. Then the solution was examined at 663.2 nm, 646.8 nm, and 470 nm and total chlorophyll content was assessed based on prior studies [25,26]. The enzyme activities of superoxide dismutase (SOD), polyphenol oxidase (PPO), and peroxidase (POD) were determined as previously described [27].

2.3. Leaf Anatomical Structure Observation

Second-developed leaves below the plantlet terminal bud of each sample were cut into small sections ($2 \times 3 \text{ mm}$) and fixed in a solution of FAA (formalin–acetic acid–alcohol) (90:5:5, V:V:V). First, gradient solutions of ethanol were used to dehydrate samples and then they were implanted in paraffin. Secondly, 1% safranin and 0.5% fast green were prepared to stain samples. Finally, samples were observed using a stereomicroscope (SMZ-168TL, MOTIC Co., Xiamen, Fujian, China), and images were captured by a Canon camera (Canon Co., Tokyo, Japan).

2.4. RNA Sample Preparation and Extraction

After 90 days of incubation, samples of leaves and roots from the three light treatments were obtained, instantly frozen in liquid nitrogen, and stored at -80°C . Three duplicates were prepared for the subsequent process using trizol reagent (Invitrogen Scientific, Inc., Carlsbad, CA, USA) to extract total RNA under the direction of the manufacturer. We employed 1%-agarose gel electrophoresis to confirm the integrity of RNA. An Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) was employed to detect the integrity of RNA with RNA integrity number (RIN). The samples with a RIN of more than eight were prepared for use in this study.

2.5. Illumina cDNA Library Preparation and Sequencing

A total of 2 μg of RNA of each leaf sample were utilized for the RNA isolate. Libraries for sequencing were created with a VAHTS mRNA-seq V2 Library Prep Kit for Illumina® (Vazyme Biotech Co., Ltd., Nanjing, Jiangsu, China). Concisely, mRNA capture beads were exploited to separate mRNA from the total RNA, and then the mRNA was fragmented and utilized for cDNA synthesis. After end repair and the addition of dA-tails, adapter ligation, purification of the ligation product, and size fractioning, nine paired-end cDNA libraries were created. After library amplification, the libraries were sequenced on an Illumina HiSeq $\times 10$ platform (Illumina, San Diego, CA, USA) and paired-end reads

were created. Illumina HiSeq sequencing in this research was performed by the Shanghai Personal Biotechnology Company (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China). The raw data was available under BioProject PRJNA592616 in the Sequence Read Archive (SRA).

2.6. PacBio Iso-Seq Library Preparation and Sequencing

The same amounts of the total RNA from tissues of leaves and roots of different light treatments were merged into one sample and then 5 µg RNA was used to prepare the SMRT library. In brief, we used the SMARTer PCR cDNA Synthesis Kit (Clontech, Mountain View, CA, USA) to synthesize the first-strand cDNA. After PCR amplification, Blue Pippin (Sage Science, Beverly, MA, USA) was used to choose the size of the amplified cDNA. An Iso-Seq library was created with the SMRTbell™ Template Prep Kit 1.0-SPv3 (PacBio, Menlo Park, CA, USA) and sequenced on a PacBio Sequel system (PacBio, Menlo Park, CA, USA). PacBio SMRT sequencing in this research was performed by the Shanghai Personal Biotechnology company (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China). The raw data was available under BioProject PRJNA PRJNA592821 in the Sequence Read Archive (SRA).

2.7. Quality Control and Transcriptome Assembly

Reads including those shorter than 50 b.p., with a predicted error rate of >20%, and with full passes lower than 0 were removed. Reads of insert (ROIs) were organized into four categories: full-length non-chimeric, full-length chimeric, non-full-length, and filtered short reads. Subsequently, the CD-HIT program [28] was used to obtain non-redundant isoforms based on sequence similarity. Only these non-redundant isoforms were utilized for further analysis.

Perl script was used to handle raw reads of Illumina with a fastq format. Reads with low quality and those containing adaptors identified by Cutadapt [29] were removed to obtain clean reads. Bowtie 2 was used [30] to create an isoform sequence index. After that, we used Trinity to compare the clean reads to the isoform sequence [31].

2.8. Gene Functional Annotation

Functional annotations were performed exploiting the BLAST toolkit ($E\text{-value} \leq 1 \times 10^{-5}$) against NCBI non-redundant protein sequences (NR), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), an evolutionary genealogy of genes: non-supervised orthologous groups (eggNOG), and the Swiss-Prot database. Gene Ontology (GO) annotation was conducted on Blast2GO based on the NR annotation [32].

2.9. Quantification of Gene Expression Levels

RSEM [31,33] was used to analyze the gene expression level. Clean data of Illumina were aligned to the SMRT sequencing data, and the mapping results provided a read-count for each gene. Taking into account the influence of the sequencing depth and unigene length on the fragments, the read-count values of all unigenes were converted into an FPKM value [34].

2.10. Identification and Functional Analysis of DEGs

Differential expression analysis was implemented by the DESeq [35] to identify DEGs between different light treatment conditions. Genes with an adjusted p -value < 0.05 found by DESeq were selected as differentially expressed, and the absolute value of $\log_2$ (Fold Change) > 1 was used as the threshold for defining significant DEGs between several groups. Volcano maps of differentially expressed genes were created by the ggplots2 R package [36].

2.11. Validation of DEGs with qRT-PCR

The real-time quantitative PCR (qRT-PCR) assay was conducted to validate the accuracy of RNA-seq results. Reverse transcription was performed using the PrimeScript™ first strand cDNA Synthesis Kit (Takara, Dalian, Liaoning, China). Primers for the qRT-PCR were designed in Primer 5 (Premier Biosoft International, Palo Alto, CA, USA). The expression of unigene (c48652_g1) was selected as the internal control. Quantitative real-time PCR was achieved by the SYBR Green method by a TIB-8600 real-time PCR System (Triplex International Biosciences, Co., Ltd., Xiamen, Fujian, China) with AceQ® qPCR SYBR® Green Master (Vazyme Biotech Co., Ltd., Nanjing, Jiangsu, China). A real-time RT-PCR reaction (20 µL) contained 10 µL of 2 × SYBR real-time PCR premixture, 1 µL cDNA, 0.4 µL of each primer, and 9.2 µL ddH₂O. The PCR setting was as follows: 95 °C for 5 min, 40 cycles at 95 °C for 15 s, and 60 °C for 30 s. A thermal denaturing step was used to generate melting curves to verify the specificity of the amplification. Three replicates were conducted in this experiment, and relative gene expression levels were analyzed by means of the $2^{-\Delta\Delta C_t}$ method [37].

2.12. Statistical Analysis

Statistical analysis was conducted on SPSS software (ver. 19.0; SPSS Inc., Chicago, IL, USA). Significant differences were analyzed by one-way ANOVA with Duncan's multiple range tests.

3. Results

3.1. Influence of Different Light Qualities on Growth Traits in *C. oleifera*

We observed *C. oleifera* plantlets grown significantly differently after 90 days under different treatments. The results showed that leaf area, leaf number, and stem diameter exposed to blue light were approximately 503.97 mm², 9.07, and 2.56 mm, respectively (Table 1), which were bigger than those plantlets displayed under white light conditions. The average height of plantlets grown under blue light was 11.24 cm, which was similar to those of plantlets exposed to white light. Nonetheless, the plantlet's height exposed to red light was smaller than those exposed to white light. The values of stem diameter exposed to red light were bigger than those plantlets exposed to white light. Plantlets exposed to red light had a similar leaf area compared with those exposed to white light. In addition, the photosynthetic indexes containing the net photosynthetic rate, stomatal conductance, intercellular CO₂, and transpiration rate were all influenced when treated with different light conditions (Table 1). Plantlets grown under blue light conditions had greater values in all photosynthetic indexes. However, the photosynthetic indexes exposed to red light were statistically similar to those exposed to white light.

Table 1. Influence of different light qualities on growth traits in *Camellia oleifera* leaves.

Parameter	White Light	Red Light	Blue Light
Plantlet height (cm)	11.24 ± 2.11 a	10.44 ± 1.51 b	11.24 ± 2.67 a
Stem diameter (mm)	2.32 ± 0.26 b	2.35 ± 0.25 b	2.56 ± 0.41 a
Leaf number	7.85 ± 2.33 c	8.43 ± 3.16 b	9.07 ± 2.79 a
Leaf area (cm ²)	4.12 ± 1.86 b	4.35 ± 1.76 b	5.04 ± 2.39 a
Net photosynthetic rate (Pn/µmol·m ⁻² ·s ⁻¹)	3.14 ± 0.35 b	3.43 ± 0.90 b	4.86 ± 0.64 a
Stomatal conductance (Gs/mmole·m ⁻² ·s ⁻¹)	0.02 ± 0.01 b	0.03 ± 0.02 b	0.05 ± 0.01 a
Intercellular CO ₂ concentration (Ci/µmol·mol ⁻¹)	424.33 ± 37.74 b	422.22 ± 30.88 b	439.00 ± 22.32 a
Transpiration rate (Tr/mmole·m ⁻² ·s ⁻¹)	0.90 ± 0.42 b	1.06 ± 0.66 b	1.47 ± 0.62 a ¹

¹ Data represent mean ± standard deviation; the different letters describe significant differences at $p \leq 0.05$ using Duncan's multiple range test.

C. oleifera plantlets growing under red, blue, and white light conditions all developed complete leaf structures (Figure 1). Under the white light conditions, two layers of palisade mesophyll (PM) were observed and were arranged regularly. Five layers of spongy cells were observed in the spongy parenchyma (SP) with large intercellular spaces. Furthermore, the upper epidermis was thicker than

the lower epidermis. Under the red light conditions, two layers of palisade mesophyll (PM) were observed, with smaller palisade cells compared with white light and were arranged regularly. Six layers of spongy cells were observed in the spongy parenchyma (SP) with medium intercellular spaces. However, under the blue conditions, four layers of palisade mesophyll (PM) were observed, which were positioned compactly and had minimal volumes. The spongy parenchyma (SP) was dispersed irregularly, with almost six layers. The intercellular spaces were the smallest compared with the red and white light conditions. Compared with leaves growing exposed to white light, plantlet leaves growing with the blue light conditions were the thickest.

Figure 1. Influence of different light qualities on the leaf structure of *C. oleifera*. (A) The influence of white light on leaf structure in *C. oleifera*. (B) The influence of red light on leaf structure of *C. oleifera*. (C) The influence of blue light on leaf structure of *C. oleifera*. UE represents the upper epidermis; PM represents the palisade mesophyll; SM represents the spongy mesophyll; IS represents the intercellular spaces; and LE represents the lower epidermis. W, R, and B represent white, red, and blue light conditions, respectively. The bar is 200 µm.

3.2. Influence of Different Light Qualities on Physiological Traits in *C. oleifera*

The chl_a, chl_b, and total chlorophyll content of *C. oleifera* leaf tissues for plantlets grown under red light conditions were greater than those grown under white light or blue light conditions. Moreover, the highest carotenoid content and chlorophyll a/b levels were also observed in plantlets growing under white light conditions; the red light sources produced the second-highest ranking, after that the blue light conditions (Figure 2A). However, the content of SOD was not influenced by different light conditions (Figure 2C). The PPO content of plantlets treated with blue light was higher than those treated with white light or red light (Figure 2B). Nevertheless, the POD content of plantlets treated with blue light was lower than those treated with white light or red light (Figure 2D). In addition, the PPO content of plantlets treated with red light was lower than those treated with white light (Figure 2B).

Figure 2. Responses of physiological processes in *C. oleifera* leaves under different light qualities. (A) Influences of different light qualities on photosynthetic pigment content in *C. oleifera* leaves. (B) Influences of different light qualities on polyphenol oxidase (PPO) content in *C. oleifera* leaves. (C) Influences of different light qualities on superoxide dismutase (SOD) content in *C. oleifera* leaves. (D) Influences of different light qualities on peroxidase (POD) content in *C. oleifera* leaves.

3.3. Influences of Different Light Qualities on the Transcriptome in *C. oleifera* Leaves

3.3.1. Statistics and Quality Assessment of the RNA-seq

For analysis of mRNA expression profiles in *C. oleifera* leaves treated with different light conditions, this study utilized the combination of PacBio SMRT and the Illumina HiSeq platform to obtain entire transcriptome profiles. Sequencing by SMART produced 429,955 reads of inserts, where 406,722 of them were the full-length non-chimeric reads and 18,585 were non-full-length reads (Supplementary Materials Table S1). The average length of the full-length non-chimeric read was 2197 bp. Overall,

sequencing by Illumina generated more than 400 million clean reads (Supplementary Materials Table S2). After removing redundant isoforms using CD-HIT [28], 131,357 non-redundant isoforms were created and all signified exclusive full-length transcripts with an average length of 2555 bp. and N50 of 2897 bp. (Supplementary Materials Table S3). Based on the results of non-redundant isoforms obtained from SMRT sequencing, an isoform sequence index was created through Bowtie 2 [30], and the clean reads from Illumina sequencing were compared using Trinity [31] to obtain unigenes; up to 96.63% of them aligned successfully to the non-redundant isoforms (Supplementary Materials Table S4).

3.3.2. Functional Annotation of Transcripts of *C. oleifera* Leaves under Different Light Conditions

To achieve the highest global annotation, all transcribed sequences from SMRT and Illumina were mapped onto five public databases: NCBI non-redundant protein sequence (NR), Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), an evolutionary genealogy of genes: non-supervised orthologous groups (eggNOG), and Swiss-Prot by BLASTX. The largest of the unigenes (116,818, or 88.93% of the total) was annotated in the NR database. Moreover, we matched a total of 49,360 (37.58%) unigenes to the three sub-categories of GO terms (Supplementary Materials Figure S5). Eventually, a total of 57,611 unigenes were mapped onto different KEGG pathways (Supplementary Materials Figure S6). Additionally, we annotated the unigenes with the eggNOG database, which categorized 115, 341 unigenes (Supplementary Materials Figure S7). Likewise, a total of 96,745 (73.65%) unigenes were aligned to the Swiss-Prot database (Supplementary Materials Table S8). The results of annotation in the five different databases are shown in Supplementary Materials Table S9. The underlying homology with sequences of distinct species were obtained; 17.15% sequences were discovered for *Vitis vinifera*, and 3.97% sequences for *Coffea canephora*. After that sequences were discovered for *Sesamum indicum* (3.91%), *Camellia sinensis* (3.8%), *Theobroma cacao* (3.73%), *Nelumbo nucifera* (3.69%), and *Ziziphus jujuba* (3.06%). Furthermore, 60.68% of sequences were similar to those of other plant species (Supplementary Materials Figure S10).

3.3.3. Differentially Expressed Gene Analysis of *C. oleifera* Leaves under Different Light Conditions

In order to reveal the gene expression patterns in *C. oleifera* leaves under different light qualities, fragments per kilobase of exon per million fragments mapped (FPKM) values were exploited to normalize the sequencing reads. Accordingly, the gene expression was compared under different light conditions. As shown in Figure 3, a wide variety of differentially expressed genes were identified in leaf tissues under different light qualities; 2948 genes were differential expressed in white light (WL) vs. red light (RL), containing 1093 up-regulated genes and 1855 down-regulated genes. Within the WL and blue light (BL) (WL vs. BL), 843 genes were differentially expressed, containing 325 up-regulated genes and 518 down-regulated genes. In the comparison of RL and BL (RL vs. BL), 1222 genes were differentially expressed, containing 846 up-regulated genes and 376 down-regulated genes.

3.3.4. DEGs Venn Diagram Analysis of *C. oleifera* Leaves under Different Light Conditions

The DEGs Venn diagram analysis within WL vs. RL, WL vs. BL, and RL vs. BL identified 19 overlapping genes in all comparisons, which could be controlled by light quality. The Venn diagram analysis further identified 240 DEGs that overlapped in WL vs. RL and WL vs. BL; these genes should be regulated by other wavelengths of light except red and blue. All of these DEGs presented similar expression patterns in WL vs. RL and WL vs. BL. We found that 352-DEGs in RL vs. BL did not overlap with WL vs. RL or WL vs. BL, which are probably regulated by red and blue light simultaneously; 28 of them presented similar expression patterns in WL vs. RL and WL vs. BL, while 138 were up-regulated by RL but down-regulated by BL compared with WL. There were 186 DEGs that were up-regulated by BL but down-regulated by RL compared with WL (Figure 4).

Figure 3. Differentially expressed gene analysis of *C. oleifera* leaves under different light conditions. (A) Differentially expressed genes in WL vs. RL. WL represents leaf tissue growth under white light conditions; RL represents leaf tissue growth under red light conditions. (B) Differentially expressed genes in WL vs. BL. BL represents leaf tissue growth under blue light conditions. (C) Differentially expressed genes in RL vs. BL. In these plots, $\log_2(\text{Fold Change})$ were plotted against the $-\log_{10}(p\text{-value})$. Genes with $p\text{-value} < 0.05$ and fold changes above 1.0 were considered to be upregulated genes, which are shown in red. Genes with $p\text{-value} < 0.05$ and fold changes below -1.0 were considered to be downregulated genes, which are shown in blue. These genes in grey did not show differential expressions.

Figure 4. Differentially expressed genes (DEGs) Venn diagram analysis of *C. oleifera* leaves under different light conditions.

3.3.5. GO Analysis of DEGs in *C. oleifera* Leaves under Different Light Conditions

The GO analysis identified 229 (WL vs. RL), 160 (WL vs. BL), and 172 (RL vs. BL) DEGs that were enriched in “cellular components” (Supplementary Materials Table S11). In WL vs. RL, the DEGs were mostly enriched in the “amyloplast (GO:0009501)”, the “extracellular region (GO:0005576)”, and a “integral component of membrane (GO:0016021)”. In WL vs. BL, the DEGs were principally enriched in the “membrane (GO:0016020)”. In RL vs. BL, the DEGs were primarily enriched in the “membrane part (GO:0044425)” and “actin cytoskeleton (GO:0015629)” (Table 2).

The analysis identified 715 (WL vs. RL), 420 (WL vs. BL), and 436 (RL vs. BL) DEGs that were enriched in “molecular function” (Supplementary Materials Table S11). In WL vs. RL, the DEGs were largely enriched in “oxidoreductase activity (GO:0016491)”, “catalytic activity (GO:0003824)”, and “naringenin–chalcone synthase activity (GO:0016210)”. In WL vs. BL, the DEGs were chiefly enriched in “dioxygenase activity (GO:0051213)”, and “oxidoreductase activity (GO:0016491)”. In RL vs. BL, the DEGs were primarily enriched in “oxidoreductase activity (GO:0016491)”, “transition metal ion binding (GO:0046914)”, and “metal ion binding (GO:0046872)” (Table 3).

Additionally, the “biological process” category included 1543 (WL vs. RL), 820 (WL vs. BL), and 1059 (RL vs. BL) DEGs (Supplementary Materials Table S11). In WL vs. RL, the DEGs were primarily enriched in the “oxidation–reduction process (GO:0055114)” and “starch biosynthetic process (GO:0019252)”. In WL vs. BL, the DEGs were primarily enriched in the “fatty acid metabolic process (GO:0006631)”. In RL vs. BL, the DEGs were chiefly enriched in the “oxidation–reduction process (GO:0055114)” and “lipid biosynthetic process (GO:0008610)” (Table 4).

Table 2. Cellular component enrichment analyses for DEGs between different light qualities.

GO Domain	GO Terms ID	Gene Ontology (GO) Term	<i>p</i> -Value	(False Discovery Rate) FDR	Up-Regulated DEGs	Down-Regulated DEGs	Total DEGs
Cellular component		WL vs. RL					
	GO:0009501	amyloplast	5.5×10^{-10}	3.4×10^{-8}	1	12	13
	GO:0005576	extracellular region	7.1×10^{-7}	2×10^{-5}	8	24	32
	GO:0016021	integral component of membrane	6.9×10^{-6}	0.00015	93	263	356
		WL vs. BL					
	GO:0016020	membrane	0.0026	0.07429	53	92	145
		RL vs. BL					
	GO:0044425	membrane part	0.00032	0.00503	131	46	177
	GO:0015629	actin cytoskeleton	0.01073	0.08725	4	0	4

Table 3. Molecular function enrichment analyses for DEGs between different light qualities.

GO Domain	GO Terms ID	Gene Ontology Term	<i>p</i> -Value	FDR	Up-Regulated DEGs	Down-Regulated DEGs	Total DEGs
Molecular function		WL vs. RL					
	GO:0016491	oxidoreductase activity	5.50×10^{-25}	6.84×10^{-22}	48	244	292
	GO:0003824	catalytic activity	1.90×10^{-23}	1.58×10^{-20}	222	842	1064
	GO:0016210	naringenin–chalcone synthase activity	1.3×10^{-18}	6.47×10^{-16}	0	14	14
		WL vs. BL					
	GO:0051213	dioxygenase activity	2.8×10^{-18}	9.8×10^{-16}	18	7	25
	GO:0016491	oxidoreductase activity	3.4×10^{-8}	3.97×10^{-6}	31	47	78
		RL vs. BL					
	GO:0016491	oxidoreductase activity	1.5×10^{-15}	1.25×10^{-12}	124	15	139
	GO:0046914	transition metal ion binding	8.3×10^{-12}	1.73×10^{-9}	83	13	96
	GO:0046872	metal ion binding	2×10^{-10}	2.78×10^{-8}	137	22	159

Table 4. Biological process enrichment analyses for DEGs between different light qualities.

GO Domain	GO Terms ID	Gene Ontology Term	<i>p</i> -Value	FDR	Up-Regulated DEGs	Down-Regulated DEGs	Total DEGs
Biological process	WL vs. RL						
	GO:0055114	oxidation–reduction process	$<1 \times 10^{-30}$	2.49×10^{-27}	36	252	288
	GO:0019252	starch biosynthetic process	7.4×10^{-16}	3.1×10^{-13}	1	20	21
	WL vs. BL						
	GO:0006631	fatty acid metabolic process	5×10^{-11}	1×10^{-8}	16	4	20
	RL vs. BL						
	GO:0055114	oxidation–reduction process	2.40×10^{-22}	4×10^{-19}	124	11	135
	GO:0008610	lipid biosynthetic process	2.4×10^{-12}	5.95×10^{-10}	36	3	39

3.3.6. KEGG Pathway Analysis of DEGs in *C. oleifera* Leaves under Different Light Conditions

Pathway analyses can promote the understanding of biological mechanisms driven by diverse genes. In this research, a KEGG pathway enrichment analysis was conducted on each comparison. In WL vs. RL, 1308 DEGs were annotated and involved 125 pathways, and the top twenty significantly enriched pathways of DEGs are presented in Figure 5, including “galactose metabolism”, “starch and sucrose metabolism”, “circadian rhythm-plant”, “glycolysis/gluconeogenesis”, and “flavonoid biosynthesis”. In WL vs. BL, 289 DEGs were annotated and involved 79 pathways, and the top twenty significantly enriched pathways of DEGs are presented in Figure 6, containing “linoleic acid metabolism”, “alpha-linolenic acid metabolism”, “sesquiterpenoid and triterpenoid biosynthesis”, “arginine biosynthesis”, and “arginine and proline metabolism”. In RL vs. BL, 556 DEGs were annotated and involved 86 pathways, and the top twenty significantly enriched pathways of DEGs are presented in Figure 7, including “galactose metabolism”, “circadian rhythm-plant”, “alpha-linolenic acid metabolism”, “amino sugar and nucleotide sugar metabolism”, and “flavonoid biosynthesis”.

Figure 5. DEG analysis by the KEGG pathway of *C. oleifera* leaves under WL vs. RL.

Figure 6. DEG analysis by the KEGG pathway of *C. oleifera* leaves under WL vs. BL.

Figure 7. DEG analysis by the KEGG pathway of *C. oleifera* leaves under RL vs. BL.

3.3.7. qRT-PCR Analysis to Validate the RNA-seq Data

To validate the data authority of the *C. oleifera* leaf transcriptome, ten unigenes were selected to examine the expression level through a real-time quantitative PCR assay. The unigene c48652_g1 was preferred as the reference gene. The information of primers designed for this qRT-PCR assay was listed in Supplementary Materials Table S12. In total, the data from qRT-PCR and RNA-seq displayed in Figure 8 had a similar trend of gene expression, which confirmed that RNA-seq results are reliable.

Figure 8. The expression results of ten randomly selected DEGs from RNA-seq and qRT-PCR in *C. oleifera* leaves under three light conditions. The y-axis on the left indicates the expression levels based on the RNA-seq data with Log₂(FPKM) (histogram), whereas the y-axis on the right indicates the relative expression levels determined by qRT-PCR with fold-changes (black line chart). The x-axis indicates the light treatments. Data shown in mean value $\pm$ standard deviation (SD).

4. Discussion

Light quality considerably influences plant phenotypes by altering numerous biological processes. Nonetheless, as an essential woody oil tree species, the literature on the molecular mechanism of growth of *C. oleifera* under different light qualities has not been reported. Therefore, we attempted to explore how different wavelengths of light affect *C. oleifera* growth at the physiological and transcriptome levels. Growth and physiological activities were observed and measured under different light treatments. In addition, both second-generation sequencing and third-generation sequencing technologies were employed to analyze the changes of the transcriptional profiles under different light conditions. Our transcriptome analysis contributes to understanding the molecular mechanisms for *C. oleifera* growth under different qualities of light.

4.1. Influence of Different Light Qualities on Growth and Physiological Processes in *C. oleifera*

In this research, the plantlets growing under blue light conditions displayed superior growth performance compared with those growing under white light and red light conditions, including increased leaf number and leaf area, greater photosynthetic capacity, and the increased promotion of chloroplast development (Table 1 and Figure 1). Leaf morphological characteristics were reported to be greatly affected by different wavelengths of light [38,39]. In this report, the greatest leaf area was found under blue light conditions (Table 1), implying that blue light promotes *C. oleifera* plantlet leaf growth. Previous studies have shown that blue light could promote increased leaf area in chrysanthemums [40], tomatoes [41], and *Platycodon grandiflorum* [42], while leaf area was scaled down under red light. Prior research indicated that distinct wavelengths of light led to the varieties of leaf area, which was triggered by changing the abundance of tubulin and dynein [12]. Additionally, gene expressions of actin, tubulin, and XET (xyloglucan endotransglycosylase) were significantly induced in grape berries when gibberellic acid (GA) was added [43].

Moreover, actin and tubulin were found participating in the promotion of cell wall relaxation and expansion [43]. In addition, XET considerably affected cell augmentation by inducing hemicellulose modification [43]. In this research, the gene expression levels of actin, dynein, and tubulin were higher under blue light conditions than those under white light or red light conditions (Figure 9A–C). However, the gene expression of XET/H (xyloglucan endotransglucosylase/hydrolase) was the highest under the red light conditions (Figure 9D). Furthermore, most leaf development-related genes, such

as defectively organized tributaries 3 (DOT3) and ADP ribosylation factor 5 (ARF5) were expressed higher under blue light conditions compared with red, but lower than white (Figure 9E). Previous research demonstrated that blue light enhanced the activities of photosynthesis in potatoes [2].

Figure 9. Expression profile of some genes in *C. oleifera* leaves under different light conditions. (A) Actin. (B) Dynein. (C) Tubulin. (D) Xyloglucan endotransglucosylase/hydrolase. (E) Leaf development-related gene. c88499_g1 represents an uncharacterized protein; c120938_g1 and c95729_g1 represent BTB/POZ domain-containing protein DOT3; c92016_g1 represents ARF5; c53158_g1 represents SRK2E; c18038_g1 represents an uncharacterized protein. (F) Chlorophyll biosynthetic process. c17633_g1, c10489_g1 and c77506_g1 represent magnesium–chelataze subunit ChlH; c61157_g1 represent geranylgeranyl diphosphate reductase; c116719_g1 and c2124_g1 represent glutamyl–tRNA reductase 1. (G) Polyphenol oxidase. (H) Peroxidase.

In this study, blue light stimulated the development of chloroplasts (Figure 1). Transcriptome data demonstrated that the expression level of genes related to the chlorophyll biosynthetic process was higher under the white light conditions when compared with red or blue. However, the microscope results supported that blue light promoted chloroplast development, which revealed that chloroplast development in *C. oleifera* is a complicated process and needs further study. In total, the expression profiles of these genes could be the reason for the greatest performance of *C. oleifera* plantlets occurring under blue light conditions.

Previous research demonstrated that different light qualities can also influence plant stress resistance by up-regulating the activities of enzymes, such as SOD and POD [44]. In our research, red light increased POD activity and blue light improved PPO activity, which is crucial for oil trees to enhance light stress tolerance. (Figure 9G,H).

4.2. Influences of Different Light Qualities on Plant Circadian Rhythm Pathway and Hormone Signal Transduction Pathway in *C. oleifera* Leaves

Biological activities that cycle in approximately twenty-four-hour intervals are called circadian rhythms [45]. Circadian rhythms allow plants to anticipate environmental cycles and to coordinate their activities with them. In this study, differing light quality affected several genes' expressions of the plant circadian rhythm pathway (Figure 10, Supplementary Materials Figure S13). Phytochrome B (PhyB) functions as a red/far-red photoreceptor participating in the regulation of de-etiolation [46–48]. Constitutively photomorphogenic 1 (COP1) is an E3 ligase mediating the degradation of numerous transcription factors, including long hypocotyl 5 (HY5) [49–55].

Figure 10. Influences of the plant circadian rhythm pathway under WL vs. RL in *C. oleifera* leaves. The red color represents up-regulated unigenes and the green color represents down-regulated unigenes.

In this research, unigenes of PhyB, COP1, and HY5 in *C. oleifera* leaves were down-regulated under red light conditions, indicating that these genes are involved in different wavelengths of light responses in *C. oleifera* (Figure 10). Recently, research indicated that HY5 not only directly affects the transcription of light-induced genes and is functionally important in the photomorphogenesis (by acts downstream of the light receptor network) but is also engaged in the signaling of light and hormones through promotion of the expression of negative regulators of auxin signaling [56–58].

Recent research showed that HY5 also acts as a shoot-to-root mobile transcription factor response to light fluctuations [59]. After transport from shoot to root, HY5 could enhance the expression level of HYH in the root [60]. It was also found that HY5 and HYH are involved in regulating the development of lateral root and gravitropism [61,62]. In this study, the HY5 gene in *C. oleifera* leaves under red light conditions showed down-regulation compared to white light (Figure 10). The reason could be that HY5 is transported from leaf to root to regulate lateral root development under red light conditions in *C. oleifera*.

Plant hormones and light quality usually cooperate to affect plant biological processes [63]. Usually, plant hormones influence light responsiveness via second messengers [64]. On the other hand, in the processes of light regulation, hormonal pathways are typically controlled by different light environments to influence plant development. Coordination between light signaling and auxin pathways have been confirmed, with phytochrome A phosphorylating auxin/indole-3-acetic acid (AUX/IAA) protein [65].

In our research, the expression genes of AUX/IAA, Gretchen Hagen3 (GH3), and small auxin-up RNA (SAUR) in *C. oleifera* leaves were up-regulated under red light conditions (Figure 11), indicating that red light could promote auxin signal transduction in *C. oleifera*. Cytokinins are involved in cell division regulation [66], especially stimulating the growth of axillary buds [67]. In the present study, regarding cytokinin metabolism, red light down-regulated the gene expression of cytokinin response 1 (CRE1) (Figure 11). Nevertheless, the gene expression of *Arabidopsis* homologs of histidine-containing phosphotransmitters (AHP) in leaves was found to be down-regulated under blue light conditions (Supplementary Materials Figure S14).

Figure 11. Influences of plant hormone signal transduction pathways under WL vs. RL in *C. oleifera* leaves. The red color represents up-regulated unigenes; the green color represents down-regulated unigenes; and the purple color represents containing both up-regulated unigenes and down-regulated unigenes.

Gibberellic acid (GA) and abscisic acid (ABA) have an antagonistic role in plant development and environmental responses. ABA inhibits plant growth while GAs enhances it. Moreover, recent research showed that ABA was induced under low red:far-red light environments to represses bud growth,

except for the terminal bud [68]. In our research, as an ABA signaling negative regulator [69], protein phosphatase (PP2C) was down-regulated in *C. oleifera* leaves under red light conditions (Figure 11). Apart from what is mentioned above, blue light and red light also affect other hormones' (ET, BR, JA) signal transduction (Figure 11, Supplementary Materials Figure S14). In total, these genes are involved in multiple hormonal signal transduction pathways and may play an important role in mediating regulation of *C. oleifera* leaf growth in response to different wavelengths of light.

4.3. Effects of Blue Light on Flavonoid Biosynthesis

Different light qualities typically change the content of secondary metabolites to affect plant developmental processes [70]. Chalcone synthase (CHS) is a catalytic enzyme that converts the 4-coumaroyl-CoA to tetrahydroxy chalcone [71]. Chalcone isomerase (CHI), naringenin 3-dioxygenase (F3H), flavonol synthase (FLS), and bifunctional dihydroflavonol 4-reductase/flavanone 4-reductase (DFR) are important enzymes in the pathway of flavonoid biosynthesis, which converts the tetrahydroxy chalcone into different flavonoid molecules. In the present study, CHS, CHI, F3H, FLS, flavonoid 3'-monooxygenase (CYP75B1), DFR, and anthocyanidin reductase (ANR) were up-regulated in *C. oleifera* leaves under blue light conditions compared with red light conditions (Figure 12), indicating that blue light could promote flavonoid biosynthesis through up-regulation of the flavonoid-biosynthesis-related genes in *C. oleifera* leaves.

Figure 12. Flavonoid biosynthesis in *C. oleifera* leaves under different light conditions. The red color represents up-regulated unigenes under blue light conditions compared with red light; the green color represents down-regulated unigenes under blue light conditions compared with red light.

5. Conclusions

Growth and physiological characteristics of *C. oleifera* “MIN 43” cultivar plantlets under white, red, and blue light conditions were analyzed, and transcriptome analysis was carried out, combining Illumina and single-molecule real-time RNA-sequencing. The growth and physiological parameter

changes indicated that blue light stimulated enhancement of the leaf area, increased the leaf number, improved chlorophyll synthesis, and increased photosynthesis. The transcriptome profiling identified a great amount of differentially expressed genes in *C. oleifera* leaves under different light conditions. Pathway analysis indicated that plant circadian rhythm and hormone signal transduction were associated with different light quality responses in *C. oleifera* leaves. The genes of PhyB, COP1, HY5, AUX/IAA, GH3, and SAUR were considered to play an important role in different light quality responses and it will be interesting to further study these genes' functions in *C. oleifera*. In addition, blue light significantly promotes flavonoid biosynthesis by changing the related genes expressed.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/11/1/91/s1>, Table S1: Transcript classification statistics, Table S2: Illumina data filtering statistics, Table S3: Non-redundant isoform statistics, Table S4: RNA Sequence map statistics, Figure S5: GO annotation, Figure S6: KEGG annotation, Figure S7: eggNOG annotation, Table S8: Swissprot annotation, Table S9: The results of annotation in five different databases, Figure S10: NR_species_distribution, Table S11: GO enrichment analyses for DEGs between different light qualities, Table S12: Primers used for qRT-PCR validation, Figure S13: Influences of plant circadian rhythm pathway under WL vs. BL in *C. oleifera* leaves, Figure S14: Influences of plant hormone signal transduction pathway under WL vs. BL in *C. oleifera* leaves.

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