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Full Length Article

Different Light Qualities Modify Morphology and Expression of *CsLHY* in *In Vitro* Plantlets of *Camillia sinensis*

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Abstract

High yield is one of the core standards of elite tea cultivars. LHY, a key component of the circadian clock, controls various biomass metabolic and developmental processes; and light is a crucial environmental factor that regulates biomass metabolism and modulates plant development and growth. Therefore, in the present study, we evaluated the effect of different light qualities via light-emitting diode (LED) on the morphogenesis and expression of *CsLHY* by qRT-PCR in *in vitro* tea plantlets. The findings suggested that the red light had an effect on bud outgrowth, blue light stimulated the content of carotenoids and significantly higher expression level of *CsLHY*, and far-red light dramatically suppressed the expression of *CsLHY* and lead to the elongation of internode and small leaf areas. The tentative correlations between the phenotypic responses and expression of *CsLHY* under different light qualities were discussed, which involved multiple light signaling pathways including phytochrome or cryptochrome, and auxin signaling. Besides the novel discoveries on the connections between light quality and the growth of *in vitro* tea plantlets, the experimental approach in this study could serve as a practical tool for induced and directed growth of tea plantlets and other in-depth study of gene function upon such light treatment. © 2018 Friends Science Publishers

Keywords: Light treatment; *CsLHY*; qRT-PCR; Circadian rhythms; Gene expression; Phytohormone

Introduction

The tea plant (*Camellia sinensis* (L.) O. Kuntze), originated in the southwestern region of China, is an important commercial crop consumed worldwide as a healthy beverage made from the processed leaves. High yield is one of the core standards of elite tea cultivars. Studies showed that tea yield correlated with many traits such as tree vigor, photosynthetic efficiency and chlorophyll content, etc. (Chen *et al*., 2013). Many genes in the secondary metabolic pathways controlling plant development and growth, resistance and tea quality have been isolated and expressed (Li *et al*., 2004; Chen *et al*., 2006). Moreover, the Expressed Sequence Tag (EST) database has been constructed through sequencing of cDNA clones from different tissues of tea plants. The blasting and assembling results indicated that 45% of these expressed sequences were full or partial sequences of novel genes (Chen *et al*., 2005). The identified functional genes were mainly in the biosynthetic pathways of biochemical components such as caffeine, tea polyphenols, amino acids, and volatile compounds which are determinant factors for cup-tea quality. However, since tea yield is influenced by regulatory networks, the molecular mechanisms underlying high yield are absent.

Studies of yield Quantitative Trait Loci (QTLs) have identified changes in regulatory networks that are involved in light and hormone signaling (Wijnen and Young, 2006; Chen, 2013). Many of these biological pathways are modulated by circadian rhythms, which are controlled by an internal circadian clock (McClung, 2006; Harmer, 2009; Nagel and Kay, 2012). The period length of this clock is approximately 24 h. The molecular basis of circadian rhythms has been intensively studied in *Drosophila*, *Neurospora*, mice, cyanobacteria, and *Arabidopsis.* Although circadian clock components are variant across the organisms, a conserved central oscillator based on feedback loops that modulate transcription has been proposed to generate the 24 h periodicity of circadian rhythms (Dunlap, 1999). Moreover, studies found that the diurnal expression of at least 30% of the transcriptome were regulated by the circadian rhythms, including molecules that were involved in photosynthesis and biomass metabolism (Michael *et al.*, 2008a, b). *LHY* (*LATE ELONGATED HYPOCOTYL*), a key component of the central oscillator, encodes highly conserved single-MYB transcriptional factors which functionally disrupt the normal circadian rhythms of the clock when expressed at high level (Huettner *et al.*, 2000). It is noteworthy that *LHY* is acutely induced by light: its transcription level displays a rapid increase reaching the sharp peak 1 h after light treatment. This is followed by a rapid decrease that leads to the lowest transcript level by 6-8 h after light irradiation (Wang *et al.*, 1997).

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Light is necessary for plant life, which leads to an array of receptors being developed in plants to monitor the light condition. A number of studies showed plants have evolved a number of cryptochrome and phytochrome photoreceptors, which not only maintain circadian clock, but also regulate massive biomass metabolic and developmental processes (Somers *et al.*, 1998; Chen *et al.*, 2004; Spalding and Folta, 2005; McWatters and Devlin, 2011). The effect of light on clock resetting typically depends on wavelength and intensity, which further evidenced the importance of light quality in circadian clock function. Light intensity can modify the stomatal index (Thomas *et al.*, 2004; Jiang *et al.*, 2011), while light quality can influence the stomatal density and index (Shimazaki *et al.*, 2007; Boccalandro *et al.*, 2009). In all, light is a crucial environmental factor that regulates biomass metabolism and modulates plant development and growth. In our knowledge, no previous study was performed to profile *CsLHY* in tea plant. Therefore, in the present study, we investigated the effect of different light qualities on morphological changes and expression of *CsLHY* in *in vitro* tea plantlets, which could be the first-hand knowledge for further study of *CsLHY* gene function.

Materials and Methods

Plant Material and Culture Conditions

A tea variety "Shuyong 1" was selected to measure the *CsLHY* rhythm, which was planted in the germplasm repository of Horticulture and Landscape College, Southwest University, Beibei, Chongqing, China in 2013. Fifteen fully expanded and hardened leaves were collected every three hours for up to 72 h in September 2016 from the most recent flush. Three biological replicates were designed, and five leaves were pooled as one sample.

To reduce environmental and genetic variation, samples for light treatments were conducted on *in vitro* tea plantlets of "Shuyong 1", which were established using axillary buds. Axillary buds were washed under running tap water first, followed by soaking in water with a few drops of detergent for 30-60 min and again rinsed under running tap water. Subsequently, they were transferred to a clean bench for further sterilization. Decontamination was performed by immersion in 75% ethanol for 15 sec, rinsing in deionized sterile water, followed by 5 min sterilization with $HgCl_2$ solution (0.1%, w/v), and finally, rinsed thoroughly using sterile distilled water.

The whole in vitro propagation was divided into 3 stages including adventitious bud induction and multiplication, adventitious bud growth and strong plantlet culture. The basal medium for experiments was a modified MS medium (modified 1/2MS for strong plantlet culture stage only) including 0.7% (w/v) agar, 30 g L^{-1} sucrose (20 g L^{-1} in strong seedling culture). For adventitious bud induction and multiplication, the sterilized explants were inoculated in basal MS medium containing 2.0 mg L^{-1} 6-benzylaminopurine, 0.2 mg L^{-1} 1-naphthaleneacetic acid. The induced adventitious buds were separated and transferred to basal MS medium with 1.0 mg L^{-1} 6-benzylaminopurine and 0.5 mg L^{-1} 1-naphthaleneacetic acid. When shoots reached about 3 cm in length, they were transferred to 1/2 basal MS medium with 0.2% activated charcoal (AC), 20 g sucrose, 0.5mg L^{-1}6-benzylaminopurine (6-BA) and 0.5 mg L^{-1} Indole-3-butytric acid (IBA) for strong seedling culture. The pH of all media were adjusted to 6.0 ± 0.2 using 1 mol L^{-1} NaOH or HCl before autoclave at 121ºC for 20 min. The culture room was exposed to artificial white light from fluorescent lamps with light intensity 30-40 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The photoperiod was 12 h a day, and its temperature was 25 ± 2ºC.

Light Treatments

After strong plantlet culture for 3 weeks, the tea plantlets were transplanted to 4 different light conditions irradiated from LED: 1. WLED, white light; 2. BLED, blue light; 3. RLED, red light; and 4. FRLED, far-red light. The LED lights were obtained from Philips Inc. (Eindhoven, the Netherlands). Light treatment chambers were separated with curtains. The reference treatment WLED was used to compare with the plantlets variants from the other three light treatments. The light intensity in each treatment was 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$. There were 30 plantlets treated in each light condition. The plantlets used for experimental variants were cultured for 6 weeks with a 12 h photoperiod.

Morphological Analysis

Fifteen leaves of the second and third leaf counting from the apex were sampled after 6 weeks of light irradiation, and the average values of the measurements were calculated along with the standard error. Total leaf areas, fresh leaf weights, and stem lengths were measured. The stem diameters were also measured at the center of stem length. Leaf areas were determined with the method described by Bailey and Mahaffee (2017).

Stomatal traits were analyzed following a nail polish print method described by Mott and Michaelson (1991). The stomatal numbers of 15 microscopic fields per leaf were used to calculate the number of stomata per mm^2 as stomatal density. The stomatal index was calculated with the following equation: number of stomata/(number of epidermal cells + number of stomata) × 100 (Zheng and Labeke, 2017).

The accumulation of chlorophylls a, b and carotenoids was analyzed following the Lichtenthaler and Buschmann protocol (2001). Briefly, Fifty milligrams fresh leaf was triturated and extracted with 80% acetone and protected from light overnight at -20ºC. The absorbance of the samples was evaluated with a spectrophotometer (Infinite 200, Tecan Group Ltd., Switzerland) at wavelengths of 470, 646 and 663 nm. The accumulation levels of pigment were

calculated as Chl a = 12.25 × A663 − 2.79 × A645; Chl b = 21.50 × A645 – 5.10 × A663, and Carotenoids = (1000 × A470 – 1.82 × Chl a− 85.02 × Chl b)/198.

Quantitative RT-PCR Analysis

To investigate the relative transcript levels of *CsLHY* under different light treatments, the second and third leaves from the apex was selected for genetic analysis. After growing for 6 weeks under the light treatments, fifteen leaves were sampled at 9:00 am. Three biological replicates were designed for each treatment, and five leaves were pooled as one sample. Samples were immediately freezed in liquid nitrogen and ground into fine powder. Total RNA was extracted with RNA simple Total RNA Kit (Tiangen, Beijing, China). First-strand cDNA synthesis were performed using 1.0 µg of total RNA as template for reverse transcriptions. qRT-PCR was conducted for transcript analyses with the synthesized cDNA using SYBR Green method. The 20 µL of qRT-PCR reaction mix included 10 µL of 2 × SYBR Premix Ex TaqTM, 0.4 µL of 50 × ROX Reference Dye, 0.5 µL of forward primer CsLHYqF (CGTCAAGTGATATGCAAGCCAC) and reverse primer CsLHYqR (GAGTGGAAGATCTTGGCGCATT), and 1 µL of diluted cDNA template. The qRT-PCR reaction program was: denaturation at 95ºC for 15 s, 60ºC for 15 s, and 72ºC for 30 sec. The melting curve was used to confirm the specific PCR amplification. The *β-actin* (HQ420251) was used as internal control by the primers CsactinF (CTAACAGAGGCACCACTCAAC) and CsactinR (TCACCCGAATCCAGCACAATA). The qRT-PCR assays were run on an ABI Prism 7300 Sequence Detection System (Applied Biosystems) according to the protocol of the SYBR Premix Ex Taq Kit (TaKaRa). Transcript levels of each gene were estimated from the Ct (cycle threshold) value, and normalized with *β-actin* gene. The relative expression differences of *CsLHY* were calculated by $2^{-\Delta\Delta ct}$ formula (Lu *et al.*, 2008).

Statistical Analysis

The samples from individual plant were analyzed in triplicates and averaged. All data were presented as the means ± SE. Statistical analyses were conducted using the SPSS Statistics v19.0 (SPSS Inc. Chicago, IL, USA). Significant differences were analyzed by one-way of variance (ANOVA) followed by Duncan's multiple-range test ($P \leq 0.05$).

Results

Effects of Different Light Treatments on the Morphology of *in vitro* Tea Plantlets

Plantlets selected for light treatments were healthy and uniformed. Significant differences were observed in the morphology of plantlets under different light conditions (Fig. 1). Plantlets grown under white light had strong stems and developed large leaves, plantlets grown under red tended to have new flush, and plantlets grown under blue light with pale leaves, while plantlets under far-red light had relative small leaves and elongated stems. Plantlets under white light were strong with high scores in all measured traits. By contrast, plantlets grown under far-red light condition resulted in a decrease in all measured traits (Fig. 2).

The total pigment content was different among the plantlets under different light treatments (Fig. 3). The plantlets grown under blue light had the highest total chlorophyll concentration ranged from 4.50 to 4.96 mg g^{-1}, while it was 3.02 to 3.15 and 2.73 to 2.81 mg g^{-1} in plantlets grown under white and far-red light, respectively. Plantlets grown under the red light condition had the lowest total chlorophyll content ranged from 1.65 to 1.72 mg g^{-1}. The carotenoids were higher in plantlets grown under blue light (5.69 to 5.87 mg g^{-1}) and white light (3.76 to 3.87 mg g^{-1}). Plantlets grown under red and far-red light condition had similar and lower amount of carotenoid content ranged from 2.41 to 2.89 mg g^{-1}. The lowest *Chl a*, *Chl b*, and highest *Chl a/b* was found in plantlets grown under red light condition, while no significant effect on plantlets grown under white, blue and far-red lights in chlorophyll content was observed. Moreover, stomatal density and index were dramatically affected by different light qualities (Table 1). Plantlets grown under white light condition showed the highest stomatal density and index. In plantlets grown under far-red light condition stomatal density decreased though index was not affected. Both plantlets grown under blue and red lights condition showed the lowest stomatal density; and the stomatal index was lowest under blue light condition.

Expression of *CsLHY* in *in vitro* Tea Plantlets under Different Light Treatment

The full-length cDNA of *CsLHY* was amplified and analyzed extensively in our lab (not published). In brief, the ORF of *CsLHY* was a 2301 bp contiguous sequence and the deduced protein contains 766 amino acid residues, which was submitted to the NCBI with GenBank ID: KX470707. To monitor the expression pattern of *CsLHY*, we used qPCR to analyse mRNA abundance. To determine the rhythmic cycling of transcript levels of clock gene *CsLHY* in leaves of tea plantlets, we examined the transcript levels of *CsLHY* every 3 h over a 72 h period under natural light condition (approximately 12:12 L:D). As shown in Fig. 4, the transcript levels of *CsLHY* were expressed rhythmically, with relatively sharp peak levels occurring in the morning around 9:00 am. The expression of *CsLHY* began to dramatically decrease after noon, finally reaching its lowest level at approximately 12 h after dawn. The expression levels of *CsLHY* increased again in the following night.

Table 1: Effects of different light qualities on the stomatal characteristics of *in vitro* tea plantlets

Light quality	Stomatal density (N mm^{-2})	Stomatal index (%)
White	104.56±1.25a	11.51±1.01a
Red	35.44±1.22c	5.78±1.05b
Blue	34.74±2.16c	3.01±0.72c
Far-red	81.05±0.82b	10.79±1.24a

Different letters indicate significant differences between values using one-way ANOVA followed by Duncan's multiple-range test at the 5% level ($p < 0.05$) for each parameter. Data given as means ± SE (n = 5)

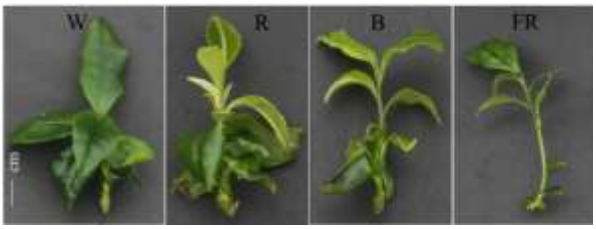

Fig. 1: Effect of different light treatments on the morphology of *in vitro* tea plantlets. White LEDs (W), red LEDs (R), blue LEDs (B), far-red LEDs (FR). Scale bar indicates 1 cm. All the samples were collected 6 weeks after light treatment

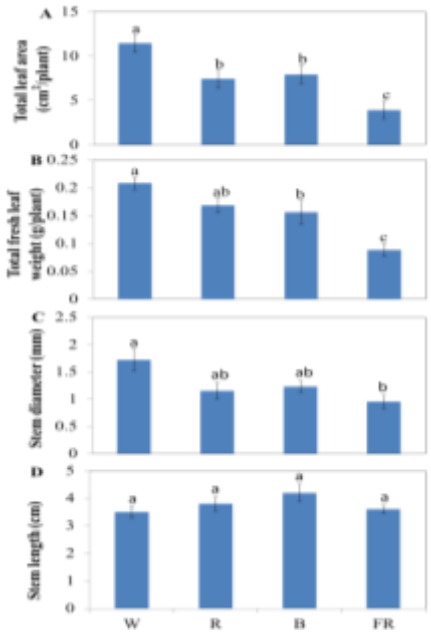

Fig. 2: Morphological analysis of *in vitro* tea plantlets under various light qualities. White LEDs (W), red LEDs (R), blue LEDs (B), far-red LEDs (FR). Each bar represents the mean. Error bars represent the standard deviation of the mean (n = 5). Different letters indicate significant differences ($P<0.05$)

To determine whether the expression level of *CsLHY* was affected by different light qualities, *in vitro* tea plantlets were transferred to red, blue and far-red LED light exposure with similar 12:12 L:D cycle for 6 weeks. Leaves of the light treated plantlets were collected at 9:00 am, and the *CsLHY* transcript levels were examined (Fig. 5). The expression of *CsLHY* was significantly influenced by different light quality. Compared to the white light condition, the expression of *CsLHY* was significantly enhanced under the blue and red light condition. Transcriptions of *CsLHY* under the blue and red light condition were 15.4 and 2.7 times greater than that under the white light condition. By contrast, the expression of *CsLHY* was dramatically depressed under the far-red light condition. Transcription level of *CsLHY* was about 28 times lower compared to that under the white light condition.

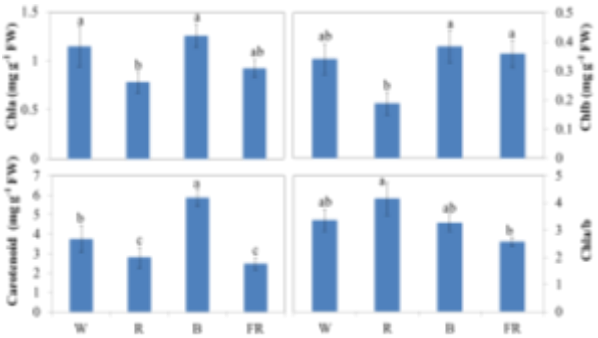

Fig. 3: Effects of light quality on chlorophyll a and b (A, B) and carotenoid (C) content and *Chl a/b* ratio (D) of *in vitro* tea plantlets. Data are presented as means ± standard error (n = 5). Different letters indicate significant differences between values using one-way ANOVA followed by Duncan's multiple-range test at the 5% level ($P < 0.05$). White LEDs (W), red LEDs (R), blue LEDs (B), far-red LEDs (FR)

Discussion

Effects of red LED on *in vitro* Tea Plantlets and Expression of *CsLHY* Gene

The *in vitro* tea plantlets under the red light condition grew new flush (Fig. 1). Previous researches have proved that bud outgrowth is under the control of a well-established hormonal network involving the biosynthesis and signaling of hormones strigolactones (SLs), cytokinins (CKs), and auxin. SLs and Auxin prevent branching, while CKs promote it (Domagalska and Leyser, 2011). In *Arabidopsis* buds grown *in vitro*, bud outgrowth was more sensitive to auxin in phytochrome B (phyB) mutant than in wild type. In addition, several auxin-responsive genes were more expressed in phyB than in the wild type, attesting that auxin signaling was elevated in phyB mutant compared to the wild type. This suggests that phyB suppressed auxin signaling to promote branching (Reddy and Finlayson, 2014). Study with *Arabidopsis* suggested phyB functioned as a main photoreceptor in regulation of hypocotyl length under long-term red light irradiation (Tepperman *et al.*, 2006).

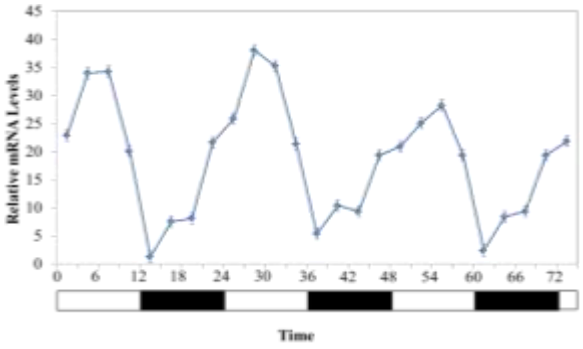

Fig. 4: The oscillation of *CsLHY* gene expression levels in tea plant under white light 12:12 L: D condition

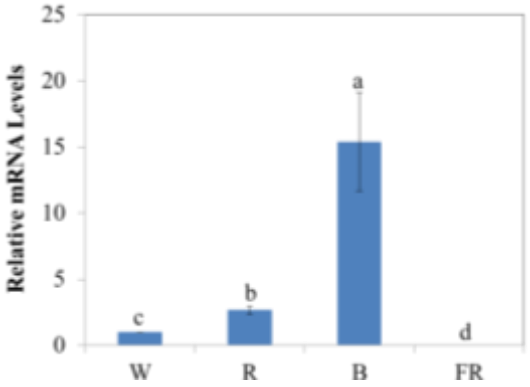

Fig. 5: Differential expression of the *LHY* gene in leaves of tea plantlets under various light qualities. Different letters indicate significant differences between values using one-way ANOVA followed by Duncan's multiple-range test at the 5% level ($P < 0.05$).White LEDs (W), red LEDs (R), blue LEDs (B), far-red LEDs (FR)

Moreover, another experiment with mutants of *Arabidopsis* found that the *LHY-TOC1*-modulated red light signal transduction is principally dependent on the phyB photoreceptor (Ito *et al.*, 2007). Taken together, these findings might indicated a regulation pattern for circadian clock-associated red light signaling, in which *LHY* negatively regulates *TOC1* (*PRR1*) in the upstream, in turn, *TOC1* functions as a positive effector of phyB in the downstream that serves as a negative regulator of auxin metabolism. Therefore, in present study, we can presume that constant red light treatment stimulated the expression of *CsLHY*, which suppressed the accumulation of auxin by phyB, lead to the bud outgrowth and new flush development.

Effects of Blue LED on Different *in vitro* Tea Plants and Expression of *CsLHY* Gene

Plantlets grown under blue light has the highest total chlorophyll and carotenoid content, which was consistent with previous findings of higher production of biomass and photosynthetic capacity under a blue light irradiance (Whitelam and Halliday, 2007; Lee *et al.*, 2014). Studies have found that pure blue light stimulated cryptochromes, phototropins, and also phytochromes, which should be the underlying mechanisms of the effect of blue light on plant development (Whitelam and Halliday, 2007). Moreover, it has been suggested that zeaxanthin acting as a blue-light receptor regulated blue light-dependent stomatal opening (Zeiger and Zhu, 1998; Frechilla *et al.*, 1999). Interestingly, in our present study, the total carotenoid of plantlets grown under blue light condition was significantly higher than that of other light treatments, which indicated a tentative genetic linkage between blue light and plant development. Furthermore, study with *Arabidopsis* suggested photomorphogenes is modulation on blue light was caused by the regulation of auxin signaling. Briefly, blue light stimulated the gene expression pattern of photoreceptors, reducing the expression of phytochrome A, phytochrome D, and crytochrome1. This lead to the upregulation of auxin response factors, which reduced the sensitivity of plant cells to auxin (Pashkovskiy *et al.*, 2016). The expression level of *CsLHY* was significantly higher in the plantlets grown under blue light condition than that of other light treatments (Fig. 3). Together with our discussion in the previous section, we presume that the upregulation of *CsLHY* was linked to the mainstream of auxin signaling by phytochromes.

Effects of Far-red LED on Different *in vitro* Tea Plants and Expression of *CsLHY* Gene

Results of anatomic analysis of *in vitro* tea plantlets under far-red light treatment showed that the internode tended to be longer and the leaf area became smaller. It was coincided with previous studies that far-red light-rich environment stimulated the stem elongation due to greater internode elongation rather than a greater number of internodes (Franklin and Quail, 2010; Hossain and Kamaluddin, 2011; Casal, 2013). These morphological changes seemed to be consistent with shade-avoidance responses (Smith, 1982). Phytochrome, the primary photoreceptor involved in red light input (Neff and Chory, 1998), detects the ratio of red/far-red to initiate a shade avoidance response. Phytochrome has two conformations, the active form (Pfr) and inactive form (Pr) and the ratio of Pfr/Ptotal serves as an indicator. In a far-red-enriched environment, the Pfr/Ptotal value is small. In terms of light quality, far-red-rich environment tend to be found in the lower canopy rather than in the upper canopy because upper leaves preferentially absorb blue and red light, which are important for photosynthesis. Therefore, leaves in the lower canopy try to avoid shady environments, and to do so, plants prioritize stem elongation over leaf growth. In the experiment here, *in vitro* tea plantlets were treated in a far-red light-rich environment. Phytochrome perceived the difference in light quality, so shade-avoidance responses were induced by changing the balance of phytochrome-photoequilibrium to the Pr form, which lead to a marked acceleration of

internode elongation, at the expense of leaf and storage organ development, such as significantly decreased fresh leaf weight, leaf area, and carotenoid accumulation (Fig. 1, 2 and 3).

Compared to the white light condition, the expression of *CsLHY* was extremely suppressed by the far-red light (Fig. 5). The interaction between circadian clock genes and photoreceptor genes has been demonstrated in several studies (Wenden *et al*., 2011). In *Arabidopsis*, phytochrome A has been found to regulate circadian clock by mediating far-red light perception. Moreover, this study has found that *LHY* down regulation observed in constant far-red light condition, and a higher expression level of their inhibitor *PRR9* was maintained by far-red light through phyA (Wenden *et al*., 2011). In present study, according to the morphological analysis, the change in transcription level of *CsLHY* may be potentially related to shade-avoidance responses caused by far-red-rich condition through phyA.

To conclude, the morphology of in vitro tea plantlets and expression of *CsLHY* were significantly affected by light quality and multiple light signaling pathways, including phytochrome or cryptochrome, and auxin signaling may be involved. It was demonstrated that the red light had an effect on bud outgrowth, blue light stimulated the accumulation of carotenoids and significant higher expression of *CsLHY*, and far-red light dramatically suppressed the expression of *CsLHY* and lead to the elongation of internode and small leaf areas. Our investigation of *CsLHY* could be the first-hand knowledge for further study of gene function.

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