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RESEARCH ARTICLE

Supplemental blue and red light promote lycopene synthesis in tomato fruits

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Abstract

Lycopene, one of the strongest natural antioxidants known and the main carotene in ripe tomato, is very important for human health. Light is well known to be one of the most important environmental stimuli influencing lycopene biosynthesis; specifically, red light induces higher lycopene content in tomato. However, whether blue light promotes lycopene synthesis remains elusive and exactly how light stimulation promotes lycopene synthesis remains unclear. We applied supplemental blue and red lighting on tomato plants at anthesis to monitor the effect of supplemental blue and red lighting on lycopene synthesis. Our results showed that supplemental blue/red lighting induced higher lycopene content in tomato fruits; furthermore, we found that the expression of key genes in the lycopene synthesis pathway was induced by supplemented blue/red light. The expression of light signaling components, such as red-light receptor phytochromes (PHYs), blue-light receptor cryptochromes (CRYs) and light interaction factors, phytochrome-interacting factors (PIFs) and ELONGATED HYPOCOTYL 5 (HY5) were up- or down-regulated by blue/red lighting. Thus, blue and red light increased lycopene content in tomatoes by inducing light receptors that modulate HY5 and PIFs activation to mediate phytoene synthase 1 (PSY1) gene expression. These results provide a sound theoretical basis for further elucidation of the light regulating mechanism of lycopene synthesis in tomatoes, and for instituting a new generation of technological innovations for the enhancement of lycopene accumulation in crop production.

Keywords: blue light, red light, lycopene, phytochromes, cryptochromes

1. Introduction

Carotenoids (e.g., β-carotene and lycopene) and their catabolites are very important for human health. β-Carotene is the major dietary precursor of vitamin A (Okoh *et al*. 1993), whose deficiency is a major public health concern. Lycopene does not have provitamin A activity, but is a good dietary antioxidant. High plasma lycopene levels are related to decreased incidence of prostate cancer (Gann *et al*. 1999). Our inability to synthesize carotenoids *de novo* forces us to rely on plants as the primary source of dietary

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carotenoids. Thus, fruits have long been an important source of carotenoids in many diets (Mcquinn *et al*. 2018). Lycopene, the main carotene accumulated in ripe tomato fruits, has attracted attention as a target for manipulation (Ip *et al*. 2013; Lian and Wang 2008).

In mature tomato fruits, the first step in carotenoid synthesis is the key-limiting step of phytoene synthase production, which is encoded by the *PSY1* (Fraser *et al*. 2002). Once synthesized, phytoene undergoes a ploy-*cis*-transformation in the course of four desaturation reactions catalyzed by phytoene desaturase (PDS) and ζ-carotene desaturase (ZDS), followed by two isomerizations facilitated by ζ-carotene isomerase (ZISO) and carotene isomerase (CrtISO), ultimately producing all-*trans*-lycopene (Fraser *et al*. 2002; Fantini *et al*. 2013). Subsequently, all-*trans*-lycopene is converted to β-carotene, the precursor of lutein and provitamin A, by the action of lycopene β-cyclase (b-lcy) (Cunningham *et al*. 1994), or converted to β-carotene by the action of epsilon cyclase (Ronen *et al*. 1999). These combined activities are tightly regulated to push carotenoid flux towards specific products in the ripe tomato fruits.

Multiple strategies have been pursued to enhance lycopene content in tomato (Fraser *et al*. 2009). Phytohormones and their signaling components functioning in tomato fruit ripening have been documented. It has been reported that abscisic acid deficiency in tomato mutant high pigment 3 (*hp3*) leads to higher fruit lycopene content (Fraser *et al*. 2009). Down-regulation of ethylene biosynthesis gene *ACO* or *ACS* resulted in reduced lycopene content (Lincoln *et al*. 1993; Yokotani *et al*. 2004). In addition, light also plays a key role in the lycopene accumulation. Tomato light-signaling mutants displayed altered fruit pigment accumulation. Tomato high pigment (*hp*) mutants *hp1* and *hp2* show elevated levels of lycopene (Kilambi *et al*. 2013). Other light signaling components, such as the E3 ubiquitin-ligase CUL4 interacts with HP1 and produces highly pigmented fruits when the latter is silenced in tomato (Wang *et al*. 2008; Tang *et al*. 2016). The E3 ubiquitin-ligase CONSTITUTIVELY PHOTOMORPHOGENIC1 (COP1) promotes degradation of the light-signaling effector ELONGATED HYPOCOTYL 5 (HY5) (Schwechheimer and Deng 2000). Down-regulation of *COP1* and *HY5* results in increased and reduced levels of carotenoids, respectively (Liu *et al*. 2004).

Plants perceive light through at least five types of sensory photoreceptors that detect specific regions of the electromagnetic spectrum. Cryptochromes (CRYs) and phototropins (PHOTs) are blue light receptors absorbing at wavelengths of 390–500 nm. Phytochromes (PHYs) function in a dynamic photo equilibrium determined by the red (660 nm) to far-red (730 nm) ratio in land plants (Möglich *et al*. 2010; Tilbrook *et al*. 2013). These photoreceptors gather photonic information and then transduce it into changes in gene expression that regulate plant development and differentiation (Jiao *et al*. 2007).

It has been proven that red light induces lycopene accumulation in tomato fruits and far-red light reverses this effect. Red light enhanced lycopene accumulation by activating PHY to inhibit the accumulation of phytochrome-interacting factor (PIF) proteins, which in turn increased phytoene synthase (*PSY*) expression (Bae and Choi 2008; Casal 2013; Leivar and Monte 2014). Although there are many reports on light regulating lycopene accumulation, how blue light affects lycopene content in undetached tomato is not well understood. The only observation worthy of mention in this regard is that the over expression of the blue light receptor *CYR1a* resulted in elevated lycopene content in tomato (Liu *et al*. 2017), and that lycopene content continuously increased from 45 to 60 days after anthesis under 2R1B treatment, i.e., when the red (660 nm):blue (460 nm) ratio was 2:1 (Xie *et al*. 2016).

Here we aimed to widen our understanding of blue light effects on lycopene content in tomato fruits, and to provide the basis for the development of a new technology for lycopene accumulation in agricultural production. Therefore, we treated tomato plants with supplementary blue and red light at anthesis to reveal the effects of blue and red light on lycopene accumulation. Color measurement and quantitative analysis by HPLC were performed to monitor lycopene accumulation and the ripening process. Moreover, gene expression levels related to blue/red light receptors, light signaling components, and lycopene synthesis were determined to provide a molecular explanation of this phenomenon.

2. Materials and methods

2.1. Plant materials and light treatments

The experiment was performed in a greenhouse at the College of Horticulture of South China Agriculture University. Tomato (*Solanum lycopersicum* L. *cv*. Micro-Tom) seedlings were cultivated on sponge blocks with a Yamasaki culture solution, which was renewed every 7 days. When tomato plants reached anthesis, the flowers were tagged every day and the plants were placed under one of the following light conditions: natural light, without any supplemental light (control); supplemental blue light (430 nm); and supplemental red light (660 nm). Supplemental light treatments were provided using LED lamps (Kedao Technology Corporation, Huizhou, China). Photosynthetic photon flux density (PPFD) was set at 50 μmol m^{-1} s^{-1} and illumination period extended from 06:00 to 18:00 h every day. Ambient temperature, ambient humidity, and total PPFD during the illumination period are provided as supplementary

data (Appendices A, B and C, respectively).

2.2. Color measurement

Skin color of tomato fruits was assessed with a spectrophotometer (Konica Minolta CM-2003d). The L*, a*, b* space and data were processed to obtain hue. Hue angle (in degrees) was calculated according to the following equation: Hue=$\tan^{-1}$(b/a), if a>0; and 180+$\tan^{-1}$(b/a), if a<0. Chroma=$(a^2+b^2)^{0.5}$ (Sagar *et al*. 2013). The hue angle value is used to monitor skin color of tomatoes: the larger the value, the greener the fruit; conversely, the smaller the value, the redder the fruit.

Color measurements were obtained in two ways: one on the vine tomato fruit, the other on the detached tomato fruits. Seven vine tomatoes were tagged at 40 days after anthesis (DAA) and their color measured every day using a spectrophotometer (Konica Minolta CM-2003d) until 56 DAA. Tomatoes were harvested at 36, 42, 48, and 54 DAA. For each time point, there were four biological replicates, consisting of nine fruits. Each fruit was measured on three different points of its surface; fruit color reported is the mean value from these three measurements.

2.3. Carotenoid extraction and measurement by HPLC

Tomatoes were harvested at 30, 36, 42, 48, and 54 DAA, and immediately flash frozen and stored at –80°C until use. Frozen pericarp (200 mg) was extracted with 1.5 mL of tetrahydrofuran:methanol (1:1, v:v), the supernatant was collected, and the precipitate extracted twice with 1 mL of tetrahydrofuran. All collected supernatant was placed in one tube and centrifuged for clarification. Next, the extract was evaporated near dryness, resuspended in methyl *t*-butyl ether:methanol (500:475, v:v), and passed through a syringe filter (GE Osmonics) prior to injection into a carotenoid column.

Carotenoid determination was carried out on an Agilent 2000 high performance liquid chromatography system with YMC (Yamamura Chemical Research, 250 mm×4.6 mm i.d.) chromatographic column. Detection of wavelength was at 450 nm and column temperature was 35°C; the mobile phase was methanol:tetrahydrofuran (THF, 75:25, v:v) at a flow rate of 1 mL min^{-1}. Individual carotenoids were separated by HPLC on a YMC column yielding lutein, lycopene, and β-carotene at 18, 43, and 30 min, respectively. Their absorbance at 450 nm was converted to mg equivalents using a standard curve of authentic lycopene. The β-carotene, lycopene, and lutein standards were purchased from Shanghai Yuanye Biotechnology Co., Ltd., China.

2.4. RNA isolation and qRT-PCR analysis

Tomatoes were harvested at 30, 36, 42, 48, and 54 DAA and immediately flash frozen and stored at –80°C until use. For each sampling time point, there were three biological replicates, each consisting of nine fruits. Total RNA was isolated from three replicates of each sample using the RNAprep Pure Plant Kit (Tiangen Biotech Co., Ltd., Beijing, China), which contained the genomic DNA elimination step. Total RNA was quantified spectrophotometrically by measuring the absorbance at 260 nm and the absorbance ratio of 260/280 nm in a Nanodop spectrophotometer (Thermo). Quality of RNA samples were evaluated by staining rRNA after size separation on 2% (w/v) agarose gels; mRNA was reverse transcribed with the M-MLV First Strand cDNA Synthesis Kit (TQ2501) of OMEGA (Omega Bio-Tek, Inc., Guangzhou, China). DNase-treated total RNA (2 g) was added and incubated at 65–70°C for 5 min, then placed on ice for at least 2 min, followed by incubation for 60 min at 42°C using oligo(dT) primer according to the manufacturer's instructions. The resulting first-strand cDNA was normalized for the expression of the housekeeping *UBQ* gene (*Solyc01g056940*). Gene-specific primers were designed and their sequences are listed in Appendix D.

To determine gene expression level, fluorescent quantitative PCR was performed using the LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland) with SYBR Premix *Ex Taq* (TaKaRa Bio, Inc., California, USA); relative expression was calculated using the expression level of housekeeping *UBQ* gene and the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001).

3. Results

3.1. Supplemental blue/red lighting accelerated fruit development and ripening

Aiming to elucidate whether blue and red light accelerate fruit development, we counted the days from anthesis to breaker stage under supplemental blue/red light and under no supplemental lighting. As shown in Fig. 1, control fruits turned yellow at 48 DAA, while blue- and red-light treated fruits reached breaker stage 6 days earlier, at 42 DAA (Fig. 1-A). At 48 DAA, fruits under blue or red lighting turned red, while control fruits were still orange. These results proved that tomatoes reached the breaker stage earlier under supplemental lighting than under no supplemental lighting. Further, hue angle values of the fruits under blue/red supplemental lighting were smaller than those of the control fruits, which suggested that supplemental light-treated fruits were consistently redder than non-treated fruits (Fig. 1-B and C).

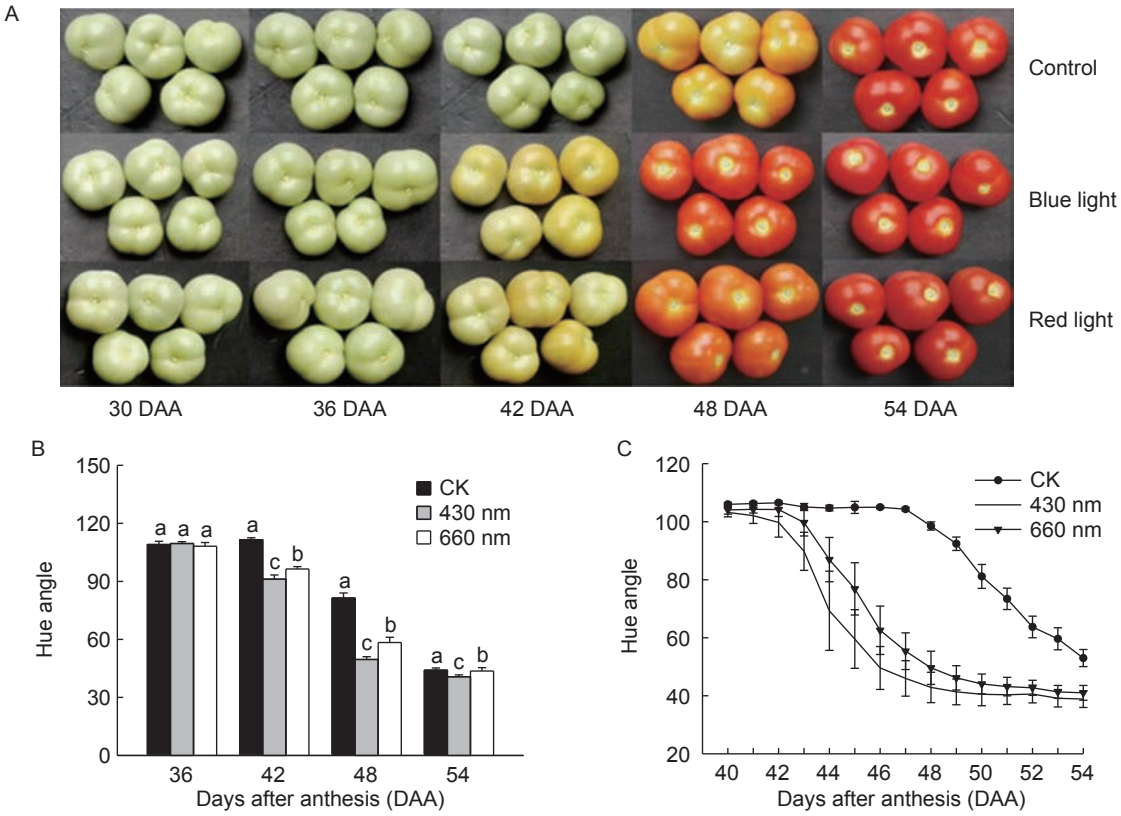

Fig. 1 Color measurement of tomato fruits under natural light or supplemental blue/red lighting. A, photographs. B, hue angle values of detached tomato fruits at 30, 36, 42, 48, and 54 DAA under control or supplemental blue/red treatment. Error bars represent standard deviations of the means of hue angle values from 36 detached tomato fruits. Statistically significant variations of mean values at different sampling points (ANOVA, $P<0.05$) are indicated with different letters. C, changes in hue angle value of non-treated and blue/red light treated tomatoes during fruit development and ripening. Error bars represent standard deviations of the means of hue angle values from seven vine tomatoes. CK, natural light; 430 nm, supplemental blue light; 660 nm, supplemental red light.

Both, blue and red light induced earlier maturing of tomatoes; thus, the breaker stage was reached earlier in the blue/red light treated fruits than in the control fruits. To determine whether the ripening process was also affected by experimental treatments, we measured the color change from 40 to 56 DAA when the control fruits became fully red. The hue angle value showed that the control fruits reached the breaker stage at 47 DAA and were fully red at 56 DAA (Fig. 2-A). Ripening of tomatoes under natural light requires about 10 days, whereas, the blue/red light treated tomatoes required only 7 days to turn fully red. This result suggested that fruits ripened 3 days earlier under blue/red lighting than under control conditions (Fig. 2-B and C).

3.2. Lycopene content increased in tomatoes growing under supplemental blue/red lighting

We investigated lycopene, β-carotene, and lutein contents of tomato fruits at 36, 42, 48, and 54 DAA. Under all light conditions, lycopene and β-carotene contents increased as the fruits progressed through ripening, whereas lutein content decreased during this process (Fig. 3-A–C). At 42, 48, and 54 DAA, lycopene content was higher in blue or red light treated fruit, whereas it remained at a similar low level in the control fruits throughout the sampling period (Fig. 3-A). β-carotene content showed a higher level in blue or red light treated fruits than in the controls (Fig. 3-B). Lutein content increased under red lighting at 36, 42, and 48 DAA, but decreased at 54 DAA (Fig. 3-C). Under blue lighting, lutein content was induced to increase only at 36 DAA, but then remained at a level similar to that in the control fruits (Fig. 3-C).

3.3. Expression analysis of lycopene synthesis and metabolism pathway genes under blue/red lighting

PSY and *ZDS* are two key genes involved in the lycopene synthesis pathway. The qPCR results showed that *PSY1* and *PSY2* were both induced by blue and red lighting (Fig. 4). *PSY1* expression level increased earlier under blue lighting, showing an increase at 36 DAA. *ZDS* also displayed an increasing expression pattern under blue/red

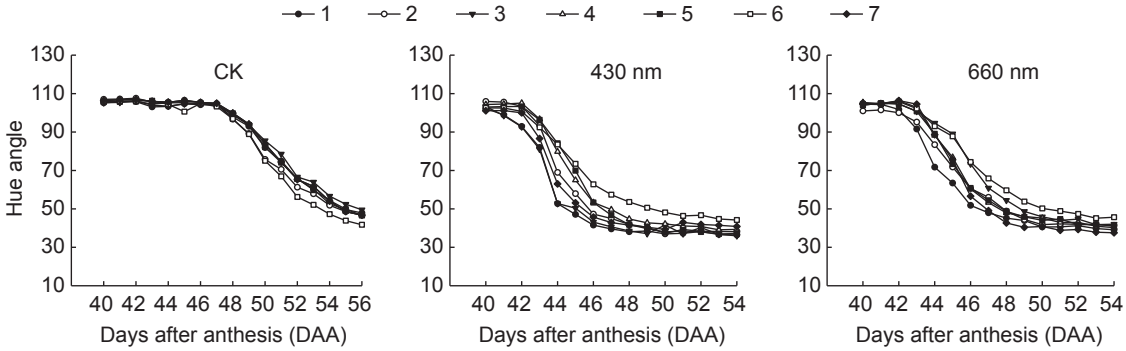

Fig. 2 Tomato fruit color variation under natural light and supplemental blue/red lighting. Changes in hue angle value of non-treated (CK, A), blue light treated (430 nm, B), and red light treated (660 nm, C) tomato fruits during fruit development and ripening. 1–7 represent seven independent tomato fruits.

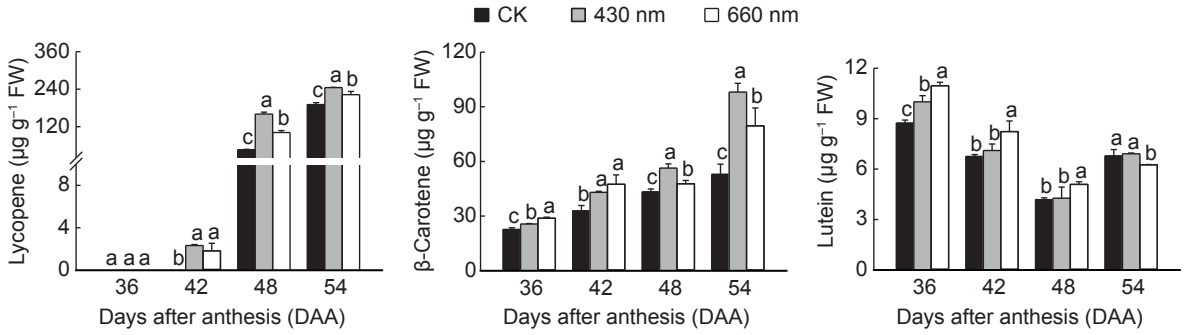

Fig. 3 Carotenoid content of fruits at 36, 42, 48, and 54 DAA under natural light and supplemental blue/red lighting. A, lycopene content. B, β-carotene content. C, lutein content. CK, natural light; 430 nm, supplemental blue light; 660 nm, supplemental red light. Error bars represent standard deviations of the means of carotenoids value from three biological replicates. Statistically significant variations among mean values at different sampling points (ANOVA, $P<0.05$) are indicated with different lowercase letters.

lighting (Fig. 4). *LCYb* and *LCYe* are two genes mediating lycopene metabolism, converting lycopene to α-carotene and β-carotene, respectively. *LCYb* expression was inhibited under blue/red lighting, while the expression of *LCYe* was induced by blue lighting but reduced by red lighting (Fig. 4).

3.4. Expression of genes related to the light signaling pathway

Aiming to explain how blue or red light may induce an increase in lycopene content in tomato fruit, we examined blue (*CRY1a*, *CRY1b*, *CRY3*), and red light receptor genes (*PHYA*, *PHYB1*, *PHYB2*), light signaling components PIFs (*PIF3*, *PIF4*), and *HY5* expression profiles corresponding to lycopene accumulation and metabolism. As shown in Fig. 5, except for *CRY1a*, all blue light receptors were inhibited by blue and red lighting at 48 DAA. *CRY1a* showed a decline in the expression level at the beginning of the maturing stage, at 36 DAA. Red light receptors *PHYA* and *PHYB2* displayed elevated expression levels at 42 DAA, whereas *PHYB1* was only induced by red light at this stage. PIFs and HY5 are light signaling components that mediate light responses in the tomato plant. The expression data showed that *HY5* was first reduced by blue and red lighting, and then increased by both at 42 DAA (Fig. 6). The expression profiles of *PIF3* and *PIF4* are distinctive. *PIF3* was suppressed by both blue and red lighting at 30 DAA, followed by an increasing expression level at 36 DAA fruit under blue lighting. *PIF4* displayed increasing inhibition from 36 to 54 DAA (Fig. 6).

4. Discussion

4.1. Red light induced lycopene content in tomato fruits

It has been reported that red light induces lycopene content of detached tomato fruits, whereas far-red lighting reverses this effect (Toledo-Ortiz *et al*. 2010; Bou-Torrent *et al*. 2015). Red light photoreceptors (PHY) play key roles in this process: the *phyA phyB phyB2* triple mutant produced white fruits completely devoid of pigments (Weller *et al*. 2000).

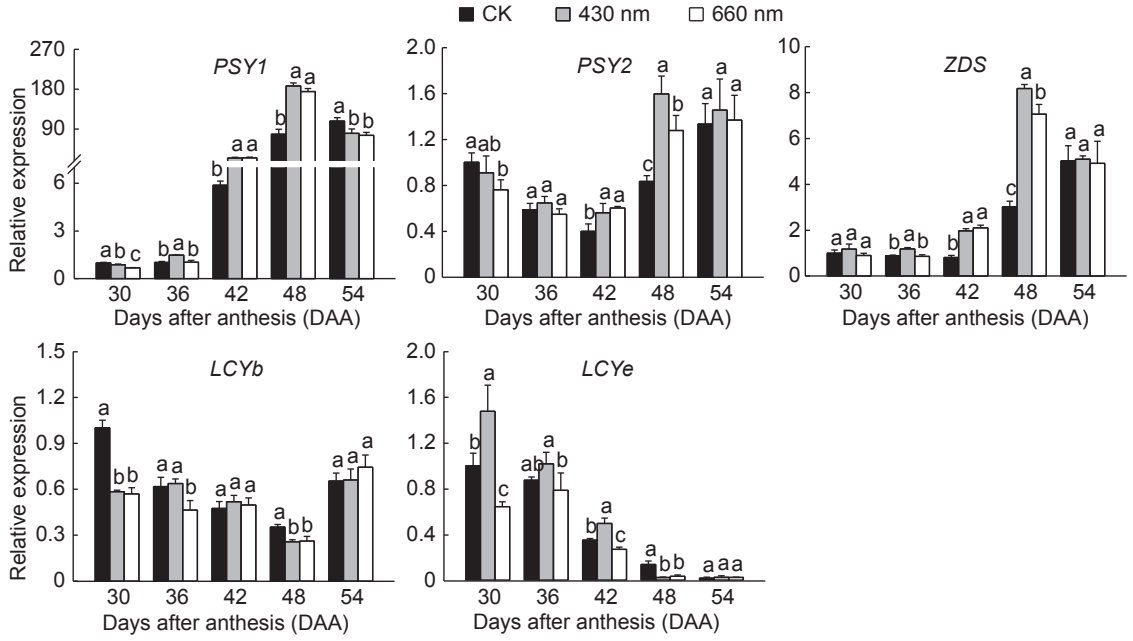

Fig. 4 Expression of carotenoid biosynthesis genes at 30, 36, 42, 48, and 54 DAA under natural or supplemental blue/red lighting. Error bars represent standard deviations of the means of three independent replicates. Statistically significant variations of expression and mean values at different sampling points (ANOVA, $P<0.05$) are indicated with different lowercase letters. CK, natural light; 430 nm, supplemental blue light; 660 nm, supplemental red light.

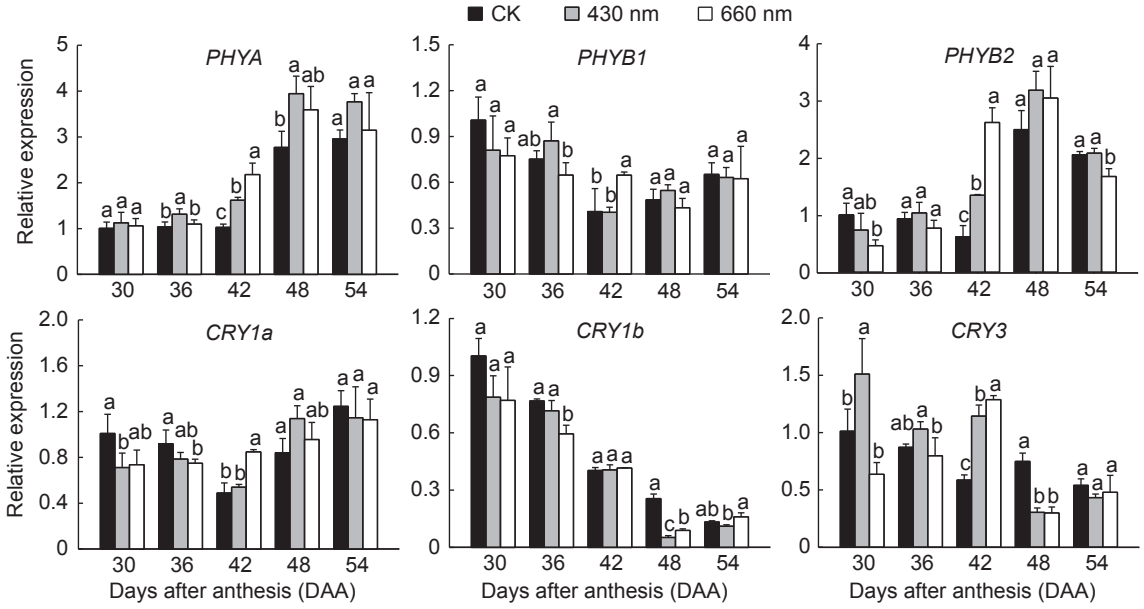

Fig. 5 Expression of red and blue light receptors at 30, 36, 42, 48, and 54 DAA in tomatoes growing under natural and supplemental blue/red lighting. Error bars represent standard deviations of the means of three independent replicates. Statistically significant variations of expression and mean values at different sampling points (ANOVA, $P<0.05$) are indicated with different lowercase letters. CK, natural light; 430 nm, supplemental blue light; 660 nm, supplemental red light.

Activation of fruit-localized PHYs with red lighting promotes carotenoid biosynthesis, while subsequent PHY inactivation by far-red light reverts it (Alba *et al*. 2000; Schofield and Paliyath 2005). PHYs have the ability of detecting the red/far-red light (R/FR) ratio. Low R/FR ratios reduce PHY activity, resulting in the accumulation of PIF, which inhibited the expression of *PSY*, the main rate-determining enzyme in the carotenoid pathway. In contrast, high R/FR ratios

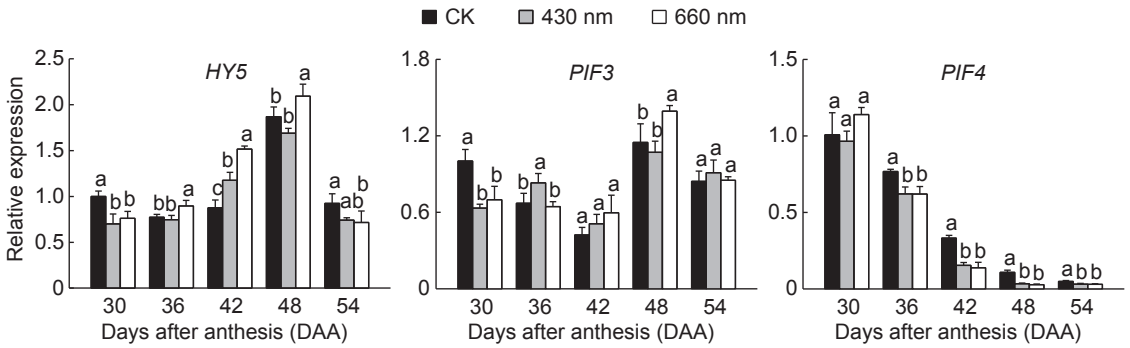

Fig. 6 Expression of light interaction transcription factors at 30, 36, 42, 48, and 54 DAA in tomatoes growing under natural and supplemental blue/red lighting. Error bars represent standard deviations of the means of three independent replicates. Statistically significant variations of expression and mean values at different sampling points (ANOVA, $P<0.05$) are indicated with different lowercase letters. CK, natural light; 430 nm, supplemental blue light; 660 nm, supplemental red light.

enhanced PHY activity, resulting in the degradation of PIF proteins, which in turn induced the expression of *PSY* (Bae and Choi 2008; Casal *et al*. 2013; Leivar and Monte 2014). In our study, supplemental red lighting on vine tomato fruits increased lycopene content (Fig. 3) and induced *PSY* expression level; meanwhile, *PHYA*, *PHYB1*, and *PHYB2* expression levels were elevated by red lighting (Figs. 4 and 5). The PHY-interacting transcription factors *PIFs* were down-regulated by red light irradiation (Fig. 6). These results indicated that red light induced lycopene content by elevating *PHY* to inhibit *PIFs*, which ultimately resulted in increased *PSY* expression.

The role of PIF1 as a direct negative regulator of *PSY* expression is antagonized by the bZIP transcription factor HY5. HY5 binds to the same promoter motif bound by PIF1, which is a G-box in the promoter of the *PSY* gene (Schwechheimer and Deng 2000). Down regulation of *HY5* in tomato results in reduced levels of carotenoids (Liu *et al*. 2004). In our study, *HY5* was induced at 36, 42, and 48 DAA in fruits growing under red light irradiation (Fig. 6). This is consistent with the induction of increased *PSY* expression and lycopene levels.

4.2. Blue light enhanced lycopene levels in tomato fruits

Blue light has numerous effects on plant development, including inhibition of hypocotyl elongation, leaf and cotyledon expansion, pigment biosynthesis, stem growth and internode elongation, control of flowering time and phototropism. However, reports on the effects of supplemental blue light on lycopene synthesis in tomatoes are scarce. We found that tomatoes turned yellow earlier and showed a higher lycopene content under supplemental blue light (Figs. 1 and 3). CRYs are blue light receptors that mainly function in photomorphogenic responses and photoperiod-dependent flowering (Lin *et al*. 1998; Yu *et al*. 2010). In tomato, the *CRY1a* over-expression lines produced fruits with higher lycopene content, but a *cry1a* mutant produced fruits with reduced lycopene levels (Liu *et al*. 2017). In our study, *CRY1a* expression was down-regulated by supplemental blue lighting at the beginning, and then it remained at a level similar to the expression in the untreated control fruits. Another blue light receptor, namely CRY3, was induced by blue light irradiation from 30 to 42 DAA (Fig. 5). This result suggested that CRY3 may play a key role in response to blue light irradiation.

Several lines of evidence suggest that the transcription factor HY5 mediates CRYs-induced gene expression in response to blue light (Liu *et al*. 2004, 2011, 2017). CRY interacts with E3 ubiquitin-ligase COP1; moreover, HY5 and COP1 act antagonistically in the regulation of tomato lycopene synthesis. COP1 promotes the degradation of the light-signaling effector HY5 (Schwechheimer and Deng 2000). Repression of *HY5* results in reduced lycopene content, while repression of *COP1* results in elevated lycopene levels (Liu *et al*. 2004). In our study, *HY5* expression was induced by blue light irradiation in tomatoes at 42 DAA (Fig. 6). This was consistent with the increased lycopene content observed, which indicated that blue light may have induced lycopene content by inducing an increase in *HY5* expression.

4.3. Blue and red light induced lutein and β-carotene contents in tomato fruits

It has been reported that red light induced lutein and β-carotene contents in tomato fruits, when compared with the dark and R/FR-exposed fruits (Schofield and Paliyath 2005). Fewer reports are available on supplemental blue light effects on lutein and β-carotene synthesis. In our study, supplemental red/blue lighting on vine tomatoes

increased both lutein and β-carotene contents (Fig. 3), but not the expression levels of *LCYb* and *LCYe*; however, *LCYe* was induced by blue lighting at 30, 36, and 42 DAA. Inhibited or unaltered expression level of *LCYb* under blue/red light and of *LCYe* under red light were also recorded in the higher pigment mutant *hp1*, which showed higher lutein and β-carotene contents than the wild type (Kilambi *et al*. 2013); conversely, other key genes in the lutein and β-carotene synthesis pathways were induced in the *hp1* mutant, such as *CYCB*, *CRTRB1/B2*, *CYP97C11*, and *CYP97A29*. Therefore, the reason for the inhibited expression levels of *LCYb* and *LCYe* in this study may be two-fold. Firstly, the gene expression level may not reflect the level of enzyme activity; thus, further examination of enzyme activity is required. Secondly, the higher lutein or β-carotene content observed under blue/red lighting may result from higher levels of other key genes in the pathway for lutein or β-carotene synthesis, such as *CRTRB1/B2*, *CYP97C11*, *CYP97A29*, and *CYCB*, whose expression levels should be determined.

5. Conclusion

In our study, supplemental blue and red light induced lycopene synthesis, consequently, lycopene content in tomatoes similarly. It is worth noting that blue light showed a much stronger effect than red light. Furthermore, gene expression analysis showed that the red light receptor PHY and the blue light receptor CRYs displayed a similar response to blue and red light. Whether this response is direct requires further investigation. The same situation was found for HY5 and PIFs genes. These results suggest that blue and red light may share some similar regulation mechanisms (Fig. 7), whereby blue and red light increased lycopene content by inducing light receptors, which modulate the activation of *HY5* and *PIFs* to mediate *PSY1* expression.

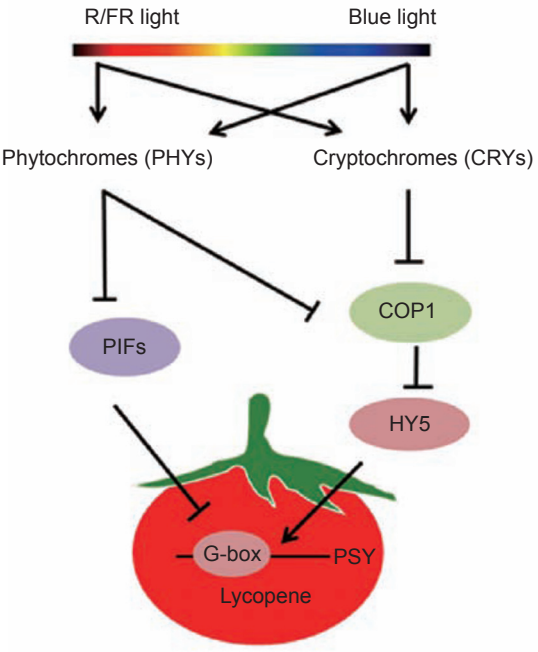

Fig. 7 Hypothetical model of blue and red light regulated lycopene synthesis network. Blue and red light induced the increase of lycopene content by two alternative pathways: either by elevating PHYs activities to inhibit PIFs proteins, which reduced *PSY* expression, or by inducing *CRYs* expression to inhibit COP1 activity; the inhibited COP1 in this case results in activation of HY5, which promotes *PSY* expression. R/FR, red/far-red light.

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Appendices associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

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