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Colored light-quality selective plastic films affect anthocyanin content, enzyme activities, and the expression of flavonoid genes in strawberry (*Fragaria* × *ananassa*) fruit

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ABSTRACT

The influence of colored light-quality selective plastic films (red, yellow, green, blue, and white) on the content of anthocyanin, the activities of the related enzymes and the transcripts of the flavonoid gene was studied in developing strawberry fruit. The results indicated that colored films had highly significant effects on the total anthocyanin content (TAC) and proportions of individual anthocyanins. Compared with the white control film, the red and yellow films led to the significant increase of TAC, while the green and blue films caused a decrease of TAC. Colored film treatments also significantly affected the related enzyme activity and the expression of structural genes and transcription factor genes, which suggested that the enhancement of TAC by the red and yellow films might have resulted from the activation of related enzymes and transcription factor genes in the flavonoid pathway. Treatment with red and yellow light-quality selective plastic films might be useful as a supplemental cultivation practice for enhancing the anthocyanin content in developing strawberry fruit.

1. Introduction

Anthocyanins are major pigments found in many plants and, combined with carotenoids or chlorophylls, are responsible for the red, purple, and blue coloration of some fruits, leaves, and seeds. In addition to their colorant properties, anthocyanins contribute to a wide range of biological activities, exhibiting anticancer, anti-inflammatory, antioxidant, pharmacological, and chemoprotective effects. Because anthocyanins are one of the principle bioactive components of strawberries, food scientists have conducted comprehensive analyses to quantify and characterize them. The anthocyanins and flavonoid biosynthetic pathway has been extensively elucidated in strawberry at the genetic, biochemical and molecular levels (Almeida et al., 2007; Carbone et al., 2009; Schaart et al., 2013). The key structural enzymes, such as phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), 4-coumarate: -CoA ligase (4CL), chalcone isomerase (CHI), chalcone synthase (CHS) and anthocyanidin synthase (ANS), leading to different intermediates and different flavonoid classes are well known (Xu et al., 2014). Moreover, the biosynthesis of anthocyanins is regulated via coordinated transcriptional control of the structural enzymes in the biosynthetic pathway by DNA binding R2R3 MYB transcription factors and interaction with MYC-like basic helix-loop-helix (bHLH) and WD40-repeat proteins (Schaart et al., 2013).

Although the genetic background of a plant is the main determinant of anthocyanin content, external environmental factors can also affect the quantitative and qualitative composition of anthocyanins in ripening fruit. The reports have shown that anthocyanin biosynthesis is highly regulated by plant hormones, light conditions, temperature, nutritional status, and biotic stressors.

Light is one of the most important environmental factors affecting anthocyanin biosynthesis in plants. The photoperiod, light intensity (quantity), and light quality (spectrum) influence anthocyanin biosynthesis in different ways (Zoratti, Karppinen, Escobar, Häggman, & Jaakola, 2014). Many studies have shown that high levels of flavonoids, especially anthocyanins, can be detected in plants under longer photoperiods. However, fruits from bilberry plants grown under 12 h of natural light per day showed a significantly higher level of anthocyanins than those from plants grown under 24 h of natural light per day (Uleberg et al., 2012). In broccoli, the opposite effect from the photoperiod was detected (Steindal, Mølmann, Bengtsson, & Johansen, 2013). The flavonoids

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quercetin and kaempferol were at their highest level at high temperatures with a 12-h photoperiod. In most plants, anthocyanin accumulation is positively related to light intensity. Kadomura-Ishikawa, Miyawaki, Takahashi, Masuda, and Noji (2015) showed that light treatment led to significantly higher total anthocyanin levels in strawberries compared to the dark control (Kadomura-Ishikawa et al., 2015). On the other hand, the decrease of anthocyanin accumulation in fruit has been found by fruit bagging, and high level of anthocyanins after debagging or exposure of shaded fruits to sunlight (Feng, Li, Maa, & Cheng, 2013).

The sunlight spectrum that affects the biological activity of plants is between 280 and 800 nm, which includes ultraviolet (UV) light (UV-A and UV-B), visible light, and far-red light. The visible light spectrum ranges 400–710 nm and is subdivided, from shortest to longest, into purple, blue, cyan, green, yellow, orange, and red wavelengths. The increase of the proportion of red and UV light has a positive effect on the increase of anthocyanin biosynthesis, while far-red light has a negative effect. Shorter wavelengths lead to the greater accumulation of flavonoids in fruits. For example, the biosynthesis of anthocyanins in postharvest strawberry can be significantly increased with blue light emitted from a light-emitting diode (LED) (Xu et al., 2014). This light treatment has also caused the increase of enzymatic activity involved in flavonoid biosynthesis, including PAL, C4H, 4CL, CHI, CHS and ANS (Xu et al., 2014; Zoratti et al., 2014). Transcript levels of early and late biosynthetic genes and transcription factor genes involved in flavonoid biosynthesis were found to be consistent with the detected metabolite levels (Kadomura-Ishikawa et al., 2015).

Strawberries are widely and highly consumed both fresh and in processed form. Due to the synergistic effects of nutrients (*i.e.*, vitamins and carotenoids) and phytochemicals (*e.g.*, polyphenols and ellagic acid), strawberries can be considered a functional food. A raised-bed hill cultivation system covered with black plastic mulch is commonly used for strawberry production. The primary benefits of black plastic mulch include the increase of early yields, moisture retention, retardment of weed growth, the control of fungal diseases, and the achievement of cleaner fruits. Other colors of mulch are also used in strawberry cultivation. Fruit weight, size, yield, aroma, phytochemical (anthocyanin, flavonoid, and phenolic) content, and foliar area are enhanced with red versus black mulch (Loughrin & Kasperbauer, 2002). This is due to the effects of reflected far-red and red light on gene expression, enzyme metabolism, and phytochrome-mediated allocation of photosynthate, with more photosynthate directed to developing fruit. In terms of marketable yield, green mulch led to higher values than that of black mulch (Medina et al., 2011). A slightly higher (10%) total anthocyanin content was found in fruits grown with brown mulch compared to white (Anttonen et al., 2006). On the other hand, plastic mulches do not always affect yield, the number of leaves, or the number of crowns of different cultivars (Locascio, Gilreath, Olson, Hutchinson, & Chase, 2005). However, there are no reports on the effect of different colored films on anthocyanin metabolism in developing strawberry fruit. In this paper, the effects on anthocyanin content, enzymes, and the expression of flavonoid genes were investigated after colored light-quality selective plastic films were used to cover strawberry fruits.

2. Materials and methods

2.1. Plant materials and light-quality treatments

Strawberry (*Fragaria* × *ananassa* Duch. 'Yueli') plantlets were planted in the greenhouse in September 2013 in Haining (120°24′E, 30°26′N), located in southern China. The levels of organic matter, total nitrogen, available nitrogen, available phosphorus, and available potassium in the soil were 3.27%, 0.221%, 169 mg kg^{-1}, 88.5 mg kg^{-1}, and 167 mg kg^{-1}, respectively. The cation exchange capacity of the soil was 14.5 me·100 g^{-1} with a pH value of 5.56. The raised bed measured 60 cm in width and 30 cm in height, and was mulched with black polyethylene film. Into each raised bed, two rows of plants were planted, spaced 20 cm apart. The drip irrigation system used irrigation tapes of 40 mm in diameter placed under the black polyethylene mulch with orifices 20 cm apart, close to the strawberry plants.

To achieve homogeneity, two secondary fruits from a strawberry inflorescence were selected for the analysis, and the other flowers and fruits were eliminated. Seven days after full bloom, the secondary strawberry fruits were covered with transparent colored filter light films (red, yellow, green, blue, and white) from December 12, 2013 to January 14, 2014. These films were composed of polyethylene strips of 0.03 mm in thickness. The radiant energy of the white filter film (control) was measured by a LI-1800 portable spectroradiometer (LI-COR, USA). The light intensity inside the films was kept consistent by covering the films with a white membrane or regulating the height of the films from the fruits. The percentage of the blue irradiance spectrum in the overall radiant energy of the red, yellow, green, blue, and white light-quality selective plastic films was 1.80%, 4.63%, 10.03%, 19.0%, and 8.10%, respectively. Temperature and humidity values were recorded with a DL-WS211 automatic recorder (Hangzhou Gsome Technology Co., Ltd, China). The trials used a random block design with the white film as the control. Each treatment had 30 plants, and the experiments were repeated three times.

Ten strawberries from each replicate were sliced into small segments, pooled, and frozen in liquid nitrogen. The strawberry slices were then ground into a powder using a high-throughput grinder (CK1000, Thmorgan, China), and each powder was transferred to a plastic bag and stored at −80 °C until analysis.

2.2. HPLC analysis of anthocyanins

Approximately 1 g of each strawberry powder was poured into 5 ml of 1% (v/v) hydrochloric acid/methanol and stored at 4 °C for 24 h. The resulting slurry was centrifuged at 12,000*g* and 4 °C for 10 min. The supernatant was then transferred to a 20 ml volumetric flask. The residue was added to 5 ml 1% (v/v) hydrochloric acid/methanol and centrifuged twice at 12,000*g* and 4 °C for 10 min. The supernatant was merged, and the contents of the volumetric flask were added to 20 ml of 1% (v/v) hydrochloric acid/methanol. The methanol extract was passed through a 0.22 μm microporous-membrane filter (Millipore, MSI, Westboro, MA).The final filtrate was stored at −20 °C until HPLC analysis.

Anthocyanins were also analyzed using the HPLC system. The solvents included (A) 10% formic acid in water and (B) HPLC-grade acetonitrile, establishing the following gradient: 0–20% B over 13 min, 20–40% B over 7 min, and 40–0% B over 5 min. The total flow rate was 1 ml min^{-1}, and the sample injection volume was 20 μl. The column temperature was 30 °C. Anthocyanin content was detected at 520 nm. Quantification was performed using an external calibration, and expressed in terms of pelargonidin-3-glucoside (Pg3G) equivalent.

2.3. PAL, C4H, 4CL, CHI, CHS and ANS enzyme extraction and assay

PAL, C4H, 4CL, CHI, CHS and ANS activity was measured as previously described (Lister & Lancaster, 1996; Xu et al., 2014). As a brief overview, 2 g of strawberry powder were added to 5 ml of extraction buffer containing 50 mM Tris–HCl buffer (pH 8.9), 15 mM β-mercaptoethanol, 4 mM magnesium chloride, 5 mM ascorbic acid, 10 μM leupeptin, 1 mM phenylmethanesulfonyl

fluoride, 10% glycerol, 5 mM EDTA, and 0.15% w/v polyvinylpyrrolidone. The homogenate was centrifuged at 13,000*g* for 20 min at 4 °C, and the supernatant was then used as a source of crude enzymes for assaying PAL, C4H, 4CL, CHI, CHS and ANS activity and the protein content. One unit of enzyme activity was equal to a change of 0.01 in absorbance per hour, expressed as U min^{-1} mg^{-1} protein. Protein was assayed with bovine serum albumin as the standard using the dye-binding method of Bradford (1976).

2.4. Gene expression

Total RNA was extracted using a modified cetyltrimethylammonium bromide (CTAB) method designed for samples rich in polyphenols and polysaccharides. Five micrograms of total RNA were used to synthesize the first-strand of cDNA with TransScript® One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, China) in a final 20 μl reaction mixture according to the manufacturer's instructions. The real-time quantitative PCR (qPCR) mixture (20 μl in total volume) included 10 μl of SYBR FastStart Essential DNA Green Master Mix (Roche, Switzerland), 0.5 μl of each primer (10 μM), and 8 μl of diluted cDNA. Subsequently, the qPCR was performed on a LightCycler® 96 real-time PCR instrument (Roche, Switzerland), initiated at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s, 50–60 °C for 10 s, and 72 °C for 30 s. Melting curve analysis was performed at 95 °C for 10 s, 65 °C for 60 s, and 97 °C for 1 s. All the qPCR reactions were normalized using the CT value corresponding to the actin gene (*FaActin*) through the $2^{-\Delta CT}$ method. No-template controls and melting curve analyses were included for each gene and PCR reaction. All analyses were repeated thrice using three replicates, and qPCR primers (see Supplementary Data – Table 1S) were designed with Primer3 software (http://primer3.sourceforge.net/).

2.5. Statistical analysis

Data were expressed as mean ± standard deviation (SD) and evaluated by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test at $p < 0.05$. All statistical analyses were performed using the Data Processes System (DPS) software package (Tang & Zhang, 2012).

3. Results and discussion

3.1. Effect of colored light-quality selective plastic films on anthocyanin content in ripened strawberry fruit

Anthocyanin content showed significant differences in fruits grown using different colored film. The average content of major individual anthocyanins is shown in Table 1, and the average composition was shown in Fig. 1. The accumulation of anthocyanin pigments in strawberries is an important indicator of ripeness and quality and has beneficial implications for human health. The major anthocyanin in Yueli was Pg3G, accounting for 70% of the total anthocyanin content (TAC), while cyanidin 3-glucoside (Cy3G) and pelargonidin 3-malonylglucoside (Pg3MG) represented

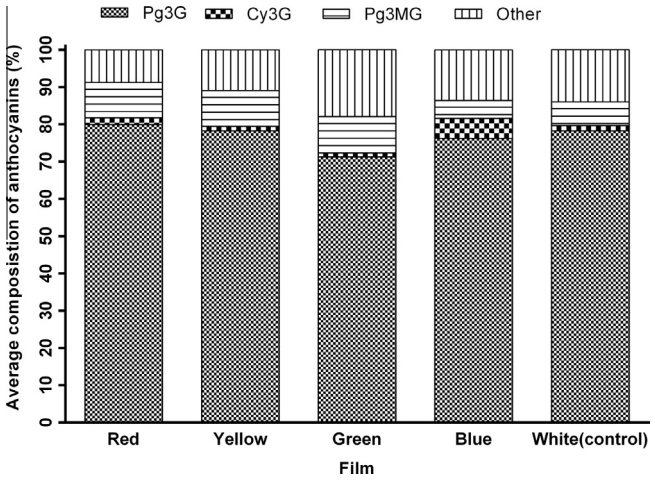

Fig. 1. The effect of red, yellow, green, blue, and white (as the control) film treatments on the average composition of anthocyanins of strawberry at the ripening stage.

smaller proportions. These findings are consistent with those of other strawberry cultivars, including Camarosa, Festival, Akihime, Elsanta, Toyonoka, and others (Aaby, Mazur, Nes, & Skrede, 2012).

Light treatment can significantly increase TAC in strawberry fruit. In general, significantly higher TAC in fruits treated with red and yellow films were observed than that of the control (white film), and significantly lower TAC after treatment with the green and blue films. Pg3G was one of the main contributors to the different effects of the films. Compared to the control, the TAC of fruit increased by 74.11% and 36.17% after treated with the red film and the yellow film, respectively. On the contrary, fruits treated with the green and blue films had TAC values 28.49% and 10.22% lower than the control, respectively. Significant differences were shown between the different films, TAC, Pg3G, and Pg3MG.

Many studies have reported that anthocyanin biosynthesis is an important process that depends on light. Apple and pear skins are influenced by light quality, and longer wavelengths increase the intensity of the red coloration (Feng et al., 2013). Consistent with our results, other researchers have also found that light quality affects strawberry fruit color (Kadomura-Ishikawa et al., 2015; Shiukhy, Raeini-Sarjaz, & Chalavi, 2014; Xu et al., 2014). Compared with fruits grown on white plastic mulch, those grown in raised beds with red plastic mulch had 31.32% higher TAC (Shiukhy et al., 2014). Blue light also caused a significant increase of the TAC of strawberry fruit during storage (Xu et al., 2014). Covering fruits with colored films improved the shortwave radiation and altered the far-red to red (FR/R) radiation ratio. Therefore, the increase in TAC with colored films could be related to greater shortwave light penetration. The positive effects of colored films on fruit quality also attributed to improvements in the use of photosynthetically active radiation for net C assimilation (Bastías & Corelligrappadelli, 2012). In contrast, light irradiation had a negative effect on anthocyanin accumulation in strawberry callus,

Table 1
Average content of individual anthocyanins and TAC for colored-film treatments on ripe strawberry fruits.

Light film	Pg3G (μg g^{-1} FW)	Cy3G (μg g^{-1} FW)	Pg3MG (μg g^{-1} FW)	Other (μg g^{-1} FW)	TAC (μg g^{-1} FW)
Red	97.76 ± 0.88 a	2.27 ± 0.18 b	11.54 ± 0.57 a	10.76 ± 0.70 a	122.33 ± 2.19 a
Yellow	74.84 ± 1.17 b	1.16 ± 0.11 c	9.18 ± 0.36 b	10.49 ± 0.27 a	95.67 ± 1.37 b
Green	35.79 ± 0.43 e	0.51 ± 0.12 d	4.92 ± 0.23 c	9.02 ± 0.09 bc	50.24 ± 0.43 e
Blue	48.05 ± 2.76 d	3.39 ± 0.10 a	3.06 ± 0.43 d	8.58 ± 0.23 c	63.08 ± 2.60 de
White (control)	54.96 ± 1.42 c	1.01 ± 0.06 c	4.47 ± 0.39 c	9.82 ± 0.52 ab	70.26 ± 1.91 cd

Values in the same column followed by a different small letter are significantly different ($p < 0.05$).

which actually produced high levels of anthocyanin in the dark (Nakamura, Takeuchi, Miyanaga, Seki, & Furusaki, 1999). It was also speculated that anthocyanin content could be related to the surrounding air temperature (Zoratti, Jaakola, Häggman, & Giongo, 2015). According to our data, the red, yellow, green, and blue films resulted in an average temperatures. 0.9 °C, 0.5 °C, 0.6 °C, and 0.3 °C higher, respectively, than the white control film (see Supplementary Data – Table 2S).

Different films also can change the proportions of individual anthocyanins in strawberry fruits (Fig. 1). The proportion of Pg3MG in strawberry fruits was most affected. Comparing with the white control film, Pg3MG was 50% higher in fruits after treatment with the red, yellow, and blue films, and 23.74% lower in fruit after treated with the blue film. On the other hand, the proportion of Pg3G, showed no significant change in fruits treated with the red, yellow, and blue films, but was decreased after treatment with the green film (from 78.23% to 71.24%). Fruits treated with the blue film had a significantly increased proportion of Cy3G (from 1.44% to 5.37%).

3.2. Effect of colored light-quality selective plastic films on PAL, C4H, 4CL, CHI, CHS and ANS activities in strawberry fruit

Anthocyanin pigments are synthesized from hexose through the shikimate, phenylpropanoid, and flavonoid pathways. PAL, C4H, 4CL, CHI, CHS and ANS play an important role in the biosynthesis and accumulation of anthocyanins (Jiang & Joyce, 2003). In the present study, we found that colored film treatments significantly affected the activity of these enzymes (Fig. 2).

PAL is a key enzyme in phenylpropanoid metabolism and catalyzes phenylalanine to cinnamic acid. This is the first committed step for the biosynthesis of the phenylpropanoid skeleton (Lister & Lancaster, 1996). Fig. 3 showed changes in PAL activity during fruit development in response to films of different colors. The PAL activity increased during fruit development and remained at high levels for all treated films, consistent with previous reports (Montero, Mollá, & Martín-Cabrejas, 1998). There were some differences among the different film treatments. Compared with fruits grown under the white control film, fruits exposed to the red film exhibited significantly higher PAL activity at all stages of development, while fruits exposed to the green film had lower PAL activity at all stages. The yellow film induced higher PAL activity at the white and red stages of development, while the blue film led to lower activity in most stages, except the red stage. Compared with the white control film, the red film increased PAL activity at the white, reddish, half-red, and red stages by 34.70%, 5.14%, 13.61%, and 34.88%, respectively. The PAL activity of fruits treated with colored films in the red stage was in accordance with the TAC accumulation at this stage (Table 1). Although fruit covered with the blue film had a lower TAC than with the white control film, there was a greater PAL activity at the red stage with this film.

C4H and 4CL modified cinnamic acid is used to produce *p*-coumaroyl-CoA, the flavonoid precursor. C4H and 4CL activity had similar trends in the development of fruits, initially increasing, then peaking at the reddish stage, and finally following a downward trend. Fruits treated with the red film had the highest C4H and 4CL activity levels of all the films, aligning with the results for anthocyanin accumulation. Fruits at the red stage covered with the red, yellow, and white control films had the highest C4H activity levels, with no significant difference between the treatments. The lowest values of C4H activity at the red stage were found using the green and blue films, with respective values of 58.15 and 53.69 U min^{-1} mg^{-1} protein. The highest values of 4CL activity were found with the red, yellow, and white control films, with no significant differences between the treatments. The lowest values of 4CL activity at the red stage were found with the green and blue films with values 20.58% and 24.12% lower, respectively, than that of the white control film.

CHS conducts the condensation of one molecule of 4-coumaroyl-coenzyme A (CoA) and three molecules of malonyl-CoA, which results in naringenin chalcone. The flavonoid pathway then diverges into side branches leading to different classes of flavonoids, including anthocyanins. This study found that red and yellow light-quality selective plastic films could increase the CHS activity, while fruits exposed to the blue film had lower ANS activity at all stages.

The first flavonoid, naringenin, is synthesized by the CHI enzyme. This study found that CHI activity levels were low at the white stage, followed by a sharp increase. The highest activity was observed at the half-red stage, which was followed by a decline. Compared with the white control film, the CHI activity of fruits treated with colored films increased significantly at the half-red stage. Only fruit treated with the blue film experienced a lower CHI activity than with the white film at the red stage.

The last step before the anthocyanin synthesis is catalyzed by ANS, which is responsible for anthocyanidin formation. ANS activity increased during fruit development and remained at high levels for all the treated films, and was consistent with the accumulation of TAC. Compared with fruits grown under the white control film, fruits exposed to the red film exhibited significantly higher ANS activity at all stages of development, while fruits exposed to the green film had lower ANS activity at all stages. Compared with the white control film, the red film increased ANS activity at the white, reddish, half-red, and red stages by 66.26%, 48.12%, 32.46%, and 54.82%, respectively.

TAC and PAL activity increased during fruit development and storage, and more rapidly in ABA-treated fruit (Jiang & Joyce, 2003). Fruits treated with gibberellic acid also experience an increased PAL activity during ripening (Montero et al., 1998). As a result, red-color development is accelerated. Blue light treatment also caused the improvement of PAL, C4H, 4CL, CHS and ANS activity in postharvest strawberry fruits (Xu et al., 2014).

3.3. Effect of colored light-quality selective plastic films on expression of flavonoid genes

To study the transcriptional profiles of flavonoid genes using qPCR, the levels of 12 structural genes leading to flavonoid biosynthesis in strawberries were investigated from the white to red stages after treatment with films of different colors (Fig. 3). The expression of flavonoid genes can be significantly influenced by the colored films.

During the fruit development process, *FaPAL* plays an important role in regulating phenylalanine ammonia-lyase to catalyze phenylalanine into cinnamic acid at the start of the flavonoid biosynthetic pathway. The expression of *FaPAL* in fruits treated with the red and yellow films was significantly greater than with other films at the red stage. Although *PAL* plays a critical role in anthocyanin synthesis, this process does not depend on *PAL* activity when precursors are sufficient (Kayesh et al., 2013).

FaC4H has a low expression in strawberries during fruit development. Treatment with colored films significantly decreased *FaC4H* expression between the reddening and ripening stages. *Fa4CL* expression experienced a declining trend during fruit development. Treatment with colored films significantly increased *Fa4CL* expression before the ripening stages. At the white stage, *Fa4CL* expression was more than doubled in fruit treated with the blue film compared to other films.

The early biosynthetic genes (*FaCHS*, *FaCHI*, and *FaF3H*) are involved in the production of dihydroflavonols, which are the common precursors of flavonols, anthocyanins, and proanthocyanins. There was an increasing trend in early biosynthetic gene

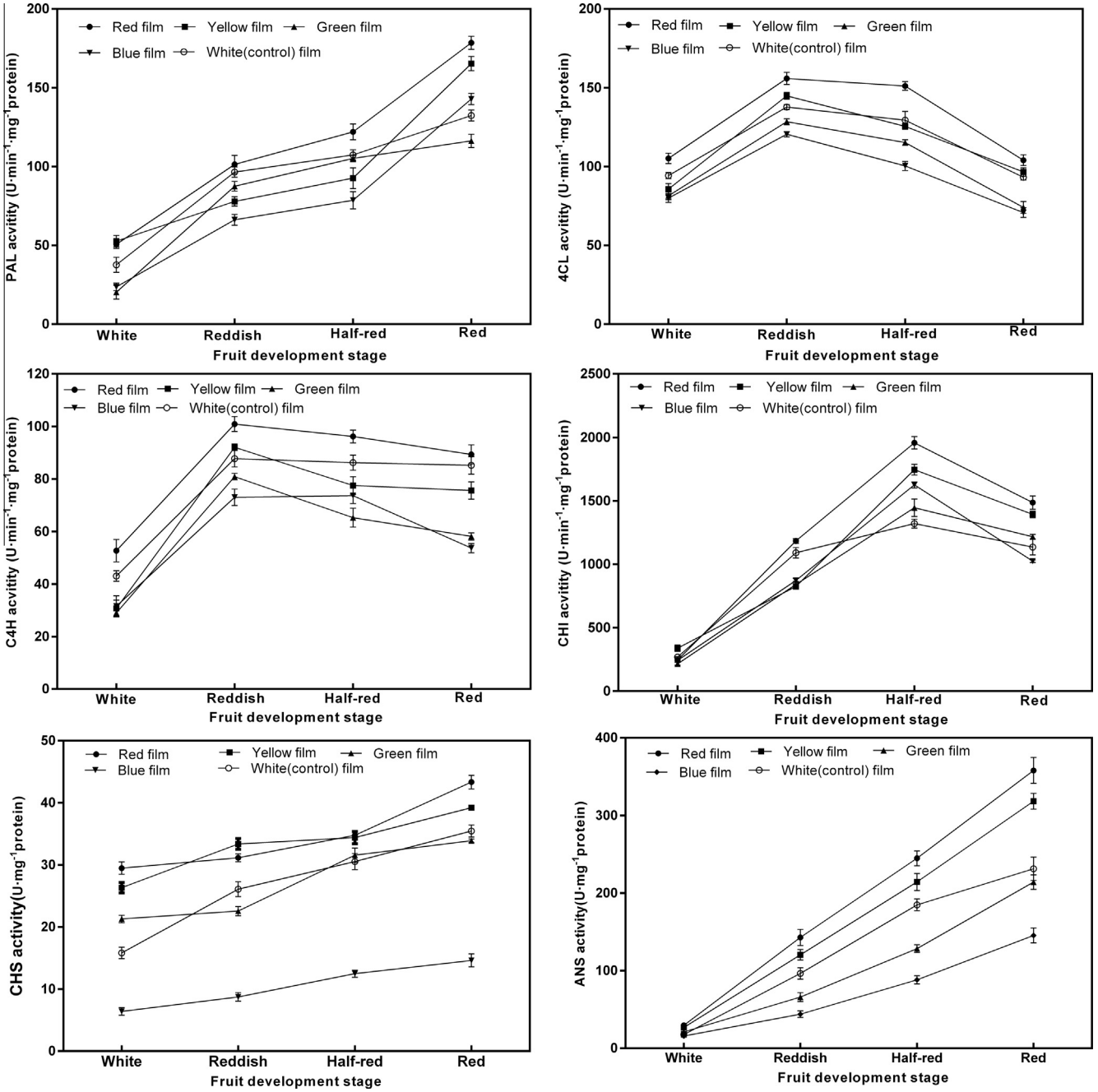

Fig. 2. The effect of red, yellow, green, blue, and white (as the control) film treatments on PAL, C4H, 4CL, CHI, CHS and ANS activity in strawberries during fruit development. Each value is shown by means ± SD. Vertical bars represent the SD of the means.

expression during fruit development, coinciding with the reddening of the fruit. Fruits treated with colored films had lower *FaCHS* transcript levels at the white stage than that of the control, while the use of the green and blue films resulted in a rapid increase in *FaCHS* transcript levels at the half-red stage. According to a previous study, light-treated harvesting fruits also maintained eight-day higher expression levels compared with fruits under the dark control, although both exhibited an increase in *FaCHS* transcript levels (Kadomura-Ishikawa et al., 2015). Fruits treated with colored films had significantly increased *FaCHI* transcript levels at the half-red and red stages. *FaF3H* exhibited an expression pattern similar to that of *FaCHI*.

Dihydroflavonols, the common precursors of flavonols and anthcoyanins, can either be oxidized by *FaFLS* to form flavonols or reduced by *FaDFR* to produce leucoanthocyanidins. At the red stage, fruits treated with the red and yellow films had a relatively lower *FaFLS* expression than fruit grown with the white film. Compared with *FaFLS*, *FaDFR* expression showed slight fluctuations during the fruit development process with a highly relative transcript level. Accordingly, treatment with colored films had slight effects on the expression of *FaDFR*. *FaANS* is the key gene in the conversion of leucoanthocyanidins to anthocyanidins. The expression of *FaANS* experienced an increasing trend during fruit development. Compared with the white film, the colored films led to significant increases in *FaANS* expression at the half-red and ripening stages.

Leucoanthocyanidins and anthocyanidins, the products of the enzymatic reactions catalyzed by DFR and ANS, can be converted into proanthocyanidins precursors by the LAR and ANR enzymes,

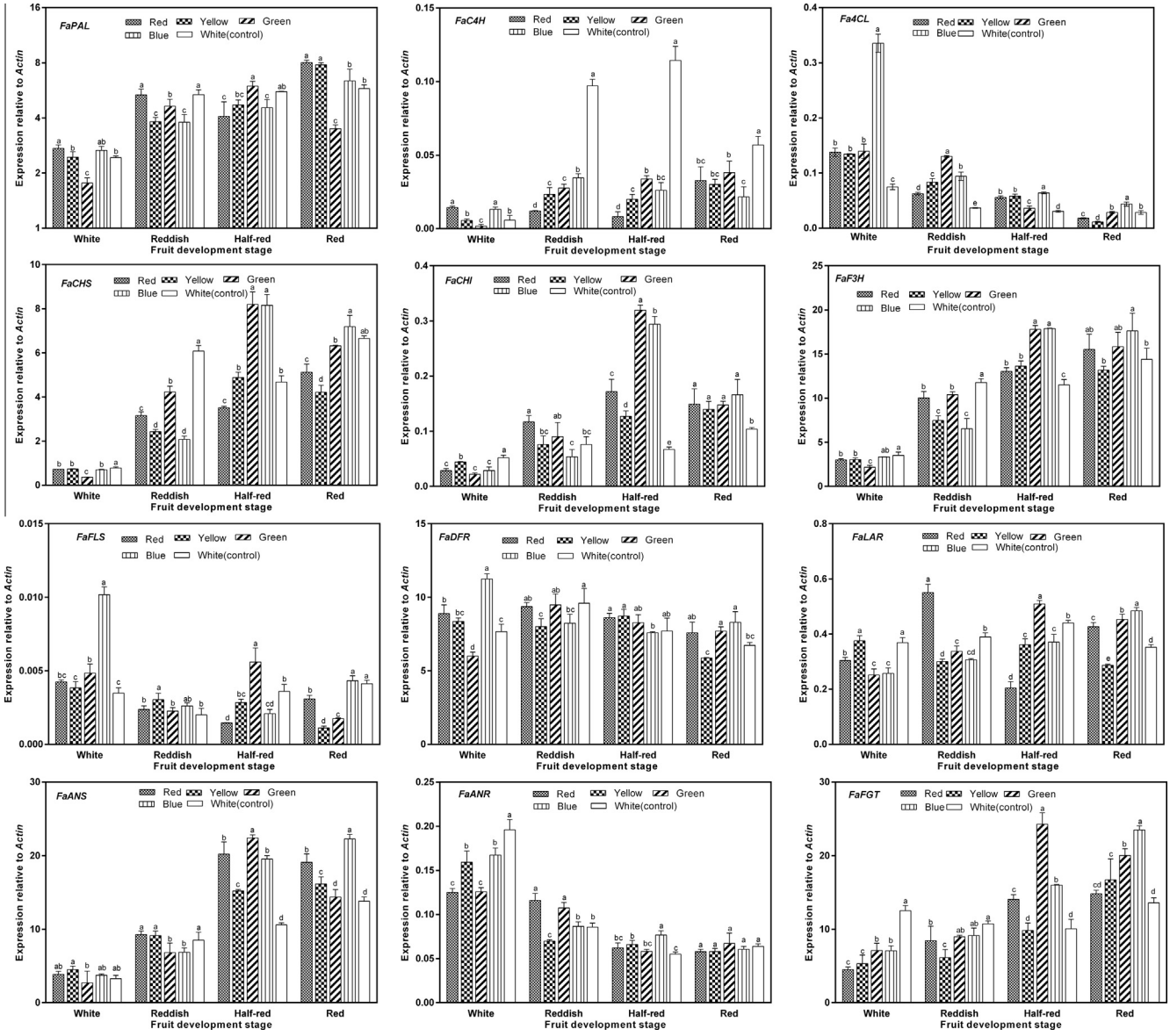

Fig. 3. The effect of red, yellow, green, blue, and white (as the control) film treatments on the expression of flavonoid biosynthetic genes in strawberries during fruit development. Each value is shown by means ± SD. Different letters on the bars indicate treatments were significantly different at $p < 0.05$.

respectively. These two reactions produce catechin- and epicatechin-based proanthocyanidins, which accounted for 44% and 56% of total proanthocyanidins, respectively (Schaart et al., 2013). Proanthocyanidins are localized throughout the fruit tissue and gradually decrease during fruit development (Almeida et al., 2007; Schaart et al., 2013). In this study, *FaLAR* expression increased initially, peaked at the half-red stage, and then followed a downward trend with the white film. Fruit treated with the red and yellow films resulted in earlier peaking of *FaLAR* expression, whereas the use of the blue film resulted in a steadily increasing expression. Fruit treated with the green film experienced a trend similar to that of the white film. *FaANR* expression coincided with proanthocyanidins accumulation during fruit development (Schaart et al., 2013). The use of colored films significantly decreased the *FaANR* transcript levels at the white stage. This effect gradually became smaller, and there were no differences at the red stage among the films.

FaFGT is the key gene in the conversion of leucoanthocyanidins to anthocyanidins. Concerning glycosylation of flavonoid end products, *FaFGT* has been found to display strong 3-O-glucosyltransferase activity on both tested anthocyanidins, while flavonols seem to be only a minor substrate. Consistent with previous reports, the strong up-regulation of *FaFGT* throughout fruit-ripening stages leads to the accumulation of anthocyanins, such as Pg3G and Cy3G, in strawberries (Almeida et al., 2007). Compared with the white film, the use of colored films in this study resulted in lower transcript levels at the white and reddish stages, in which fruits were just beginning to turn red. However, treatment with colored films significantly increased *FaFGT* expression at the half-red and ripening stages.

These results suggest that treatment with films of different colors can regulate the expression of structural genes (*i.e.*, *FaPAL*, *FaCHS*, *FaCHI*, *FaF3H*, *FaANS*, and *FaFGT*) involved in the flavonoid biosynthesis pathway in strawberries. Accordingly, the TAC of fruits treated with the red and yellow films was higher than with the white film, while the green and blue films led to significantly lower TAC than that of the white film. The proportions of individual anthocyanins in strawberry fruits were also influenced.

3.4. Effect of colored light- quality selective plastic films on expression of transcription factor genes

In addition to the structural genes, the expression of anthocyanin biosynthetic genes are specifically induced by a ternary protein complex composed of R2R3-MYB, bHLH, and WD40 (MBW complexes), which are well-conserved in higher plants (Li, 2014; Schaart et al., 2013; Xu, Dubos, & Lepiniec, 2015). These proteins have been shown to control multiple enzymatic steps in the biosynthetic pathway responsible for the production of flavonoids, important secondary metabolites. In *Arabidopsis thaliana*, the MBW complexes are composed of AtTT2 (AtMYB123), AtTT8 (AtbHLH042), and AtTTG1. In strawberries, *FaMYB9*/*FaMYB11*, *FabHLH3*, and *FaTTG1* are the respective functional homologues of *AtTT2*, *AtTT8*, and *AtTTG1* (Schaart et al., 2013). *FaMYB10* plays a major role in the regulation of flavonoid/phenylpropanoid metabolism during the ripening of *Fragaria* × *ananassa* fruits, while *FaMYB1* plays a key role in down-regulating the biosynthesis of anthocyanins and flavonols (Aharoni et al., 2001; Paolocci et al., 2011). *FaMYB5* and *FabHLH3*Δ are also considered negative regulators (Schaart et al., 2013). To determine the levels of transcription factor genes during strawberry fruit development after treatment with colored films, the expressions of *FaMYB10*, *FaMYB1*, *FaMYB5*, *FaMYB9*, *FabHLH3*, and *FaTTG1* were measured using qPCR (Fig. 4).

FaMYB10 was defined as a true, specific receptacle-specific gene that is related to ripening and is not or lowly expressed in other strawberry vegetative tissues. The expression of *MYB10* can be induced by high light intensity (Lin-Wang et al., 2010). Because of the low light intensity in this study, the expression of *FaMYB10* was very low in the early developmental stages of the fruit but increased substantially during the ripening stages, coinciding with the reddening of the fruit. Fruits treated with the yellow film had a significantly greater expression of *FaMYB10* at the white stage than with the white film, while no significant differences were exhibited between the other films. At the reddish stage, fruits treated with the red and green films had remarkably greater *FaMYB10* expression than with the white film. At the half-red stage, the accumulation of the transcript of *FaMYB10* with the colored films was significantly greater than with the white control film. There was no significant difference among the films at the red stage, with the exception of the blue film. Overall, these results suggest that the activation of *FaMYB10* by colored films may occur before the red stage. *FaMYB10* transcript levels have also been found to increase significantly with light, indicating that *FaMYB10* expression is positively regulated by light after fruit harvesting (Kadomura-Ishikawa et al., 2015). However, *FaMYB10* only affected Cy3G content but not Pg3G content in strawberry fruits, suggesting the role of *FaMYB10* was different before and after harvest.

FaMYB1, which is expressed in the red ripe strawberry fruit similarly to *FaMYB10*, plays a key role in negatively regulating the metabolic levels of anthocyanins and flavonols in various flavonoid biosynthetic branches (Aharoni et al., 2001; Lin-Wang et al., 2010; Paolocci et al., 2011). It counterbalances the activity of the endogenous transcriptional MBW complex, promoting proanthocyanidin biosynthesis via the catechin and epicatechin branches and repressing transcription in order to balance the levels of anthocyanin pigments produced in the latter stages of strawberry fruit maturation (Aharoni et al., 2001). *FaMYB1* showed an expression pattern similar to that in the literature, with the highest transcription level at the ripe fruit stage (Aharoni et al., 2001; Lin-Wang et al., 2010). This study found that fruits treated with colored films experienced increased *FaMYB1* expression after turning reddish and had the highest expression at the red stage. In contrast, a high light intensity cannot induce the expression of *FvMYB1* in woodland strawberries (Lin-Wang et al., 2010).

FaMYB5 is another known negative regulator in the biosynthesis of flavonoids (Schaart et al., 2013). Unlike *FaMYB1*, the expression of *FaMYB5* experienced slight fluctuations during fruit development and treatment. Compared with the white control film, the colored films led to increases in the transcription level at the red stage.

In strawberries, *FaMYB9*, *FabHLH3*, and *FaTTG1* are components of the MBW complexes that regulate the biosynthesis of anthocyanins and flavonols (Schaart et al., 2013). *FaMYB9* expression significantly increased during all development stages in fruits treated with the red and yellow films. Consistent with this result, fruits treated with the red and yellow films had greater TAC accumulation than that of other films. However, the expression of *TT8* (homologues of *FabHLH3*) was significantly reduced under low light in *Arabidopsis* leaves (Rowan et al., 2009). The present results

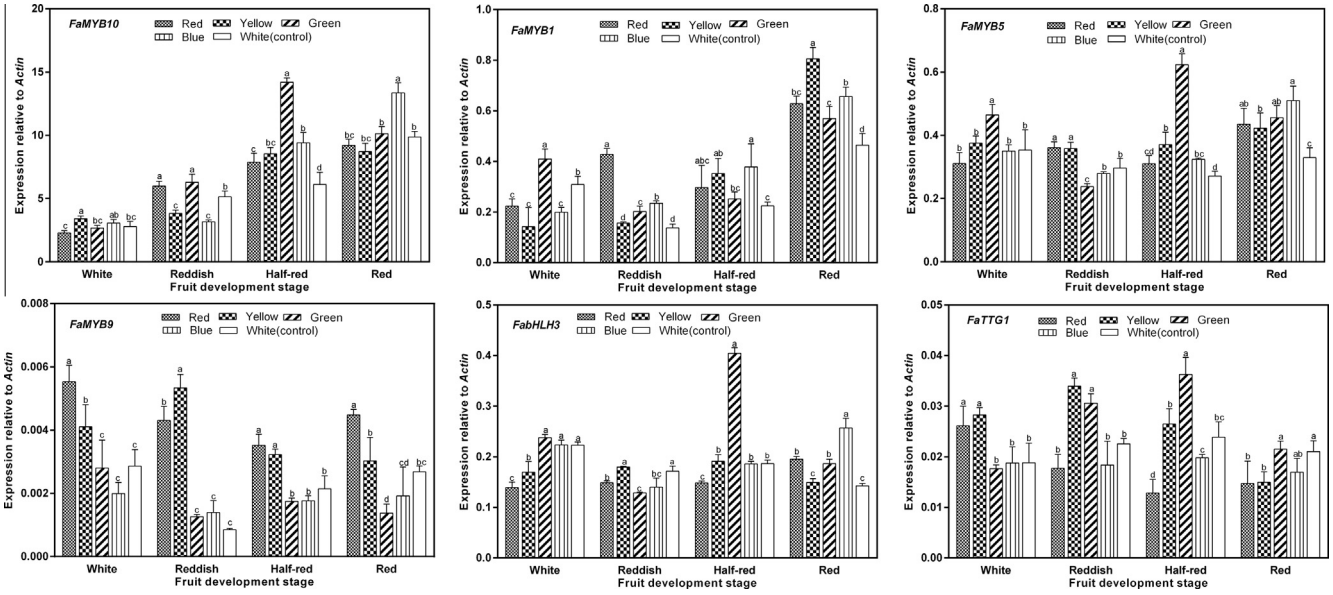

Fig. 4. The effect of red, yellow, green, blue, and white (as the control) films on the expression of transcription factor genes in strawberries during fruit development. Each values is shown by means ± SD. Different letters on the bars indicate treatments were significantly different at $p < 0.05$.

showed that transcript levels of *FabHLH3* experienced slight fluctuations during strawberry fruit development. Treatment with colored films had a slight influence on the expression of *FabHLH3*, and the green films in particular led to greater expression at the half-red stage. The expression of *FaTTG1* also had slight fluctuations during the entire fruit development process. Fruits treated with the yellow and green films experienced increased *FaTTG1* transcription levels at the reddish and half-red stages. In nectarines, bHLH3 but not WD40, was shown to be up-regulated after light treatment (Zoratti et al., 2014).

Additionally, some post-translational modifications of MBW proteins have been reported to fine-tune the transcriptional activity of MBW complexes (Li, 2014; Xu et al., 2015). These proteins participate in the flavonoid pathway via directly interacting with the components of the MBW complex. MYBL2 can interact with different bHLHs (*e.g.*, MYC1, EGL3, GL3, and TT8) by competing with R2R3-MYBs to inhibit the activity of the MBW complex and thus negatively regulate anthocyanin biosynthesis in response to developmental and environmental stimuli. SPL9 negatively regulates anthocyanin accumulation via destabilizing the MBW complex. TT1 interacts with TT2 and positively regulates proanthocyanidin accumulation in endothelial cells of the seed coat. TCP3 can enhance flavonoid biosynthesis by strengthening MBW activity through its interaction with both R2R3-MYBs and MYBL2.

4. Conclusions

This study found that colored light-quality selective plastic films, especially red and yellow films, can affect anthocyanin content in strawberries by altering related enzymes, the flavonoid pathway and transcription factor genes. Treatment with red and yellow light-quality selective plastic films might be useful as a supplemental cultivation practice for developmental strawberry fruit to improve anthocyanin content.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2016.02.077.

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