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ORIGINAL ARTICLE

Synergistic effects of light and temperature on anthocyanin biosynthesis in callus cultures of red-fleshed apple (*Malus sieversii* f. *niedzwetzkyana*)

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Abstract We established a red callus from the leaves of a red-fleshed apple individual, which was a hybrid offspring of the cross between *Malus sieversii* f. *niedzwetzkyana* and *Malus domestica* cv. 'Fuji'. We analyzed callus growth and anthocyanin biosynthesis/metabolism under different combinations of temperature and light conditions. Incubation in darkness resulted in decreased anthocyanin accumulation, while it promoted callus growth. Exposure to light and low temperature (16 °C) induced the expression of *MYB10* and *bHLH3/33*, which are responsible for coordinating the regulation of anthocyanin biosynthesis, as well as the expression of other structural genes. Treatments with light and high temperature (32 °C) induced *MYB16* expression, which repressed anthocyanin biosynthesis. Additionally, low temperature (16 °C) inhibited the expression of *MYB111*. We analyzed the expression patterns of MYB and bHLH transcription factor genes by quantitative real-time polymerase chain reaction. Our data suggest that light-induced regulation of anthocyanin biosynthesis is primarily caused by altered *MYB10* transcript levels, while temperature-induced regulation is the result of changes to the expression of *bHLH3/33*, *MYB16*, *MYB17*, *MYB111*, and other repressors. In conclusion, we investigated the reciprocal effects of light and temperature on anthocyanin biosynthesis in red-fleshed apple calli. Our findings may provide a theoretical basis for breeding red-fleshed apple varieties with high anthocyanin contents.

Keywords *Malus sieversii* f. *niedzwetzkyana* · Callus · Anthocyanin · Light · Temperature

Introduction

Anthocyanins are water-soluble natural pigments that provide color to various plant tissues and organs (Horbowicz et al. 2008). They also function as natural antioxidants that provide protection from free radicals and other harmful substances. Additionally, anthocyanins enhance vascular elasticity, prevent cardiovascular disease, and protect the liver from damage (Winkel-Shirley 2001; Regan et al. 2001; Schaefer et al. 2008; Butelli et al. 2008). In apple (*Malus domestica* Borkh.), the health benefits of flavonoids and anthocyanins have been investigated (Szankowski et al. 2009; Balasuriya and Rupasinghe 2012). The anthocyanin content in the pulp of most cultivated apples is very low and unstable (Nie et al. 2010). *Malus sieversii* f. *niedzwetzkyana* is a red-fleshed variant of *M. sieversii*. Its branches, leaves, flowers, fruit skin, and pulp are all red, with extremely high anthocyanin concentrations and considerable health benefits (Wang et al. 2010). Therefore, investigating the mechanism regulating anthocyanin biosynthesis in *M. sieversii* f. *niedzwetzkyana* is warranted, and may promote the breeding of red-fleshed apple varieties with high anthocyanin contents. Additional studies may also provide novel information regarding the genetics and diversity of cultivated apple species.

Nan Wang and Zongying Zhang have contributed equally to this work.

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Anthocyanin biosynthesis is a process involving the coordinated expression of transcription factors (TFs) and structural genes (Dixon and Steele 1999; Takos et al. 2006). The MYB (*ZmC1*) and bHLH (*ZmR* and *ZmB*) TFs regulating anthocyanin biosynthesis were first detected in maize, and were subsequently isolated from petunia and *Arabidopsis thaliana* (Paz-Ares et al. 1988; Chandler et al. 1989; Quattrocchio et al. 1993; Borevitz et al. 2000). Investigations focused on anthocyanin biosynthesis in apple species occurred after these initial studies. The *MdMYB1* and *MdMYBA* TFs were first isolated from apple skin, and were observed to regulate anthocyanin biosynthesis (Takos et al. 2006; Ban et al. 2007). The expression of *MdMYB1* and *MdMYBA* is strongly induced by light. The *MdMYB10* TF, which regulates color development in red apples, was isolated and identified from a red-fleshed apple cultivar (i.e., 'Red Field'). Its regulatory activities require *MdbHLH3* and *MdbHLH33* (Espley et al. 2007).

In addition to genotype effects, environmental factors (e.g., light and temperature) play a crucial role in the accumulation of anthocyanins and endogenous hormones (Chandler et al. 1989; Quattrocchio et al. 1993; Borevitz et al. 2000; Solfanelli et al. 2006). In *A. thaliana*, the expression of *MYB* (*PAP1* and *PAP2*) and *bHLH* (*TT8*, *EGL3*, and *GL3*) TF genes involved in anthocyanin biosynthesis is induced by light (Cominelli et al. 2008). In contrast, high temperature can inhibit the expression of *EGL3*, *TTG1*, and *TT8*, thus inhibiting the biosynthesis and accumulation of anthocyanins (Rowan et al. 2009). Low temperature and light intensity have a synergistic effect on the expression of genes in the flavonoid biosynthesis pathway in grape berry skin (Azuma et al. 2012). In apple fruit skin, high intensity light can induce the expression of *MdMYB1* and promote the accumulation of anthocyanins (Takos et al. 2006). Low temperatures and UV-B irradiation can significantly increase the expression of related genes to promote the accumulation of anthocyanins in apple fruit skin (Ubi et al. 2006). Results of a previous study indicated that high temperatures inhibit *MYB10* expression, resulting in a decrease in the biosynthesis and accumulation of anthocyanins (Lin-Wang et al. 2011).

To date, the light- and temperature-regulated mechanisms controlling anthocyanin biosynthesis have been partially characterized. However, because of variabilities in growth position, developmental phase, and nutritional status, it is difficult to accurately analyze the effects of environmental factors on anthocyanin biosynthesis in fruits. Furthermore, light, temperature, and other environmental conditions cannot be controlled during field experiments, and exactly which TFs are regulated by environmental conditions is currently unclear. Consequently, there have been few reports describing the relationship between light and temperature effects on anthocyanin accumulation in *M. sieversii* f. *niedzwetzkyana* red callus tissue. How structural genes and the MYB and bHLH TFs respond to various combinations of light and temperature to regulate anthocyanin biosynthesis has not been comprehensively investigated.

We used *M. sieversii* f. *niedzwetzkyana* germplasm available from the Luntai National Fruit Germplasm Resources Garden (Xinjiang Academy of Agricultural Science) as the parents to generate hybrids in 2006. Wang et al. (2010) studied a red-fleshed apple individual ('Zihong 1') obtained from segregating populations, and determined that its flesh contains extremely high anthocyanin contents. Ji et al. (2015) induced callus formation using a red-fleshed apple strain, and observed that increasing auxin concentrations can significantly inhibit anthocyanin biosynthesis. In this study, we generated a red callus using the leaves of 'Zihong 1' apple trees. The red callus responded to environmental changes. This callus tissue represented an uncommon material for studying the regulation of anthocyanin biosynthesis in red-fleshed apples. We investigated the effects of different temperature and light conditions on anthocyanin metabolism in red callus tissue, as well as the expression of related genes. Our objective was to generate novel molecular information regarding anthocyanin biosynthesis regulated by light and temperature conditions, which may be useful for the breeding of new red-fleshed apple cultivars.

Materials and methods

Plant materials and callus induction

The F_1 hybrid population of the cross between *M. sieversii* f. *niedzwetzkyana* and *M. domestica* cv. 'Fuji' was grown at the Tai'an Hengling Fruit Tree Breeding Base of Shandong Agricultural University (36° 260 N, 117° 290 E). We used young 'Zihong 1' leaves as explants for callus cultures (Fig. 1a). The red callus culturing method was based on a published procedure (Ji et al. 2015). The callus induction medium consisted of Murashige and Skoog medium supplemented with 0.6 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 0.5 mg l^{-1} thidiazuron, 30 g l^{-1} sugar, and 7 g l^{-1} agar. The pH was adjusted to 5.8 ± 0.1. Surface-sterilized leaves were first cultured at 24 °C for 15 days in darkness, and then cultured under light (16-h light/8-h dark; intensity: 1000–2000 lx). The red callus was finally induced and subcultured every 20 days (Fig. 1c).

Determination of callus growth

The callus/cell cultures (fresh weight: 0.15 g) were transferred to new culture flasks containing Murashige and

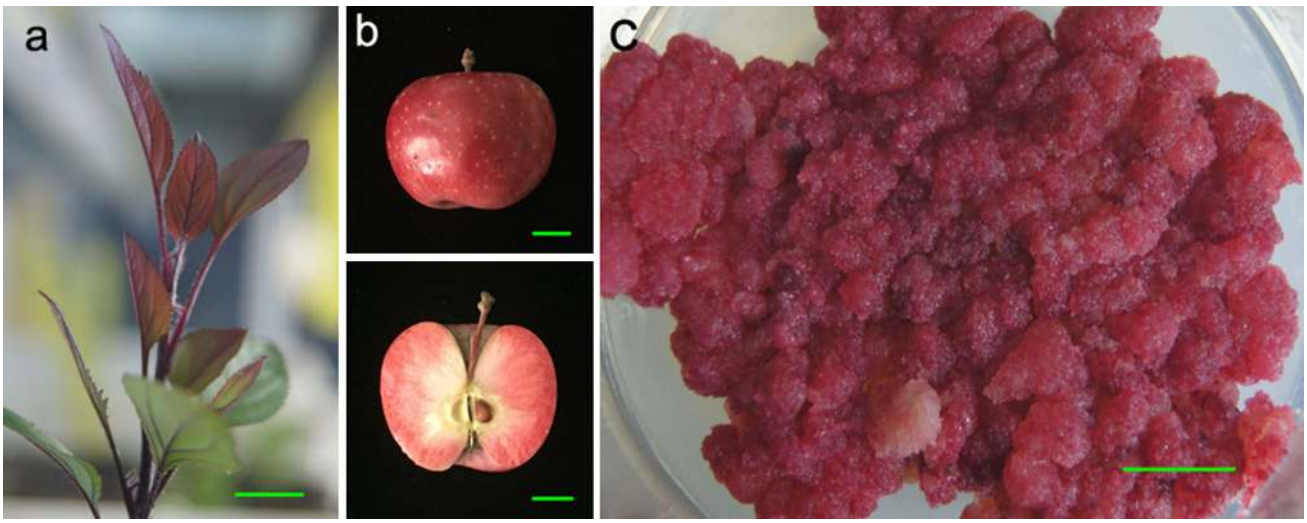

Fig. 1 Establishment of red-fleshed apple callus. **a** Leaves of red-fleshed apples used as explants for callus culture. *Bar* 1 cm. **b** Red-fleshed apples in the F_1 population. *Bar* 1 cm. **c** Red callus induced from red-fleshed apples. *Bar* 1 cm

Skoog medium. The calli were incubated under the following temperature and light conditions: 16 °C/light, 24 °C/light, 32 °C/light, 16 °C/dark (shade treatment), 24 °C/dark, and 32 °C/dark. Other growth parameters were the same for all cultures. The callus was harvested at 0, 5, 10, 15, and 20 days after the initiation of the cultures. Three culture flasks per treatment (i.e., biological replicates) were collected at each time point. The callus fresh weight was determined before samples were frozen in liquid nitrogen and stored at −80 °C until analyzed.

Measurement of relative anthocyanin contents

Callus samples frozen with liquid nitrogen were ground into a fine powder, and 0.5 g ground material was incubated in 15 ml 1 % (v/v) HCl-methanol for 24 h at 4 °C in darkness. Samples were centrifuged at 8000×*g* for 10 min, and the upper aqueous phase was subjected to spectrophotometric analysis at 530 nm using a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan). Relative anthocyanin contents were calculated as follows: absorbance (at 530 nm)/fresh weight (g).

HPLC analysis of anthocyanin extracts

Anthocyanin was extracted from 1 g powdered callus tissue. Samples were incubated in 5 ml 1 % (v/v) HCl-methanol for 2 h at 4 °C in darkness, and then centrifuged at 8000×*g* for 15 min. The upper aqueous phase was saved, and the pelleted material was incubated in 5 ml of 1 % (v/v) HCl-methanol for 1 h at 4 °C in darkness. Samples were centrifuged as before, and the two upper aqueous phases were combined and concentrated using a RE-52AA rotary evaporator (YaRong, Shanghai, China). Concentrated samples were rinsed in 2–3 ml methanol, transferred to 10-ml test tubes, and centrifuged at 8000×*g* for 20 min. The upper aqueous phase was diluted with methanol to 5 ml and filtered through an organic filter membrane (pore size: 0.2 μm). The HPLC analysis of anthocyanin extracts was conducted using an ACQUITY UPLC System (Waters Corporation, USA) with a BEH C18 chromatographic column (100 mm × 2.1 mm). The column particle size was 1.7 μm. Samples were eluted at a column temperature of 45 °C using a flow rate of 0.3 ml min^{-1}. The mobile phases consisted of solvent A (acetonitrile) and solvent B [formic acid/water, 1:500 (v/v)] in the following gradient: 0–0.1 min, 5 % solvent A; 20 min, 20 % solvent A; 22 min, 80 % solvent A; 21 min, 5 % solvent A; and 25 min, 5 % solvent A. The HPLC eluates were monitored spectrophotometrically (530 nm).

Phylogenetic analysis and protein sequence alignment of transcription factors

A total of 24 MYB TFs from apple and other plant species (e.g., *A. thaliana*, *Fragaria ananassa*, *Vitis vinifera*, *Zea mays*, and *Petunia hybrida*) were used for phylogenetic analysis and protein sequence alignment. Half of the TFs were reported to promote anthocyanin biosynthesis, while the other half were known repressors of anthocyanin biosynthesis. Full-length TF protein sequences were obtained from the NCBI database, and aligned using DNAMAN software. Phylogenetic analyses were conducted using MEGA5.1 software with 1000 bootstrap replicates after aligning sequences with ClustalW (opening = 10, extension = 0.2).

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated from callus tissues using an RNAprep Pure Plant kit (Tiangen, Beijing, China). The concentration and quality of the purified RNA were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). First-strand cDNA was synthesized from 1 μg total RNA using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Hanover, MD, USA). The cDNA samples were stored at −20 °C until used.

The quantitative real-time polymerase chain reaction (qRT-PCR) primers were designed using the Beacon Designer 7 program (Table S1). Primers were synthesized by Sangon Biotech (Shanghai, China) and purified by polyacrylamide gel electrophoresis. The qRT-PCR was conducted using tenfold diluted cDNA samples as templates, the SYBR Green PCR Master Mix (TransGen Biotech, Beijing, China), and the iCycler iQ5 system (Bio-Rad, Hercules, CA). The *MdActin* gene served as an internal control, and the relative quantities of mRNA were calculated using the $2^{-\Delta\Delta Ct}$ method of the IQ5 2.0 program (Livak and Schmittgen 2001).

Results

Comparative analysis of callus growth under various conditions

We observed significant differences in the growth of the red calli exposed to different light conditions and temperatures (Fig. 2). Callus growth was greatest at 24 °C/dark, followed by 24 °C/light, 32 °C/dark, 32 °C/light, 16 °C/dark, and 16 °C/light. The growth was inhibited at 32 and 16 °C, indicating that high and low temperatures can inhibit callus growth, although the inhibitory effect was particularly evident at the lower temperature. The fresh weights of calli incubated at 16 °C for 20 days were eightfold lower than the fresh weights of calli incubated at 24 °C. Additionally, we observed that at all temperatures, the fresh weight of calli grown in darkness was higher than that of calli incubated under light. This finding indicated that a lack of light was conducive to cell division and callus growth.

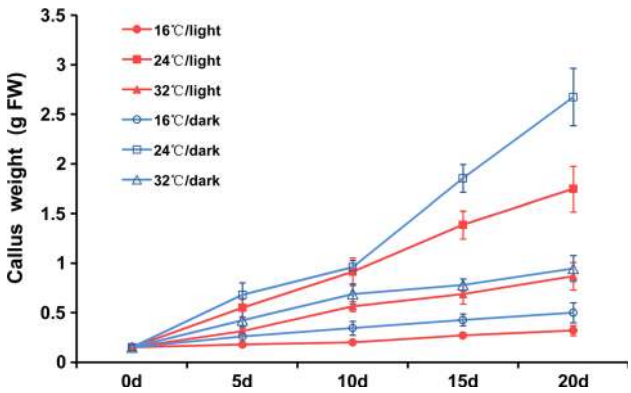

Fig. 2 Callus weight changes under different culture conditions. During the 20-day culture period, calli were harvested and weighed on days 0, 5, 10, 15, and 20

Comparative analysis and measurement of dynamic anthocyanin levels

To investigate the dynamic anthocyanin levels during the 20-day culture period, the absorbance (at 530 nm) of anthocyanin extracts was measured at different time points. We observed significant differences in the callus pigment and anthocyanin contents (Fig. 3). After a 20-day incubation under light, anthocyanins had considerably accumulated at 16 °C, and the calli appeared purple. As the temperature increased, the redness of the calli faded (Fig. 3a). All calli incubated in darkness were yellow. The relative anthocyanin contents over 20 days are presented in Fig. 3b. The anthocyanin contents continuously decreased in calli incubated in darkness. In contrast, in calli grown under light, the anthocyanin levels initially decreased, but then slowly increased. The callus anthocyanin levels on day 20 were highest at 16 °C/light, followed by 24 °C/light, 32 °C/light, 16 °C/dark, 24 °C/dark, and 32 °C/dark. These results suggest that under the same light condition, low (16 °C) and high (32 °C) temperatures can promote and inhibit anthocyanin biosynthesis, respectively. The anthocyanin content of calli incubated in darkness was very low, especially at 32 °C, and the relative content on day 20 was only 0.06, which was 27-fold lower than that of calli incubated under light.

A scatter diagram was generated to analyze the correlation between relative anthocyanin content and callus growth rate (Fig. 4). Higher callus anthocyanin contents tended to correspond with lower callus growth rates, suggesting there was a negative correlation between callus anthocyanin content and growth rate.

HPLC analysis of callus cyanidin 3-*O*-galactoside content

There is currently very little diversity regarding apple anthocyanins. Cyanidin 3-*O*-galactoside is the main apple anthocyanin, accounting for 80 % of the total anthocyanin content (Treutter 2001). Therefore, the callus cyanidin-3-galactoside contents were determined (Fig. 5a). According to our HPLC analysis, the retention time of cyanidin-3-

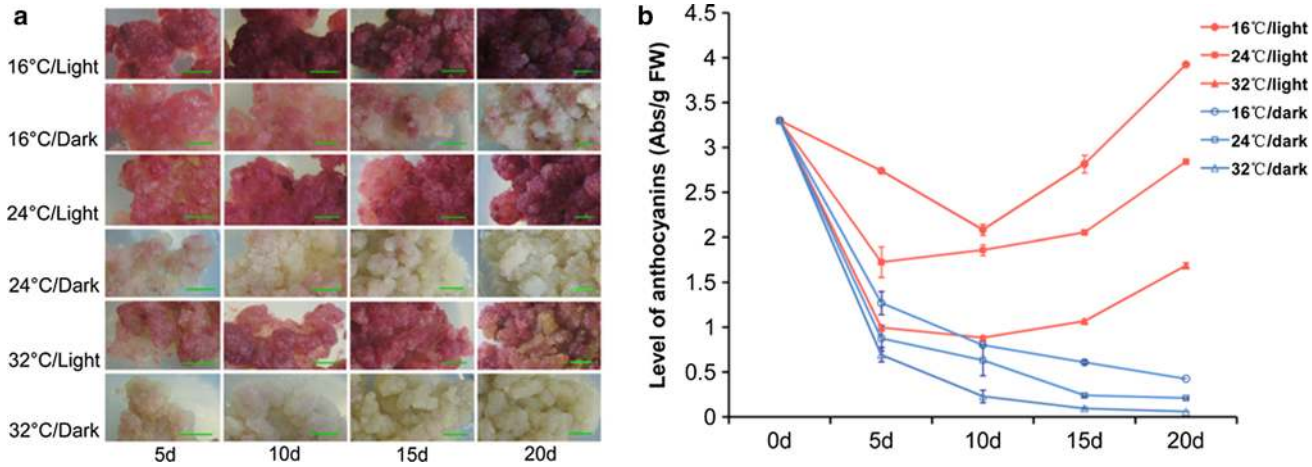

Fig. 3 Relative callus anthocyanin contents at different temperature and light conditions. **a** Changes in callus color under different conditions and culture stages. *Bar* 1 cm. **b** Relative anthocyanin contents over 20 days. The relative anthocyanin content was calculated as follows: absorbance (at 530 nm)/fresh weight (g)

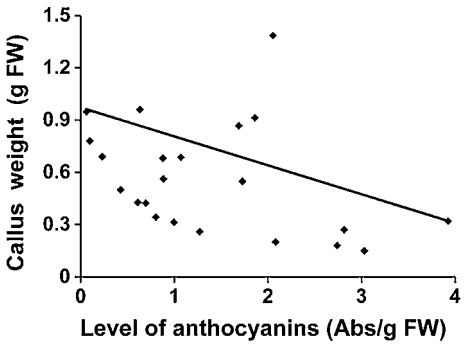

Fig. 4 Correlation between relative anthocyanin content and callus growth rate

galactoside was about 13 min (Fig. 5b). Figure 5c provides a close-up view of the region indicated by a red box in Fig. 5b. There was significant variability among the peak areas for the various treatments, with the largest differences observed for calli incubated at 16 °C/light (a), followed by calli grown at 24 °C/light (b), 32 °C/light (c), 16 °C/dark (d), 24 °C/dark (e), and 32 °C/dark (f). These results were consistent with the differences in anthocyanin contents. The absolute cyanidin-3-galactoside content was calculated by comparing the peak areas of treated calli with those of standard samples. We observed that calli incubated at 16 °C/light accumulated the most cyanidin-3-galactoside (up to 695.55 μg g^{-1}). This abundance was 1.8-fold higher than that of calli cultured at 24 °C/light, and threefold higher than that of calli grown at 32 °C/light (Table 1).

Phylogenetic analysis and multiple sequence alignment of MYB transcription factors

Many studies have revealed that the anthocyanin biosynthesis pathway is affected by MYB TFs, which can function as inducers or repressors. In *A. thaliana*, *MYB75* and *MYB90* promote anthocyanin biosynthesis, and the expression of their genes is induced by light (Borevitz et al. 2000). In contrast, *MYB3*, *MYB4*, and *MYB6* suppress anthocyanin accumulation (Zhou et al. 2015). These known *A. thaliana* MYBs were used as queries in BLAST searches of various plant genomes, including the apple genome, to identify MYB homologs (Fig. 6). The phylogenetic tree indicated that *MdMYB10* and *MdMYB1* are homologous to *PyMYB10*, *VvMYBA1*, and *AtMYB75*, which positively regulate anthocyanin biosynthesis. A multiple sequence alignment revealed that the anthocyanin biosynthesis-promoting TFs contain the R2R3 domain and bHLH motif (Fig. 6c). We also determined that *MdMYB16*, *MdMYB17*, and *MdMYB111* are homologous to *PyMYB6*, *VvMYB4*, *AtMYB3*, and *AtMYB4*, which negatively regulate anthocyanin biosynthesis. According to a multiple sequence alignment, these TFs contain the subgroup 4 ($pdLNL^{D}/_{E}L$) suppression motif (Stracke et al. 2001) (Fig. 6d).

Expression levels of anthocyanin pathway genes and related transcription factors

We analyzed the expression levels of six structural genes from the anthocyanin biosynthesis pathway (i.e., *CHI*, *CHS*, *F3H*, *LDOX*, *UFGT*, and *DFR*) in calli exposed to different culture conditions for 20 days (Fig. 7). Under the same light conditions, all of these structural genes were most highly expression at 16 °C. The expression levels in calli incubated at 16 °C/light were 1.2–2-fold higher than in calli incubated at 24 °C. Additionally, the expression levels were 2–10-fold higher at 16 °C/light than at 32 °C. In contrast, the expression levels of structural genes (except *CHI*) were significantly inhibited at 32 °C. The *CHS*, *F3H*,

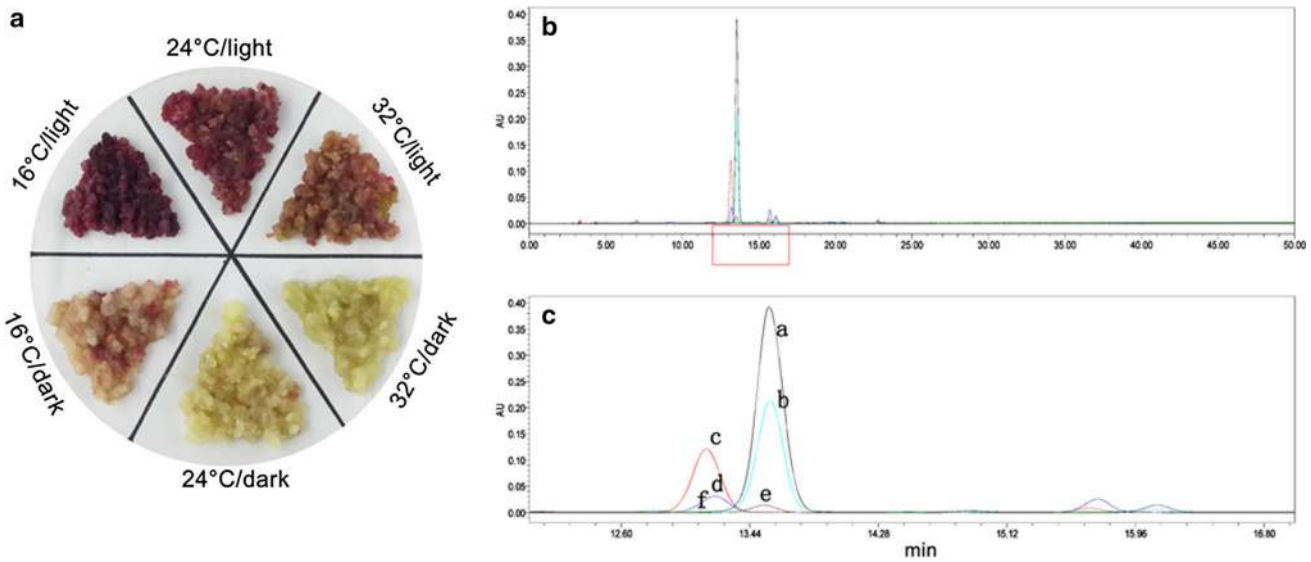

Fig. 5 HPLC chromatograms of anthocyanins from calli grown under different culture conditions. **a** Comparison of the colors of calli treated with different conditions for 20 days. **b** HPLC graph of the cyanidin-3-galactoside content of calli exposed to different treatments. **c** Close-up view of the region in the *red box* in (B), with (*a*)–(*f*) indicating the following treatments: (*a*) 16 °C/light, (*b*) 24 °C/light, (*c*) 32 °C/light, (*d*) 16 °C/dark, (*e*) 24 °C/dark, and (*f*) 32 °C/dark

Table 1 HPLC datas and cyanidin 3-*O*-galactoside content of each callus

Sample	Retention time (min)	Peak area	Concentration (ug/ml)	Cyanidin 3-*O*-galactoside content (ug/g)
Standard sample[a]	13.444	3,464,065	100.00	–
16 °C/light	13.566	4,818,866	139.11	695.55
24 °C/light	13.573	2,618,632	75.59	377.97
32 °C/light	13.158	1,602,026	46.25	231.23
16 °C/dark	13.213	413,458	11.94	59.68
24 °C/dark	12.866	55,156	1.59	7.96
32 °C/dark	12.912	14,398	0.42	2.08

[a] Cyaniding 3-*O*-galactoside standards (Sigma chemical, St, Louis, USA)

LDOX, *UFGT*, and *DFR* expression levels at 32 °C were approximately 7-, 2-, 3-, 3-, and 2-fold lower than at 24 °C, and 11-, 3-, 5-, 5-, and 3-fold lower than at 16 °C, respectively. In calli incubated in darkness, the expression levels of all of the analyzed structural genes were relatively low.

To validate the regulatory mechanism of TFs during anthocyanin biosynthesis under different light and temperature conditions, the expression levels of MYB TF (*MYB10*, *MYB16*, *MYB17*, and *MYB111*) and bHLH TF (*bHLH3* and *bHLH33*) genes were analyzed by qRT-PCR (Fig. 8). The *MYB10* transcript levels were higher in calli incubated under light than in calli grown in darkness (i.e., approximately 17-, 6-, and 13-fold higher at 16, 24, and 32 °C, respectively). Additionally, temperature also affected the *MYB10* expression levels under light, with 16 °C inducing the highest expression levels and 32 °C inhibiting expression. *MYB16*, *MYB17*, and *MYB111* were speculated to be anthocyanin biosynthesis repressors. The highest *MYB16* expression level was observed at 32 °C/light, and was about 2–3-fold higher than at other temperature and light conditions. *MYB111* expression was inhibited by low temperature under light and dark conditions, which promoted the accumulation of anthocyanins. The *MYB17* expression levels were higher in calli incubated in darkness, with 32 °C inducing the highest expression levels. BHLH is another important TF family involved in regulating anthocyanin biosynthesis. The *bHLH3* and *bHLH33* expression levels were highest at 16 °C, and were approximately twofold higher than at other temperatures (Fig. 8).

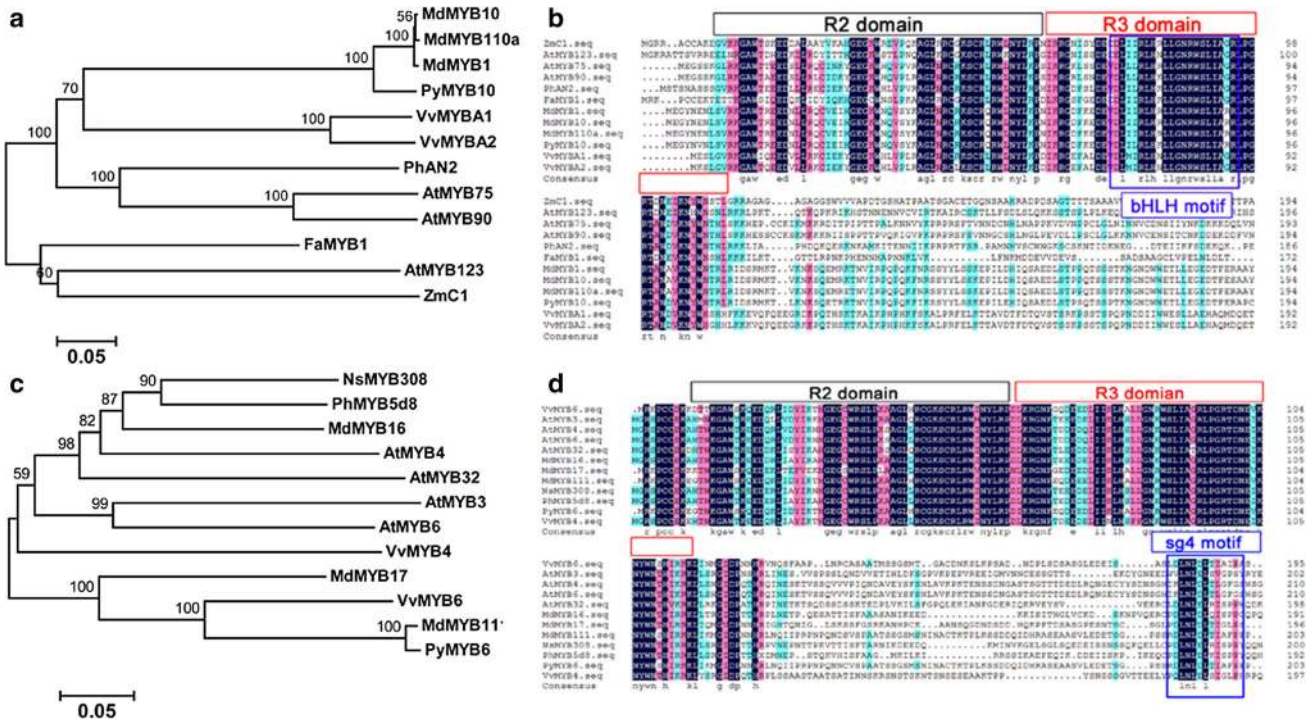

Fig. 6 Phylogenetic analysis and multiple sequence alignment of MYB transcription factors from various plants. **a** Phylogenetic analysis of MYB transcription factors reported to promote anthocyanin biosynthesis. **b** Phylogenetic analysis of MYB transcription factors reported to inhibit anthocyanin biosynthesis. **c** Multiple sequence alignment of MYB transcription factors. All of the anthocyanin biosynthesis-promoting transcription factors contained the R2R3 domain and bHLH motif. **d** Multiple sequence alignment of transcription factors that negatively regulate anthocyanin biosynthesis. These transcription factors contained the subgroup 4 (pdLNL-$^{D}/_{E}$L) suppression motif

Discussion

Effect of light and temperature on callus growth and anthocyanin biosynthesis

Apple is one of the most widely cultivated fruit trees in the world, with many cultivars producing fruits with a red peel (Chen et al. 2010). Fruit color is an important apple trait, and largely determines the market value of apple crops. Anthocyanins are the main pigments affecting the color and nutritional value of apple fruits, and there has been considerable interest in their synthesis and regulation among apple researchers (Takos et al. 2006; Ubi et al. 2006; Espley et al. 2007; Lin-Wang et al. 2011). As a type of antioxidant, anthocyanins have important implications for human health (Smith et al. 2000; Wang and Mazza 2002; Chun et al. 2004). However, anthocyanins accumulate only in the red peel of cultivated apple fruits, with very little (or none at all) detected in the white fruit flesh (Nie et al. 2010). Most studies on anthocyanins have concentrated on the apple peel, which is not the main part of consumers to eat. Meng et al. (2016) studied anthocyanin biosynthesis in red-skinned cultivars at different fruit development stages, and suggested that the expression of the responsible genes is induced by light. Wang et al. (2006) observed that anthocyanin contents of apple peels increase significantly following exposure to continuous light. Other researchers determined that UV-B irradiation can induce anthocyanin biosynthesis in apple peels, and the regulatory effect of UV-B is enhanced at low temperatures (Ubi et al. 2006).

In this study, we developed a red callus culture system using red-fleshed apple 'Zihong1'. The generated calli exhibited consistent growth and development, making them appropriate for studies on anthocyanin biosynthesis regulated by light, temperature, and other environmental factors. We observed that incubations in darkness significantly inhibited the accumulation of anthocyanins. The anthocyanin content of calli grown in darkness was only 4–10 % of that of calli exposed to light. Low temperatures are conducive to anthocyanin biosynthesis. The anthocyanin content was significantly higher at 16 °C than at 24 or 32 °C. These findings are consistent with the results of previous studies, indicating that temperature and light regulate anthocyanin biosynthesis not only in red-skinned cultivated apples, but also in red-fleshed apples and red callus tissue. Thus, anthocyanin metabolism may be relatively stable in response to different temperatures and light

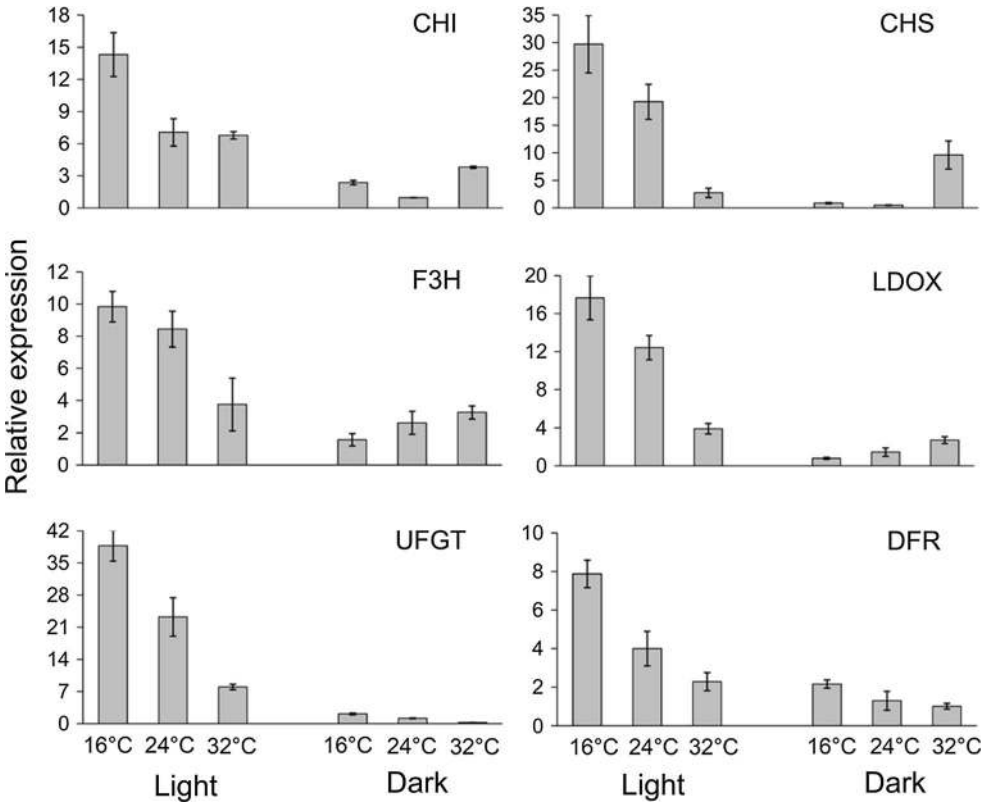

Fig. 7 Relative expression levels of the anthocyanin biosynthesis pathway structural genes under different culture conditions. *CHS* Chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *DFR* dihydroflavonol 4-reductase, *LDOX* leucoanthocyanidin dioxygenase, *UFGT* UDP-flavanone-3-*O*-glucosyltransferase. *MdActin* was used as an internal control

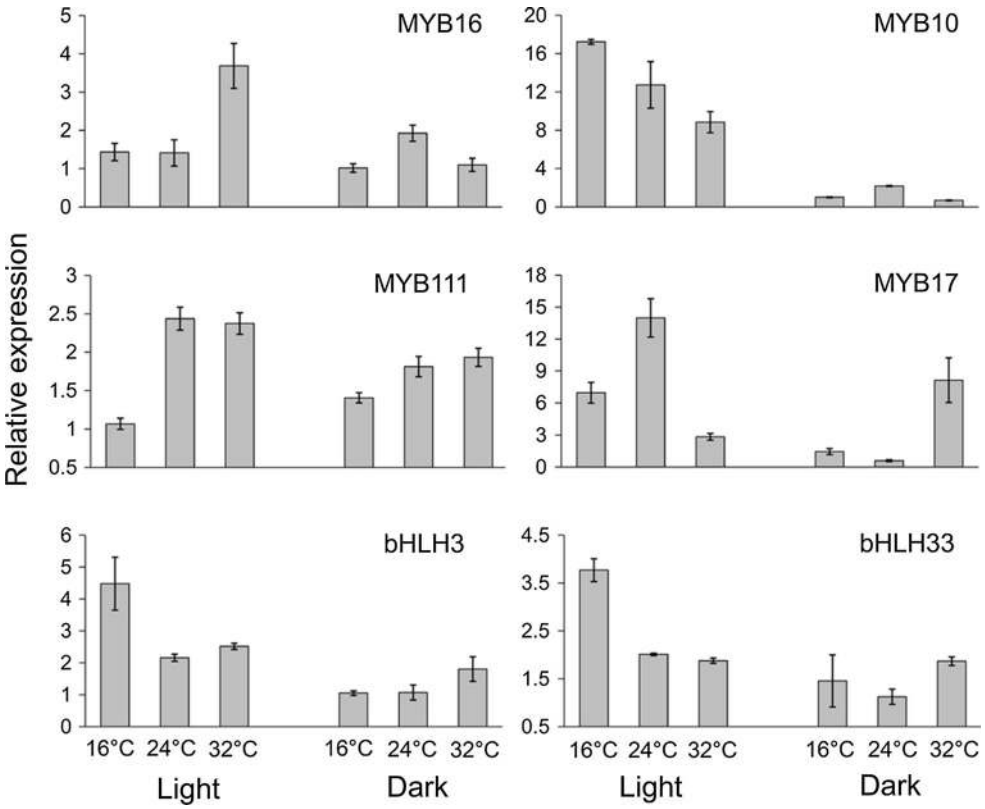

Fig. 8 Relative expression levels of MYB and bHLH transcription factor genes related to anthocyanin biosynthesis under different culture conditions

conditions, and is not influenced by different genotypes or tissues.

Interestingly, Ji et al. (2015) studied anthocyanin accumulation in the same red callus at different concentration of auxins and/or cytokinins, and suggested that anthocyanin accumulation decreased with increased auxin (NAA and 2,4-D) concentration, while nitrogen deficiency could reverse the inhibition of anthocyanin synthesis by auxins. Both growth regulators and nitrogen affected the primary metabolism of the plant. Growth accumulation and secondary metabolite require different conditions to induce a shift from the growth state to the metabolite production state (Simões et al. 2012). Our results confirmed this statement, and found that callus growth is influenced by temperature and light treatments, and that callus growth and anthocyanin content are negatively correlated to a certain extent. Therefore, there may be a competitive relationship between the growth state and the metabolite production state in plant. This is consistent with the apple fruit developmental process in which the fruit first increases in size during the primary growth stage, and then secondary metabolic activities lead to the accumulation of anthocyanins.

Regulatory effects of light and temperature on related transcription factors and structural genes

Light and temperature can induce the expression of related TF and structural genes to regulate anthocyanin metabolism. In apple skin, *CHS*, *ANS*, *F3H*, *DFR*, and *UFGT* have been isolated and identified (Honda et al. 2002). Among these genes, *ANS* and *UFGT* have important functions during anthocyanin biosynthesis. Their expression is induced by UV-B irradiation and low temperatures in apple fruit skin (Kondo et al. 2002; Kim et al. 2006). In in vitro culture systems, some studies have suggested that light irradiation can induce anthocyanin production in *Catharanthus roseus* (Hall and Yeoman 1986), *Centaurea cyanus* (Kakegawa et al. 1987), *Perilla frutescens* (Zhong et al. 1993), Strawberry (Sato et al. 1996) and other plant species. In contrast, the effect of temperature on anthocyanin synthesis was more variable. The highest anthocyanin accumulation was at 30 °C when compared to lower temperatures in callus cultures of *Daucus carota* (Narayan et al. 2005), while low temperature enhanced both anthocyanin accumulation and *CHS* expression in petunia flower (Shvarts et al. 1997). In this study, we focused on the expression of TF and structural genes in calli treated with different combinations of temperatures and light conditions. The *ANS*, *F3H*, *CHS*, *DFR*, and *UFGT* expression patterns based on our data are consistent with those of previous studies. The expression level of the five genes was higher under light than in darkness, and increasing temperatures resulted in decreasing expression levels. We observed that the expression of *CHI* was induced by low temperature. Additionally, its expression was not inhibited by high temperature, unlike the expression of *CHS* and *F3H*. The expression levels of *LDOX*, *DFR*, and *UFGT* decreased with increasing temperature.

A previous study on TFs concluded that light is an essential factor influencing the color of apple fruit skin (Takos et al. 2006). Li et al. confirmed that MdMYB1 protein stability is enhanced by exposure to light, leading to increased anthocyanin biosynthesis in apple skin (Li et al. 2012). Azuma et al. (2012) reported that low temperature and light have a synergistic effect on the expression of the flavonoid biosynthesis pathway genes in grape berry skin. Tian et al. (2015) found that low temperatures induced the expression of *McMYB10*, *McbHLH3/33* and *McTTG1*, leading to anthocyanin accumulation in crabapple leaves. In this study, we observed that *MdMYB10* expression was up-regulated in calli cultured under light, while the expression was very low in calli grown in darkness, which is in agreement with earlier findings regarding anthocyanin biosynthesis. Furthermore, we revealed that temperature can also regulate *MdMYB10* expression, as indicated by the fact its expression level decreased with increasing temperatures.

The bHLH TFs and the negative regulatory factors, *MYB16*, *MYB17*, and *MYB111*, were unaffected by light, but they were sensitive to temperature. The mechanism underlying the regulation of anthocyanin biosynthesis by temperature is relatively complex. Low temperatures increase the expression level of anthocyanin biosynthesis genes in *Z. mays*, *V. vinifera*, *Citrus sinensis*, and other plants (Christie et al. 1994; Lo Piero et al. 2005; Mori et al. 2005). Additionally, *MdbHLH3* promotes anthocyanin accumulation and fruit coloration in apples at low temperatures (Xie et al. 2012). In this study, *bHLH3* and *bHLH33* were highly expressed in calli grown at 16 °C/light, implying that the expression of bHLH TF genes may be induced by low temperature, but not light, to regulate anthocyanin accumulation. Heating apple fruits rapidly decreases the expression levels of *MYB10*, which is responsible for coordinating the development of red fruit skin color (Lin-Wang et al. 2011). We observed that high temperatures inhibited *MYB10* expression. Additionally, some transcription repressors regulating anthocyanin biosynthesis were also affected by temperature. For example, *MYB16* was highly expressed at 32 °C/light, indicating that high temperatures up-regulate *MYB16* expression, leading to inhibited accumulation of anthocyanins. However, this regulatory mechanism did not apply to calli incubated in darkness. In contrast, the high temperature-induced expression of *MYB17*, and subsequent inhibition of anthocyanin accumulation, occurred in calli

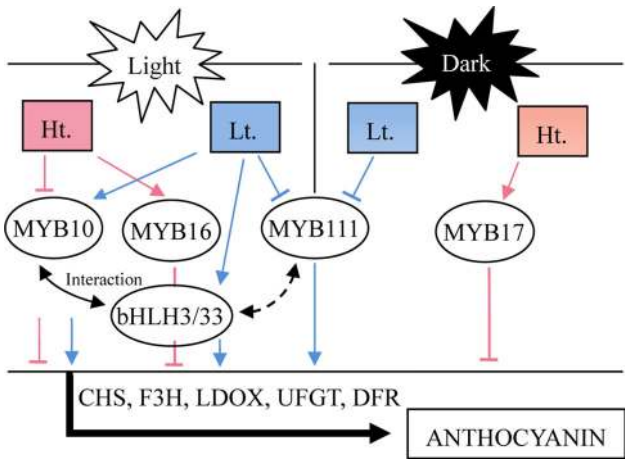

Fig. 9 Model of the regulatory effects of light and temperature on anthocyanin biosynthesis. *Ht* High temperature, *Lt* low temperature

grown in darkness. *MYB111* expression was repressed by low temperature in calli incubated under light and in darkness, which promoted anthocyanin accumulation. Based on these results, we considered the effects of different combinations of temperatures and light conditions on the mechanism regulating anthocyanin biosynthesis. We developed a model summarizing our findings regarding the effects of light and temperature on anthocyanin biosynthesis in apple (Fig. 9).

Conclusion

The results presented herein indicate that light is the main environmental factor influencing anthocyanin biosynthesis. It affects the accumulation of anthocyanins by regulating the expression of *MYB10*. Temperature is a secondary factor that helps regulate anthocyanin biosynthesis by mediating the production of bHLH TFs, MYB16, MYB17, and other repressors.

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Authors contribution Conceived and designed the experiments: NW ZYZ XSC. Performed the experiments: NW. Analyzed the data: NW ZYZ. Contributed to the writing of the manuscript: NW ZYZ XSC.

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