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Different coloration patterns between the red- and white-fleshed fruits of *malus* crabapples

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ABSTRACT

The red crabapple is a rare type of fruit in the *malus* family that is important for apple breeding because it contains large amounts of anthocyanins/flavonoids. However, the molecular mechanism underlying anthocyanin/flavonoid accumulation in the red peels and flesh of the fruits has not been fully explored. Here, we analyzed flavonoid accumulation and the transcript levels of the main structural genes and transcription factors in fruits under sun-exposed and shaded conditions in two crabapple varieties with different coloration characteristics. Our results show that varietal differences in the flavonoid pathway mainly affect the accumulation of anthocyanins and flavanols. Shading treatment induced a significant decrease in the concentration of anthocyanins but increased the levels of flavanols. Quantitative real-time PCR analysis shows that the transcript levels of the primary structural genes, except for *McF3′H* and *McANR*, were higher in the ever-fleshed fruits than in the white-fleshed fruits. Additionally, several MYB TFs have been suggested to be involved in the regulation of the coloration patterns in response to shading. Data analysis indicated that changes in the flavonoid accumulation were associated with the transcription of the structural genes regulated by MYB transcription factors, providing new insights into horticultural plant breeding.

1. Introduction

Flavonoid compounds, such as flavones, flavonols, flavanols, and anthocyanins, originate from several branches of the phenylalanine metabolism pathway in plants (Jaakola, 2013). The types and quantities of flavonoids in fruit directly affect fruit coloration phenotype. Colorful fruits have features that not only protect the fruit from stress but also provide diverse benefits for human health, including strong antioxidant activities, anti-cancer activities and anti-atherosclerotic effects (Batra and Sharma, 2013; Gafrikova et al., 2014; Grassi et al., 2010; McKay et al., 2015; Woo and Kim, 2013).

Plant flavonoid synthesis is regulated by primary structural genes and transcription factors (Fig. S1) (Koes et al., 2005). The main structural genes are chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), flavonol 3′-hydroxylase (*F3′H*), flavonol synthase (*FLS*), dihydroflavonol 4-reductase (*DFR*), leucoanthocyanidin dioxygenase (*ANS*), uridinediphosphate-glucose (*UFGT*), leucoanthocyanidin reductase (*LAR*), and anthocyanidin reductase (*ANR*) (Jaakola, 2013). In fruits of different colors, the composition, structure, and metabolism of flavonoid compounds are diverse, which may be caused by distinct patterns of gene expression and regulation (Ali et al., 2011; Degu et al., 2014;Henry-Kirk et al., 2012). During cranberry fruit development, the flavonols and proanthocyanin biosynthesis branches shows obvious competition for substrates with the anthocyanin biosynthesis branch (Vvedenskaya and Vorsa, 2004). The flavanol biosynthesis branch is also known to compete for substrates with the anthocyanin branch in fruits such as grapes and apples (Han et al., 2012; Kayesh et al., 2013). MYB transcription factors regulate the transcription levels of the flavonoid biosynthetic genes (Jaakola, 2013). In apple, *MdMYB10* in skin and *MYB110a* in flesh were identified as important TF that are

Abbreviations: CHS, chalcone synthase; F3H, flavanone 3β-hydroxylase; F3′H, flavonoid 3′-monooxygenase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, uridinediphosphate (UDP)-glucose: flavonoid 3-O-glycosyltransferase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; TF, transcription factor.

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responsible for anthocyanin accumulation, respectively (Ban et al., 2007; Umemura et al., 2013). The different apple skin patterns can be explained by the differential accumulation of *MdMYB10* transcripts (Telias et al., 2011). However, apple *MdMYB22* plays crucial roles in increasing flavonol concentrations by enhancing the expression of *FLS* (Peng et al., 2013). Several MYB transcription factors, such as *FaMYB5* and *VvMYBPA*, were also found to regulate the flavanol branch by controlling the expression of *LAR* and *ANR* in grapes and strawberries, respectively (Heppel et al., 2013; Schaart et al., 2013).

Environmental factors, especially light conditions, affect fruit coloration and alter the distribution of flavonoid compounds. In grape skin, increased anthocyanin levels have been observed via the up-regulation of *DFR* under light treatment, whereas the anthocyanin levels were significantly decreased under dark treatment (Azuma et al., 2012). In addition, low light reduced *MdMYB1* expression via an ubiquitin-dependent pathway that involves *MdCOP1*, leading to decreased anthocyanin biosynthesis in apples (Li et al., 2012), and it decreased the accumulation of flavonols in grape skin through *VvMYB12*-mediated reduction in expression of *VvFLS* (Matus et al., 2009).

Nowadays, increasing attention has been devoted to flavonoid biosynthesis in fruits, while the flavonoid accumulation and its molecular mechanisms associated with the different fruit coloration patterns remains unclear. The *malus* crabapple is one of the most important germplasm resources in ornamental landscaping, food processing and nutritional products. The extracts of crabapple tissue have been associated with anti-oxidative activities and inhibition of fatty acid synthase (Wei et al., 2009). Here, we chose two typical colored fruits: *M.cv.* 'Royalty' fruits (red peel and red flesh throughout development), and *M.cv.* 'Flame' fruits (white flesh, a green peel during the immature period and then a partial red flush on the sun-facing side at maturity). During fruit coloration, the flavonoid accumulation, the expression levels of related biosynthetic genes were studied to reveal the molecular mechanism of flavonoid accumulation in different fruit coloration patterns.

2. Materials and methods

2.1. Plant materials

The plant materials used for the experiments included two *malus* crabapple cultivars: (1) *malus* cv. 'Royalty', a red-fleshed cultivar, and (2) *malus* cv. 'Flame', a white-fleshed cultivar. Eight-year-old trees grafted on *malus hupehensis* var. *pingyiensis* were planted in the Crabapple Germplasm Resources Nursery at the Beijing University of Agriculture. All of the samples were immediately frozen in liquid nitrogen and stored at −80 °C and/or freeze-dried for RNA isolation and HPLC analysis.

2.2. Shading treatment

For 'Royalty' and 'Flame', three healthy trees of each cultivar with similar growth potential were chosen for sun-exposed (control) or shading treatment. Before treatment, braches of each group in four fixed directions were marked for sampling. For the shade group, the trees were covered with a single layer of sunshade net (Ketai Plastic Products Factory, Wenan, China) before the fruit-coloring stage. For the control (sun-exposed) group, the trees were grown under natural light. Testing showed that the light intensity under the sunshade net was 30–40% of that under direct sunlight. Three biological replications were performed. For both control trees and shaded trees, 15–20 fruits of each cultivar were collected at 15, 35 and 50 days after shading (DAFS) from the marked branches. The sun-side peels and flesh of fruits collected at each time point were separated for experimental testing.

2.3. HPLC analysis

The crabapple fruit samples were freeze-dried and coarsely ground. A total of 0.8 g of fruit powder from each sample was extracted using 10 mL of extraction solution (methanol:water:formic acid:trifluoroacetic acid = 70:27:2:1) at 4 °C under dark conditions for 72 h. During this period, the mixture was shaken every 3 h, and the liquid was separated from the solid matrix via filtration through sheets of qualitative filter paper. The supernatant was further filtered through a 0.22 μm Millipore™ filter (Billerica, MA, USA). The flavonoid compounds were quantified using an HPLC 1100-DAD system (Agilent Technologies, Waldbronn, Germany). A solid-phase extraction cartridge (500 mg, 3 mL) C18 Supelclean ENVI-18 column (Pretech Instruments, Sollentuna, Sweden) operating at 25 °C was used to purify the residue, which was dissolved in 5 mL of water, and the filtrate was evaporated at 30 °C (Revilla and Ryan, 2000). The column was successively rinsed with water and methanol. The anthocyanins were separated from the other flavonoid compounds via HPLC, and the mobile phase consisted of trifluoroacetic acid:formic acid:water (0.1:2:97.9) as solvent A and trifluoroacetic acid:formic acid:acetonitrile:water (0.1:2:48:49.9) as solvent B, with a flow rate of 0.8 mL min^{-1}.

The gradient elution program was as follows: 30% B at 0 min, 40% B at 10 min, 55% B at 50 min, 60% B at 70 min and 30% B at 80 min (Zhang et al., 2014). Detection was performed at 520 nm for the anthocyanins and at 350 nm for the flavones, flavonols and flavanols. All of the samples were analyzed in triplicate, and three biological replicates were performed.

2.4. qRT-PCR analysis

Quantitative RT-PCR was performed in 20 μL reactions using ~2 μL of 10× diluted cDNA template. qRT-PCR was performed using the SYBR® Premix Ex Taq™ II (Perfecta Real Time) (Takara, Ohtsu, Japan) on a CFX96™ Real-Time System (BIO-RAD, Hercules, CA, USA). The qRT-PCR program was initiated with a preliminary step of 3 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 59 °C for 30 s, and 72 °C for 30 s. The data were analyzed with Bio-Rad CFX Manager software, and the values presented are the mean ± standard deviation (SD) of three biological replicates. The differences in gene expression were calculated using the $2^{-\Delta\Delta Ct}$ analysis method, and the transcription levels were determined via relative quantification using the *malus 18S ribosomal RNA* gene as the internal control (Lu et al., 2015). The primers for qRT-PCR were designed as described in Table S1.

2.5. Statistical analysis

All of the data were analyzed using one-way ANOVA followed by a *t* test to compare the differences among the experimental sets at $p = 0.05$ [Data Processing System (DPS) version 7.05].

3. Results

3.1. Differential flavonoid accumulation leads to color differences between cultivars

To confirm the coloration phenotypes and flavonoid biosynthesis during the selected fruit development stages in 'Royalty' and 'Flame', we analyzed the flavonoid contents and proportions via HPLC. The results showed a significant variation in the coloration patterns between cultivars (Fig. 1).