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The *Malus* crabapple transcription factor McMYB10 regulates anthocyanin biosynthesis during petal coloration

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ABSTRACT

Anthocyanins are responsible for the pigmentation of flowers in the *Malus* crabapple, and anthocyanin accumulation is based on the expression level of both anthocyanin biosynthetic and regulatory genes. Here, we analyzed the chromatic parameters of the petals and the expression levels of anthocyanin biosynthetic pathway genes and the *McMYB10* transcription factor in two cultivars with different flower colors during flower development. The results showed that the anthocyanin content and expression levels of anthocyanin-related genes in the cultivar ('Royalty') with red petal were much higher than those in the cultivar ('Flame') with a lightly pigmented petal. We also overexpressed the *McCHS*, *McANS*, and *McMYB10* genes in tobacco. The transgenic plants harboring *35S*::*McCHS* or *35S*::*McANS* had an altered flower color, with the petals turned into red, compared with the control with pink petals. Moreover, plants transformed with *35S*::*McMYB10* appeared to exhibit stronger anthocyanin accumulation, showing deeper red petal color compared with the *McCHS*- and *McANS*-overexpressing lines. All tested anthocyanin biosynthetic genes, especially *NtDFR* and *NtANS*, showed higher expression levels in the *McMYB10*-overexpressing lines than in the control lines. These results suggested that the transcription factor *McMYB10* plays an important role in anthocyanin accumulation during petal coloration in the *Malus* crabapple, at least partially through transcriptional regulation of its downstream genes in the anthocyanin biosynthetic pathway.

1. Introduction

Anthocyanins, which belong to the flavonoid subclass, are a class of secondary metabolites that contribute to the red coloration of many fruits, vegetables, flowers and seeds in higher plants (Field et al., 2001; Honda and Saito, 2002). In flowers, anthocyanins attract pollinators; in fruit skin, they attract animals to aid in seed dispersal (Regan et al., 2001; Schaefer et al., 2004).

Two groups of genes are required for anthocyanin biosynthesis: structural genes that encode the enzymes directly participating in the biosynthetic reactions, and transcription factor genes, whose products regulate the expression of these structural genes, and then the accumulation of flavonoid metabolites. Transcription factors can act as activators or repressors of structural gene expression and mediate increases or decreases of anthocyanin content (Broun, 2004).

MYB transcription factors have been shown to play an important role in the transcriptional regulation of anthocyanins. The regulatory function of MYB also appears to be specific to structural genes, which act either early or late in the anthocyanin biosynthetic pathway (Davies and Schwinn, 2003). In recent studies, the behavior of *MdMYB10* suggests that it is responsible for the red color of apple fruit, and anthocyanin biosynthetic genes are regulated by the *MdMYB10* transcription factor (Espley et al., 2007). Further analysis revealed that a structural difference in the promoter region is responsible for the expression difference of the *MdMYB10* gene between the white-flesh and red-flesh apples (Espley et al., 2009). Two homologous genes of *MdMYB10*, *MdMYB1* (Takos et al., 2006a,b) and *MdMYBA* (Ban et al., 2007), have been reported that they have the same function as *MdMYB10* in apple skin. In addition to fruit, flower coloration is also regulated by MYB TFs. In *Petunia*, a MYB-related gene, *AN2*, is required for anthocyanin production in flowers (Quattrocchio et al., 1998), and a bHLH protein, AN1, interacts with AN2 to activate anthocyanin biosynthesis (Spelt et al., 2000). In *Antirrhinum*, the *Rosea1*, *Rosea2*, and *Venosa* genes encode MYB-related transcription factors with different specificities in regulating the expression of target genes encoding the enzymes of anthocyanin biosynthesis, and these transcription factors can activate anthocyanin pigmentation in flowers (Schwinn et al., 2006). In gerbera, *GMYB10* led to strongly enhanced accumulation of anthocyanin pigments as well as to an altered pigmentation pattern in transgenic plants (Laitinen et al., 2008).

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Fig. 1. Flower development series of two cultivars *Malus* crabapples. Four stages were examed in this study. Stages: (1) six days before full bloom, (2) three days before full bloom, (3) one day before full bloom, (4) full bloom. Two cultivars: top, 'Royalty'; bottom, 'Flame'.

Malus crabapples are one of the most important ornamental and economic germplasm resources, providing abundant plant landscape species and favorable research material to exploit the mechanism of color formation (Tian et al., 2011). *Malus* crabapples have diverse colors of leaves, flowers and fruits in different varieties, especially the flower color, which is an important ornamental trait of *Malus* crabapples. As shown in Fig. 1, petals of the 'Royalty' variety are red throughout development, while the petals of 'Flame' transition from pink to white during development (Shen et al., 2012).

To explore the molecular mechanism of red flower coloration in the *Malus* crabapple, we tested the anthocyanin content and the expression levels of anthocyanin biosynthetic genes and the transcription factor gene *McMYB10* in two cultivars of crabapples with different petal colors. We also overexpressed the *McCHS*, *McANS*, and *McMYB10* genes in tobacco. The results showed that *McMYB10* is associated with flower coloration in the *Malus* crabapple, and the expression level of *McMYB10* is the genetic basis of red flower coloration.

2. Materials and methods

2.1. Plant material

Flowers of *Malus cv*. 'Royalty' and *Malus cv*. 'Flame' were collected at different developmental stages (1, six days before full bloom; 2, three days before full bloom; 3, one day before full bloom; 4, full bloom). Only the petals were used for research. Wild-type and T_2 transgenic plants of tobacco (*Nicotiana benthamiana*) were grown in a greenhouse, and flowers at the full-bloom stage were collected for study. All samples were frozen in liquid nitrogen and stored at −80 °C.

2.2. HPLC analysis of anthocyanin and flavonoid content

Anthocyanins and flavonoids were extracted using a solvent containing methanol:water:formic acid:trifluoroacetic acid (70:27:2:1, v/v). The supernatants were filtered through a 0.22 μm Millipore™ (Billerica, MA, USA) filter before use. Anthocyanins in the samples were analyzed using an HPLC1100-DAD system (Agilent Technologies, Waldbronn, Germany). Detection was performed at 520 nm for anthocyanins and 350 nm for flavonoids. A solid-phase extraction cartridge (500 mg, 3 ml) C18 Supelclean ENVI-18 cartridge was used for separation at 30 °C, and elution was carried out using a mobile phase consisting of solvent A, trifluoroacetic acid:formic acid:water (0.1:2:97.9) and solvent B, trifluoroacetic acid:formic acid:acetonitrile:water (0.1:2:35:62.9) at a flow rate of 0.8 ml min^{-1}. The elution program followed the procedure described by Wu and Prior (2005) with several modifications: solvent B started at 30% and increased linearly stepwise to 35% at 5 min, 40% at 10 min, 50% at 30 min, 55% at 50 min, 60% at 70 min, and 30% at 80 min.

2.3. Quantitative real-time PCR analysis

Total RNA from flower tissues was extracted using an RNA Extract Kit (Aidlab, Beijing, China) according to the manufacturer's instructions. DNase I (TaKara, Japan) was added to remove genomic DNA, and the samples were then subjected to cDNA synthesis using the Access RT-PCR System (Promega, USA) according to the manufacturer's instructions. The full-length cDNA sequences of *McPAL*, *McCHS*, *McCHI*, *McF3H*, *McF3′H*, *McFLS*, *McDFR*, *McANS*, *McUFGT* and *McMYB10* were isolated by 5′ and 3′ RACE with SMARTer RACE cDNA Amplification Kit (Clontech Laboratories, Inc.) according to the manufacturer's instructions. The expression levels of those genes were analyzed using quantitative real-time PCR (RT-qPCR) with SYBR Green qPCR Mix (Takara, Japan) and Bio-Rad CFX96 Real-Time PCR Systems (BIO-RAD, USA), according to the manufacturers' instructions. The expression levels of *NtCHS*, *NtCHI*, *NtF3H*, *NtF3′H*, *NtDFR*, and *NtANS* were analyzed using the same techniques. The primers in this paper were designed by NCBI Primer BLAST and listed in Supplemental Table 1.qPCR analysis was carried out in a total volume of 20 μl containing 9 μl of 2× SYBR Green qPCR Mix (Takara, Japan), 0.1 μM specific primers (each), and 100 ng of template cDNA. The reaction mixtures were heated to 95 °C for 30 s, followed by 39 cycles at 95 °C for 10 s, 59 °C for 15 s, and 72 °C for 30 s. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products.

2.4. Expression vector construction and tobacco transformation

Three pairs of primers were designed to amplify the entire coding sequences of *McCHS*, *McANS* and *McMYB10* using cDNA from the flower of 'Royalty' as the template. The primers for *McCHS* contain SpeI and Kpn restriction enzyme sites, the primers for *McANS* contain XhoI and Kpn restriction enzyme sites, and the primers for *McMYB10* contain BamHI and SacI restriction enzyme sites. The coding regions of all three genes were amplified into the pBI121 vector. The primers used are listed in Supplemental Table 1.

The *Agrobacterium* strain LBA4404 containing these vectors was used to transform tobacco (*Nicotiana benthamiana*) 'W38′ according to the leaf disk method (Horsch et al., 1988). Transgenic plants were selected based on kanamycin resistance. The T2 progeny of transgenic plants were used for further analysis and compared with wild-type non-transformed lines grown in the same conditions.

All data were analyzed using one-way ANOVA followed by Duncan's SSR test (shortest significant ranges) to compare differences among the experimental sites at $P < 0.05$ (Microsoft Excel 2003 and Data Processing System (DPS) software 7.05).

3. Results

3.1. Analysis of the differences in anthocyanin content between 'Royalty' and 'Flame'

To understand the flavonoid composition and variation in the *Malus* crabapple, we investigated the level of flavonoid accumulation in two different flower color cultivars. The results showed that cyanidin is the main coloration anthocyanin component in