The Structure and Methylation Level of the *McMYB10* Promoter Determine the Leaf Color of *Malus Crabapple*

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Abstract. Anthocyanins are protective pigments that accumulate in plant organs such as fruits and leaves, and are nutritionally valuable components of the human diet. The MYB10 transcription factor (TF) plays an important role in regulating anthocyanin biosynthesis in *Malus crabapple* leaves. However, little is known about how the promoter regulates *McMYB10* expression and influences the substantial variation in leaf anthocyanin accumulation and coloration that is observed in different crabapple cultivars. In this study, we analyzed leaf coloration, anthocyanin levels, and the expression levels of *McMYB10* in the leaves of 15 crabapple cultivars with three leaf colors at various development stages, and showed that the expression of *McMYB10* correlates positively with anthocyanin accumulation. We also examined the relationship between the number of R6 and R1 elements in the *McMYB10* promoters of the different cultivars and the pigmentation of the new buds of spring-red cultivars, as well as the methylation level of the *McMYB10* promoters at different development stages in three representative crabapple cultivars. The ratio of R6/R1 minisatellites in the promoters correlated with the color and anthocyanin accumulation in new crabapple buds, and we concluded that the differences in promoter structure and methylation level of the *McMYB10* promoters coordinately affect the leaf color of crabapple cultivars.

Anthocyanins are a class of pigments that contribute to the red, blue, and purple colors of many organs and tissues in a wide range of plant species (Field et al., 2001; Honda and Saito, 2002). Anthocyanins also have diverse other functions, including providing protection against abiotic stresses, particularly ultraviolet irradiation, and biotic factors, such as phytophagous insects, as well as attracting pollinators (Misra et al., 2010; Page et al., 2012). Moreover, as they have antioxidant properties that can help prevent cancer, as well as cardiovascular and neurodegenerative

diseases, they are considered beneficial to human health (Boudet, 2007; Martin, 2013).

Many aspects of the molecular genetics and biochemistry of the anthocyanin biosynthetic pathway have been well characterized. For example, the genes in encoding chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase, flavonoid 3'-monooxygenase, dihydroflavonol 4-reductase, anthocyanidin synthase, uridine diphosphate glucose-flavonoid 3-o-glucosyltransferase, flavonol synthase, leucoanthocyanidin reductase, and anthocyanidin reductase, which are all components

of the biosynthetic pathway, have been characterized in several plant species, including *Arabidopsis thaliana* (Winkel-Shirley, 2001), *Petunia hybrida* (Beld et al., 1989), and *Malus crabapple* (Shen et al., 2012; Tian et al., 2011), as well as in species that bear fleshy fruits, such as apple (*Malus domestica*) (Chagné et al., 2013) and grape (*Vitis vinifera*) (Cavallini et al., 2014).

Other studies have shown that anthocyanin biosynthetic genes are regulated by a transcriptional activation complex consisting of R2R3-MYB and bHLH TFs, as well as WD40 family proteins (Bai et al., 2011; Hichri et al., 2011; Zhao et al., 2013). The MYB TF family, which is one of the largest plant TF families, has been reported to play a particularly important regulatory role. For example, the anthocyanin pathway in apple is controlled by the MYB TFs, *MdMYB1*, *MdMYB10*, and *MdMYBA*, which are allelic to each other. The protein sequence of *MYBA* is 100% and 98% identical to *MYB1* and *MYB10*, respectively, and functional assays have shown that these genes are key regulators of anthocyanin accumulation and fruit coloration (Ban et al., 2007; Espley et al., 2007; Takos et al., 2006). A recent study also showed that *MYB110a*, a paralog of *MYB10*, is associated with a red-flesh cortex phenotype in apple, and that *MYB10* and *MYB110a* have conserved functions in some cultivars, but differ in their expression patterns during fruit maturity (Chagné et al., 2013). The pear (*Pyrus pyrifolia*), peach (*Prunus persica*), and mangosteen (*Garcinia mangostana* L.) *MYB10* homologs have also been shown to regulate fruit anthocyanin biosynthesis (Feng et al., 2010; Palapol et al., 2009; Ravaglia et al., 2013). In addition, we previously identified MYB TF, named *McMYB10*, which is expressed in the petals and leaves of *Malus crabapples*, and that has the same function as *MdMYB10*, *MdMYBA*, and *MdMYB1* (Jiang et al., 2014; Tian et al., 2015). Genetic transformation and DNA-binding assays demonstrated that the *McMYB10* TF functions to promote anthocyanin accumulation by regulating the expression of the crabapple *McF3'H* gene in the flavonoid pathway, thereby promoting red leaf and petal coloration (Tian et al., 2015).

Subsequent analysis showed that a rearrangement of the 23-base pair (bp) sequences in the promoter region of *MdMYB10* was responsible for the difference in gene expression between the white- and red-flesh apples (Espley et al., 2009). Specifically, the R1: *MdMYB10* promoter has a single *MdMYB10* binding motif, and is only present in white-fleshed apples, whereas the R6: *MdMYB10* promoter, which is present in red-fleshed apples, has five additional tandem repeats of the *MdMYB10* binding motif. This allelic rearrangement in the promoter of *MYB10* has generated an autoregulatory locus, and this autoregulation mechanism is sufficient to account for the increase in *MYB10* transcript levels and subsequent ectopic accumulation of anthocyanins throughout the plant (Espley et al., 2009). In a previous study, we

found the same structural difference in the McMYB10 promoters, where six repeated minisatellites and a dinucleotide microsatellite were found in the promoter region of the an ever-red leaf crabapple cultivar, but only one minisatellite and a dinucleotide microsatellite were present in an evergreen leaf cultivar (Tian et al., 2015).

Leaf color is a key determinant of the commercial value of many ornamental plant species, and *Malus* crabapples varieties show a diverse range of leaf, flower, and fruit and leaf colors. There are three kinds of *Malus* crabapples leaf-color cultivars, including the “ever-red-leaf,” the “ever-green-leaf,” and the “spring-red-leaf” cultivars (Tian et al., 2011; Tai et al., 2014), and so crabapple ornamental apple germplasm is a valuable model for studying plant pigmentation mechanisms. We reported that R6 and R1: McMYB10 promoters are present in the ever-red and ever-green-leaf cultivars, respectively (Tian et al., 2015). However, the promoter structures of McMYB10 in cultivars with red leaves in the spring (spring-red-leaf), and the relationship between the number of R6 and R1 minisatellites in the promoter and anthocyanin accumulation in the leaves, are still unknown.

To investigate the role of the McMYB10 promoter in leaf color formation, we analyzed its structure and distribution in three cultivars with different leaf colors. Specifically, we examined the relationship between the number of R6 and R1 elements in the McMYB10 promoters and the pigmentation of new buds in cultivars that have red leaves in the spring. We also examined the promoter methylation at different development stages to investigate its effects on anthocyanin accumulation and *McMYB10* expression. We describe here difference in promoter structure and minisatellite copy

number, as well as the methylation level of the McMYB10 promoters, in the context of crabapple leaf coloration.

Materials and Methods

Plant materials and growth conditions. Five-year-old trees of the ever-red-leaf cultivar *Malus* cv. Royalty; cultivars with leaves that are red in the spring (spring-red; ‘Radiant’, ‘Indian Magic’, ‘Prairifire’, ‘Red Begonia’, ‘Red Splendor’, ‘Kelsy’, and ‘Strawberry Jelly’); and the ever-green-leaf cultivars Spring Snow, Donald,

Dolgo, Snowdrift, Red Jade, Flame, and Jewelberry grafted on *Malus hupehensis* were planted in the Crabapple Germplasm Resources Nursery in the Beijing University of Agriculture. We selected five trees of each 15 cultivars that showed similar growth and collected leaf samples from annual branches growing in all four compass directions on the outer edge of each canopy as five biological replicates. The average weight of each leaf at 6 d was 0.1–0.2 g and at 30 d was 0.8–1.0 g. Thirty leaves were collected in each tree of 15 cultivars and at each of the five different

Fig. 1. Five leaf developmental stages with different colors used for the analyses. *Malus* cv. Royalty is an ever-red-leaf cultivar; ‘Radiant’, ‘Indian Magic’, ‘Prairifire’, ‘Red Begonia’, ‘Red Splendor’, ‘Kelsy’, and ‘Strawberry Jelly’ are spring-red-leaf cultivars; ‘Spring Snow’, ‘Donald’, ‘Dolgo’, ‘Snowdrift’, ‘Red Jade’, ‘Flame’, and ‘Jewelberry’ are ever-green-leaf cultivars. Scale bars = 1 cm.

Table 1. Primer sequences used in this study.

Accession number	ID	Sequence (5’-3’)	Analytical use
EF528482	APX-F	ATCAAGCAACAGTTC	Copy number qRT-PCR
	APX-R	GGGGAAGTGTGCTT	
AB557643	R6 + R1-F	GCATTGCCTCTCATCTCTAC	Copy number qRT-PCR
	R6 + R1-R	CAGCAAAACCCAAAATCCCT	
AB557643	R6-F	CTGGTTAGACTGGTAGCT	Copy number qRT-PCR
	R6-R	AGACTCCAGAAAGACACC	
JX162681	MYB10M-F	GTTAGTTTGTAAATAGATGATAGGT	Methylation analysis
	MYB10-R	AACAATTAATTTCAAAATAAAAACTAC	
AB557643	PMYB10-F	CTTATGGTGGTCAAAGATGTGTGTT	Promoter cloning
	PMYB10-R	CCACAGAAGCAAACACTGACAAG	
JX162681	MYB10-F	GGCGCATGATCTTGGCGACAGT	qRT-PCR
	MYB10-R	ACGCCACCACAAACGTCGTCG	
AB638619	MdActin-F	GCTCCCGAAGAACACCCAG	qRT-PCR
	MdActin-R	GGACCTCCAATCCAGACA	

qRT-PCR = quantitative real-time reverse transcription polymerase chain reaction.

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developmental stages (stage 1, 3 d after budding; stage 2, 9 d after budding; stage 3, 15 d after budding; stage 4, 21 d after budding; stage 5, 30 d after budding) (Fig. 1). Of each leaf samples, 2 and 1 g were used for RNA extraction and high-pressure liquid chromatography (HPLC) analysis, respectively. At stage 1, 1 g of leaf samples was used for DNA extraction.

HPLC analysis. Crabapple leaves (≈ 0.8 – 1.0 g fresh weight) were extracted with 10 mL extraction solution (methanol:water:formic acid:trifluoroacetic acid = 70:27:2:1) as previously described (Tian et al., 2015) at 4 °C in the dark for 72 h, with shaking every 6 h. Each sample was filtered through sheets of qualitative filter paper and the filtrates were then passed

through 0.22- μ m reinforced nylon membrane filters (Billerica, MA). The samples were then analyzed by HPLC on an HPLC1100-DAD system (Agilent Technologies, Waldbronn, Germany), using trifluoroacetic acid:formic acid:water (0.1:2:97.9) as mobile phase A, and trifluoroacetic acid:formic acid:acetonitrile:water (0.1:2:48:49.9) as mobile phase B. The gradients used were as follows: 0 min, 30% B; 10 min, 40% B; 50 min, 55% B; 70 min, 60% B; and 80 min, 30% B and detection was performed at 520 nm for anthocyanins and 350 nm for flavonols (Revilla and Ryan, 2000) (Supplemental Fig. 1). All samples were analyzed in five biological replicates.

Cloning and sequence analysis of the *McMYB10* promoter region. To analyze the

differences in *McMYB10* promoter sequences between the different cultivars, genomic DNA was isolated from leaves using the Plant Genomic DNA Kit (TIANGEN Biotech Co., Ltd, Beijing, China). Cloning primers PMYB10-F and PMYB10-R were designed as previously described (Tian et al., 2015) (Table 1). Polymerase chain reaction (PCR) products were cloned into the pMD-19T vector and sequenced.

Quantitative real-time PCR analysis. Total RNA was extracted from crabapple leaves using an RNA Extraction Kit (Aidlab, Beijing, China) according to the manufacturer's instructions. DNase I (TaKaRa, Ohtsu, Japan) was added to remove genomic DNA, and the samples were then subjected to cDNA

Table 2. High-pressure liquid chromatography (HPLC) analysis of anthocyanin (cyanidin) levels in the leaves of 15 crabapple cultivars.

Leaf phenotypes	Cultivars/phyllotaxis	1 ($\mu\text{g}\cdot\text{g}^{-1}$)	2 ($\mu\text{g}\cdot\text{g}^{-1}$)	3 ($\mu\text{g}\cdot\text{g}^{-1}$)	4 ($\mu\text{g}\cdot\text{g}^{-1}$)	5 ($\mu\text{g}\cdot\text{g}^{-1}$)
Ever-red cultivar	Royalty	623.36 $\pm$ 6.56 aA	702.60 $\pm$ 17.14 aA	710.88 $\pm$ 27.13 aA	231.45 $\pm$ 3.47 bA	16.31 $\pm$ 0.98 cG
Spring-red cultivars	Radiant	398.19 $\pm$ 3.66 aB	313.91 $\pm$ 3.18 bC	123.35 $\pm$ 2.27 cB	48.66 $\pm$ 0.57 dD	34.96 $\pm$ 1.60 eC
	Indian Magic	282.57 $\pm$ 0.35 aC	235.70 $\pm$ 0.88 aD	39.20 $\pm$ 0.79 bD	26.05 $\pm$ 0.12 cF	19.39 $\pm$ 0.59 dF
	Prairifire	434.46 $\pm$ 1.01 aB	51.70 $\pm$ 0.62 dF	64.25 $\pm$ 0.24 bC	53.61 $\pm$ 0.08 cC	45.24 $\pm$ 0.77 eB
	Red Begonia	153.33 $\pm$ 0.78 aD	22.99 $\pm$ 0.70 bG	14.26 $\pm$ 0.41 dE	14.53 $\pm$ 0.35 dG	19.43 $\pm$ 0.41 cF
	Red Splendor	155.20 $\pm$ 2.18 aD	85.35 $\pm$ 0.72 bE	70.95 $\pm$ 1.11 dC	78.91 $\pm$ 0.38 cB	55.30 $\pm$ 0.73 eA
	Kelsy	151.59 $\pm$ 22.19 bD	111.05 $\pm$ 9.02 aB	58.11 $\pm$ 3.04 cC	29.62 $\pm$ 0.11 dE	28.53 $\pm$ 0.78 dD
	Strawberry Jelly	55.02 $\pm$ 0.52 aE	51.94 $\pm$ 0.86 aG	30.94 $\pm$ 0.03 aD	28.53 $\pm$ 0.78 bE	25.44 $\pm$ 0.91 cE

1, 3 d after budding; 2, 9 d after budding; 3, 15 d after budding; 4, 21 d after budding; 5, 30 d after budding. Error bars indicate the mean $\pm$ SE of five replicate reactions. Small letters and capital letters (in column) indicate significance at $P < 0.05$ and $P < 0.01$, respectively, according to Duncan's multiple range test.

Fig. 2. Expression profiles of *Malus* crabapple *McMYB10* during five leaf developmental stages. Real-time polymerase chain reaction was used to analyze *McMYB10* expression patterns in the leaves of 15 crabapple cultivars (names on the x axis). The *18S* gene was used as the reference gene. 1 to 5 represent leaf developmental stages 1 to 5. Error bars on each bar indicate the mean $\pm$ SE of three replicate reactions.

cultivars (Fig. 3A). Interestingly, the R6 and R1 promoters were both present in the spring-red crabapple cultivars, whereas the R6 or R1 promoter sequences were specific for the ever-red- or ever-green-leaf cultivars, respectively. The results indicate that the presence of both R1 and R6 promoter in spring-red cultivars have allowed the leaves show both red and green color during the developmental stages (Fig. 3B). These genetic polymorphisms were consistent with the leaf phenotypes.

Number of R6 and R1 minisatellites in the new buds of spring-red cultivars. Since the R6 promoter sequence determined the red leaf color, we investigated whether a difference in the number of R6 minisatellites might also explain the differences in the red color and anthocyanin content in new buds compared among different spring-red cultivars, by analyzing the promoter sequences of the 15 crabapple cultivars using qRT-PCR. We could only detect either the R6 or the R1 promoter sequences in the ever-red and ever-green-leaf cultivars, respectively. In contrast, the R6 and R1 promoters were both present in the spring-red cultivars, but in different ratios (Table 3; Supplemental Fig. 2). To further confirm the relationship between the number of R6 and R1 elements and new bud color and anthocyanin content, we calculated the ratio of R6/R1 and the flavonoid content as a ratio of anthocyanins/flavonols (Table 4). We observed that R6/R1 ratio had a similar variation trend as the anthocyanins/flavonols ratios at leaf development stage 1, with a correlation coefficient of 0.953 (Fig. 4). This result was consistent with the R6/R1 ratio determining the color and anthocyanin accumulation in new crabapple buds.

Methylation level of the *McMYB10* promoter is associated with green leaf color in spring-red cultivars. Several studies have shown that methylation may play a role in the regulation of MYB10 expression (Telias et al., 2011; Wang et al., 2013), and a CpG island has been identified in the *McMYB10* promoter. To investigate whether methylation affects the anthocyanin accumulation in crabapple leaves, we measured the methylation level of the *McMYB10* promoter in three typical crabapple cultivars: the ever-red cultivar Royalty, the ever-green cultivar Flame, and the spring-red cultivar Radiant. We analyzed the methylation level of cytosine in the *McMYB10* promoter by BSP in the -1288 to -1595 bp region. The methylation level of the promoter gradually decreased during leaf development in 'Royalty' and 'Flame', but increased during development stages 1–3 and decreased in the last two developmental stages in 'Radiant'. Analysis of correlation coefficient indicated a clear negative relationship between the methylation level and the expression level of *McMYB10* (Fig. 5). These data suggested that the methylation level of the *McMYB10* promoter in crabapple leaves can affect gene expression, and that differences in R6 and R1 copy numbers, together with variation in degree of methylation of the *McMYB10*

Table 3. Number of R6 and R1 promoter elements in 15 crabapple cultivars.

Leaf phenotypes	Cultivars	Number of R6 elements	Number of R1 elements
Ever-red cultivar	Royalty	7	0
Spring-red cultivars	Radiant	5	3
	Indian Magic	4	4
	Prairifire	3	1
	Red Begonia	3	3
	Red Splendor	2	4
	Kelsy	11	4
	Strawberry Jelly	7	2
	Spring Snow	0	4
	Donald	0	10
	Dolgo	0	14
Ever-green cultivar	Snowdrift	0	10
	Red Jade	0	7
	Flame	0	3
	Jewelberry	0	5

Table 4. The content of anthocyanins and flavonols, as well as the number of R6 and R1 promoter elements, in 15 crabapple cultivars.

Cultivars	Cyanidin-3- <i>o</i> -glucoside ($\mu\text{g}\cdot\text{g}^{-1}$)	Flavonol ($\mu\text{g}\cdot\text{g}^{-1}$)	Number of R6 elements	Number of R1 elements
Radiant	398.19 $\pm$ 3.66	1,192.26 $\pm$ 7.12	5	3
Indian Magic	282.57 $\pm$ 0.35	918.9 $\pm$ 5.36	4	4
Prairifire	434.46 $\pm$ 1.01	876.34 $\pm$ 4.59	3	1
Red Begonia	153.33 $\pm$ 0.78	526.43 $\pm$ 3.71	3	3
Red Splendor	155.20 $\pm$ 2.18	1,349.31 $\pm$ 3.56	2	4
Kelsy	151.59 $\pm$ 22.19	262.41 $\pm$ 5.66	11	4
Strawberry Jelly	55.02 $\pm$ 0.52	93 $\pm$ 3.16	7	2

Fig. 4. The ratio of R6/R1 correlates positively with the content of anthocyanin/flavonol in leaf developmental stage 1 in spring-red-leaf cultivars. (A) The variation in the anthocyanin/flavonol content ratio in leaf developmental stage 1 in spring-red-leaf cultivars. (B) The variation in the R6/R1 ratio in leaf development stage 1 in spring-red-leaf cultivars.

promoter, coordinately regulate color change in spring-red cultivars.

Discussion

Anthocyanins are a class of secondary metabolites that contribute to the red, blue, and purple colors of a range of leaves, flowers, and fruits. Previous studies have

shown how a rapid red coloration response to light and low temperature is likely to be caused by an elevation in the expression of *MYB10* genes, driving anthocyanin biosynthesis gene expression and, consequently, anthocyanin pigmentation in *Malus* plants (Ban et al., 2007; Tian et al., 2015). The *MYB10* gene is allelic to a peel-expressed gene, *MdMYB1* or *MYBA* (Ban et al., 2007;

Fig. 5. Correlation between the methylation and expression level of *McMYB10* in three representative cultivars with different leaf colors. (A) The methylation level of the *McMYB10* promoter and the expression level of *McMYB10* in 'Royalty'. (B) The methylation level of the *McMYB10* promoter and the expression level of *McMYB10* in 'Radiant'. (C) The methylation level of the *McMYB10* promoter and the expression level of *McMYB10* in 'Flame'.

Lin-Wang et al., 2010; Takos et al., 2006), and genetic studies suggests that these alleles are the major controllers of apple peel (Ban et al., 2007; Zhu et al., 2011) and flesh and foliage color (Espley et al., 2007). *MdMYB11/MYBA* expression is induced by light and cold temperature, whereas the *MYB10* transcript levels are suppressed at higher temperatures (Ban et al., 2007; Lin-Wang et al., 2010; Takos et al., 2006). *McMYB10* play an important role in leaf and petal coloration, by promoting the expression of downstream genes in crabapple (Jiang et al., 2014; Tian et al., 2015). When *MYB10* sequences from diverse apple cultivars were sequenced, no differences were found in the encoded protein sequences; however, differences in the expression of *MYB10* between apple cultivars were associated with differences in its promoter (Espley et al., 2009).

In a previous study, a 23-bp repeat motif in the upstream regulatory region of alleles of *MYB10* was found to present only in red-fleshed apples and red-leaf crabapples

(Espley et al., 2009). Moreover, this promoter allele was shown to be responsible for the increased accumulation of anthocyanins, and the number of repeat units correlates with an increase in transactivation by the *MYB10* protein (Espley et al., 2009). In our study, we determined the *McMYB10* promoter structures of 15 crabapple cultivars, which collectively represent three types of leaf color. The ever-green-leaf cultivars tested contained only the R1 promoter element and the ever-red-leaf cultivar had only the R6 promoter element, whereas the spring-red cultivars had both the R1 and R6 promoter elements. In a previous study, red-fleshed apple cultivars were reported to have both R6 and R1, or R6 only (Espley et al., 2009); however, we conclude that in crabapple, the presence of both minisatellite types results in a spring-red phenotype (Fig. 3). Based on these results, we conclude that the absence of the minisatellite R6 missing from the ever-green cultivars is the predominant reason for their green leaf

color, whereas the presence of both the R6 and R1 elements in the spring-red-leaf cultivars allows them to have both red and green leaves simultaneously. In addition, the ever-red-leaf cultivar Royalty, which only had an R6 element, showed a red color in its leaves, even in late developmental stages.

Extant apple chromosome homologies are derived from a putative nine-chromosome ancestor (Velasco et al., 2010). Each doublet of the eight apple chromosomes (3–11, 5–10, 9–17, and 13–16) is principally derived from one ancestor. The *MYB10* and *MYB1* genes are located at very similar positions on linkage group 9, and it was concluded that these genes were allelic (Lin-Wang et al., 2010; Zhu et al., 2011). Additionally, *MYB110a*, a paralog of *MYB10* was recently, located to chromosomes 17 (Chagné et al., 2013). These MYB TFs maybe share a common ancestor and coordinately regulated the red color formation in apple. However, the different numbers of R6 and R1 promoters in apple genome, and the functions of R6 and R1 promoter numbers on coloration are still unknown. PCR analysis showed that both minisatellites were present in spring-red crabapple cultivars, and that the anthocyanin levels in new buds from these cultivars were different. We speculated that the ratio of R6 and R1 satellites might determine the color of the new buds, and indeed the qRT-PCR results showed a similar trend in R6/R1 with the content of anthocyanins/flavonols in leaf development stage 1 of different cultivars. This is consistent with the dosage of the R6 and R1 elements playing an important role in the coloration of new crabapple buds (Fig. 4). At present, R6 and R1 promoter sequences are used as molecular markers to determine the fruit color (Zhang et al., 2014) and leaf color in apple breeding. Furthermore, the ratio of R6/R1 can be used as a key evaluation factor to analyze the color of the new buds in spring-red-leaf crabapple hybrids. Interestingly, most crabapple cultivars are diploid and the ratio of R6/R1 or R1/R6 is not duple 2, and there may be copy number variation of the two promoters in specific cultivars as a consequence of long-term evolution and hybridization.

DNA methylation is a critical feature of epigenetic regulation in many eukaryotes (Wang et al., 2013). Several studies have shown that the methylation pattern of the *MYB10* promoter affects plant color change. One example is fruit of the Honey Crisp cultivar, where a specific methylation pattern caused a striped pigmentation (Telias et al., 2011). A comparison of the methylation level in the pear (*Pyrus communis*) *PcMYB10* promoter between the MRB (Max Red Bartlett) and MRB-G (MRB green-skinned sport) varieties revealed a correlation between methylation and the green-skin phenotype (Wang et al., 2013). We therefore predicted that methylation might also affect the expression of *McMYB10* in crabapple leaves, and indeed our results showed a significant negative relationship between DNA methylation in the *McMYB10* promoter and *McMYB10* transcript levels. In MRB and MRB-G pear fruits, and in

red- and green-striped apple cultivars, there are no sequence differences in the MYB10 promoter sequences, while the significant methylation levels differ (Wang et al., 2013). However, significantly promoter sequence difference and methylation level variation were exist simultaneous in spring-red-leaf crabapple cultivars. We conclude that the promoter sequence and methylation level of *McMYB10* coordinately regulate the *McMYB10* expression (Fig. 5). Meanwhile, the main reason for the methylation levels in the spring-red cultivars were much higher than that in the other two leaf phenotype types of cultivars will trigger a series of future research projects to elucidate the mechanisms governing methylation levels in the spring-red cultivars.

Variation in methylation patterns has the potential to play a role in phenotypic plasticity as a response to environmental stress, and the difference in promoter sequences is mainly caused by evolutionary selection and mutation. Most of the ever-red- and spring-red-leaf crabapple cultivars originated from Europe and the United States, whereas most of the ever-green-leaf cultivars originated from China and other Asia countries. Differences in *McMYB10* methylation levels and promoter sequences between the three leaf color types likely reflect environmental/geographic adaptations and differences in the evolutionary selection pressure; however, these have yet to be identified.

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