MrMYB44-Like Negatively Regulates Anthocyanin Biosynthesis and Causes Spring Leaf Color of Malus ‘Radiant’ to Fade From Red to Green

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The “Spring-red-leaf” crabapple cultivar has young red leaves and mature green leaves. However, the mechanism of anthocyanin biosynthesis in crabapple leaves in spring remains unknown. In this study, Illumina RNA sequencing (RNA-Seq) was performed on Malus ‘Radiant’ leaf tissues in different stages of development. Twenty-two genes in the anthocyanin biosynthesis pathway and 44 MYB transcription factors (TFs) were significantly enriched among differentially expressed genes (DEGs). Three R2R3-MYB TFs in subgroup 22 of the MYB TF family, MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3, were highly expressed in green leaves according to RNA-Seq and quantitative real-time quantitative PCR results. Their expression levels were negatively correlated with anthocyanin content. In transient assays, overexpression of MrMYB44-like1, MrMYB44-like2, or MrMYB44-like3 inhibited anthocyanin accumulation and reduced pigment in leaf disks of M. ‘Radiant’ and fruit peels of M. domestica ‘Fuji.’ When the conserved region of the three MrMYB44-like s was silenced, the anthocyanin biosynthesis pathway was activated and pigments increased in both tissues. Moreover, bimolecular fluorescence complementation assays showed MrMYB44-like s interacted with MrWRKY6 to form protein complexes that regulated anthocyanin biosynthesis.

Keywords: anthocyanin, transcriptome, MYB44-like, WRKY6, spring leaf color, transcription factors, crabapple

INTRODUCTION

Malus crabapple (Malus Mill.) is an ornamental deciduous tree or shrub in the family Rosaceae. It is a popular decorative plant in landscapes because of its pleasing form and attractive colors (Cui et al., 2018). Malus ‘Radiant’ is a “Spring-red-leaf” crabapple cultivar with young red leaves and green mature leaves, indicating complex changes in coloration. This quality trait is primarily determined by its metabolite composition (Yang et al., 2018).

Anthocyanins are the main pigments in flowers, leaves, and fruits and generate characteristic red, blue, and purple hues (Winkel-Shirley, 2001; Jaakola, 2013). Bright organ colors derived from anthocyanin accumulation directly determine the ornamental value of plants (Xie et al., 2012).

The anthocyanin metabolic pathway includes anthocyanin biosynthesis and degradation. Anthocyanin biosynthesis is derived from branches of the phenylalanine pathway (Koes et al., 2005; Petroni and Tonelli, 2011). Major enzymes include phenylalanine ammonia lyase (PAL),
cinnamate-4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3′-hydroxylase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanin synthase (ANS), and UDP-glucose: flavonoid 3-O-glu-cosyltransferase (UFGT). The enzymes encoded by the genes are responsible for biochemical reactions in anthocyanin biosynthesis (Honda et al., 2002). Anthocyanin degradation also affects color change. Loss of red pigmentation may be caused by increases in actively induced anthocyanin degradation, as well as termination of anthocyanin biosynthesis and dilution by growth (Oren-Shamir, 2009). Enzymes in three common families were recently found to participate in anthocyanin degradation, including β-glucosidase (BGLU), polyphenol oxidase (PPO), peroxidase (PER), and laccase (LAC) (Ying et al., 2018).

Expression of genes in the anthocyanin biosynthesis pathway is primarily regulated by a series of transcription factors (TFs). The MYB family of TFs is important in the anthocyanin biosynthesis pathway because it activates or inhibits genes by directly or indirectly binding the cis-acting element of DNA (Chen L. et al., 2019). As activators, MYB TFs usually form protein complexes with bHLH and WD40 (Albert et al., 2011). For example, the Aft (MYB) protein interacts with SIJAF13 (bHLH) and SIAN11 (WDR) forming an MBW complex and enhancing anthocyanin content in tomato (Solomonum lycopersicum) (Yan et al., 2020). There has been a recent notable focus on MYB repressors (Ma and Constabel, 2019). The MYB repressors can act directly on promoters of structural genes (Yan et al., 2021), like NtMYB3 represses promoter activity of NtFLS in Chinese narcissus (Narcissus tazetta L. var. chinesis) (Anwar et al., 2019) and MdMYB6 inhibits the promoter activity of MdANS and MdGSTF12 in apple (Malus domestica) (Xu et al., 2020). They can also passively repress anthocyanin biosynthesis by interacting with bHLH proteins to compete with MYB activators in the MYB–bHLH complex, thereby reducing their activation capability. For example, MdMYB15L interacts with MdbHLH33 in Malus (Xu et al., 2018); VvMYB4b interacts with VvMYC1 (bHLH) in Vitis vinifera (Cavallini et al., 2015); and MtMYB2 interacts with MtTT8 (bHLH) in Medicago truncatula (Jun et al., 2015).

Moreover, other TF families also regulate anthocyanin biosynthesis via interaction with MYB TFs, like AtSPL9, negatively regulates anthocyanin accumulation through destabilization of a MYB-bHLH-WD40 transcriptional activation complex in Arabidopsis (Gou et al., 2011); PyWRKY26 interacts with PybHLH3 to target the PyMYB114 promoter resulting in anthocyanin accumulation in red-skinned pear (Pyrus L.) (Li et al., 2020); IbNAC56a and IbNAC56b interact with IbMYB340 and IbbHLH2 to positively regulate anthocyanin biosynthesis by binding to the IbANS promoter in sweet potato (Ipomoea batatas) (Wei et al., 2020).

Fading color in spring leaves is common, but for crabapple, the mechanism of changes in leaf coloration in natural conditions remains unclear. In this study, contents of anthocyanin metabolites and expression of differentially expressed genes (DEGs) in young red and green mature leaf tissues of M. 'Radiant' were analyzed to determine the key genes involved in anthocyanin biosynthesis. Then, the function of candidate key TFs was determined. This study increases understanding of the mechanism of pigmentation underlying color changes in leaves and therefore will facilitate breeding plants with desirable color traits.

**MATERIALS AND METHODS**

**Plant Material**

*Malus 'Radiant'* plants were grown in the crabapple germplasm nursery of Northwest A&F University, Yangling, China, under natural conditions. During growth, leaf phenotype gradually changes during development and can be divided into four stages (S1–S4, Figure 1A). Tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until RNA extraction and total anthocyanin and high-pressure liquid chromatography (HPLC) analyses.

**Leaf Color Measurement**

Colors of fresh leaves were measured with a CR-400 chroma meter (Konica Minolta, Tokyo, Japan). Parameters L*, a*, and b* were determined. Parameter L* indicates lightness (ranging from 0 to 100). Positive and negative values of a* are standard for red and green, respectively, and those of b* represent yellow and blue, respectively (both ranging from −60 to +60) (Mcguire, 1992; Liu et al., 2016). Means were based on five independent biological replicates with three technological replicates.

**Measurement of Total Anthocyanin Contents**

Total anthocyanins were extracted with a methanol–hydrochloric acid (HCL) solution (Lee and Wicker, 1991). Leaf samples (0.5 g) were incubated in 10 ml of methanol and 0.1% HCL (v/v) at 4°C for 48 h in the dark. Mixtures were centrifuged at 6,000 rpm for 3 min, and supernatants collected. Absorbance was measured at 350, 620, and 657 nm, in triplicate. Relative anthocyanin content was determined as follows:

\[ A = (A_{530} \text{ nm} - A_{620} \text{ nm}) - 0.1(A_{650} \text{ nm} - A_{620} \text{ nm}) \]

Total anthocyanin content was normalized to the fresh weight (FW) of each sample.

**High-Pressure Liquid Chromatography Analysis**

Accurately weighed leaf samples (0.5 g) were leached with 10 ml of methanol at 4°C for 48 h in the dark and then stirred by ultrasonic waves for 1 h. Mixtures were centrifuged at 6,000 rpm for 3 min at 4°C and then set aside. Materials were separated using a Shimadzu LC-2030C liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Inertsil C-18 column (5.0 mm particle size, 4.6 mm × 250 mm). The HPLC separation was performed as previously described by Han et al. (2020a). The respective structure was confirmed by comparison with a standard using LC–MS as described by Li et al. (2007). Measured...
**Meng et al. MrMYB44-Like Negatively Regulates Anthocyanin Biosynthesis**

FIGURE 1 | Phenotype and total anthocyanin content at different stages of spring leaf development in *Malus* 'Radiant.' (A) Leaf color at four stages (S1–S4). (B) *L* ∗, *a* ∗, and *b* ∗ values of leaves at four stages. (C) Total anthocyanin content at four stages (FW, fresh weight). Error bars represent the standard errors. Different lowercase letters indicate significant differences among treatments according to one-way ANOVA test (*P* < 0.05).

Results were compared with retention time and standard curve of the reference substance. Three biological replicates were analyzed in this section.

**RNA Sequencing Data Analysis**

Extraction of total RNA, library construction, RNA sequencing (RNA-Seq), RNA assembly, and DEG analysis of *M. 'Radiant'* leaves (S1 and S4, each with three biological replicates) were performed by Gene Denovo Biotechnology Co., (Guangzhou, China) as described previously (Han et al., 2020b). *Malus × domestica* HFTH1 Whole Genome version 1.0\(^1\) was used to conduct transcriptome alignment.

All DEGs were mapped to the Gene Ontology (GO) database\(^2\) and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways database.\(^3\) Gene numbers were calculated for every term, and significantly enriched GO terms in DEGs, compared with the genome background, were defined by hypergeometric test with FDR ≤ 0.05 as a threshold.

\(^1\)https://www.rosaceae.org/species/malus_x_domestica_HFTH1/genome_v1.0
\(^2\)http://www.geneontology.org/
\(^3\)http://www.kegg.jp/kegg

**Quantitative Real-Time PCR Analysis**

Total RNA was isolated following the method described previously. Approximately 1 µg of total RNA was used for first-strand cDNA synthesis using a PrimeScript\textsuperscript{TM} RT reagent kit with a gDNA Eraser (TaKaRa Bio Inc., Shiga, Japan). Gene specific primers were designed with Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, CA, United States) and primer sequences are shown in Supplementary Table 1.

The relative expression level of DEGs in the samples was determined using 2 × SYBR real-time PCR mixture kit (BioTeKe, Beijing, China) on the StepOnePlus real-time PCR system (Applied Biosystems, Waltham, MA, United States). 18S rRNA (DQ341382) was used as the internal control. All experiments were performed with three independent biological replicates and three technical replicates.

**Construction of Expression Vectors**

To construct *MrMYB44-like1/2/3* overexpression vectors, *MrMYB44-like1/2/3* cDNA sequences without a termination codon were inserted between *BamHI* and *SalI* of pCAMBIA2300-green fluorescent protein (GFP) vectors (35S: GFP). In addition,
a 152-bp fragment of MrMYB44-like3 (411–563 bp), which is highly conserved in MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3, was inserted between Kpn1 and Xhol of pTRV2 vectors to suppress MrMYB44-like gene expression. A Seamless Cloning and Assembly Kit (Novoprotein, Shanghai, China; primers are listed in Supplementary Table 1) was used to produce overexpression vectors 35S:MrMYB44-like1/2/3: GFP and gene silencing vector pTRV2-MrMYB44-like. Then, recombinant plasmids 35S:MrMYB44-like1/2/3: GFP, pTRV2-MrMYB44-like, pCAMBIA2300-35S: GFP empty vector, and pTRV1 and pTRV2 empty vectors were introduced into Agrobacterium tumefaciens GV3101 by the heat shock method.

Subcellular Localization

The 35S:MrMYB44-like1/2/3: GFP vectors described in the last section were used to identify subcellular localization of MrMYB44-likes. Recombinant (35S:MrMYB44-like1/2/3: GFP) and control (pCAMBIA2300-35S: GFP) vectors were introduced into 5-week-old Nicotiana benthamiana leaves by agroinfiltration. Infiltrated plants were grown for over 72 h in a growth chamber, and the GFP fluorescence of samples was observed under a confocal laser-scanning microscope (TCS SP8; Leica, Wetzlar, Germany).

Agroinfiltration in Fruit of Malus domestica ‘Fuji’

Procedures for overexpression and suppression of MrMYB44-likes by agroinfiltration in apple fruit were according to Li et al. (2012) with some modifications. Agrobacterium tumefaciens carrying pC2300-MrMYB44-like1/2/3 and empty pC2300 vectors were injected into the peel of freshly bagged apples using a needle-less syringe. Injected apples were treated in darkness at 4°C for 7 day and then transferred to 24 h of continuous white light (200 µmol m⁻² s⁻¹) with supplemental UV-B (280–320 nm) at 17°C in a growth chamber for 4 day.

To suppress MrMYB44-like expression, separate A. tumefaciens cultures containing pTRV1 and pTRV2-MrMYB44-like were mixed in a 1:1 ratio and then infiltrated into the fruit skin of freshly bagged apples using a needle-less syringe. Injected apples were treated as described above.

Agroinfiltration in Leaf Disks of Malus ‘Radiant’

Overexpression, suppression, and empty vector injections were prepared as described previously. Agroinfiltration in leaf disks was performed according to procedures described by Dai et al. (2012) with some modifications. Leaves (S1) of M. ‘Radiant’ were collected from trees grown in natural conditions. One-centimeter-diameter disks were taken from the center of leaves with a hole punch. Leaf disks were placed in a bacterial suspension solution and infiltrated under vacuum at 0.5 MPa for 15 s. After release of the vacuum, disks were washed in deionized water twice and kept in deionized water for 3 day at 4°C and then at 24°C for 3 day.

Bimolecular Fluorescence Complementation Assays

Constructs to investigate interactions were produced in pSPYNE-35S and pSPYCE-35S vectors by using bimolecular fluorescence complementation (BiFC) assays. The cDNAs without stop codons of MrMYB44-like1/2/3 were cloned into pSPYNE-35S, and those of MrbHLH3 and MrWRKY6 were cloned into pSPYCE-35S. Primers used for plasmid construction are listed in Supplementary Table 1. Constructs were transformed into A. tumefaciens by the heat shock method. Five-week-old Nicotiana benthamiana leaves were infiltrated with the mixed A. tumefaciens. Fluorescence signals were detected 72 h after infiltration using an inverted laser-scanning microscope with a 40 × water-immersion objective (TCS SP8; Leica, Wetzlar, Germany).

RESULTS

Analysis of Pigment Levels and Differentially Expressed Genes in Spring Leaves

Leaves of M. ‘Radiant’ changed from red to green during spring growth (Figure 1A). The values of chromatic parameters L* and b* increased gradually and peaked in S4 (Figure 1B). By contrast, values of a* decreased to negative in S3 and reached the lowest value in S4. To investigate physiological changes in leaves of different colors, total anthocyanin content was determined by extraction with methanol–HCL. Total anthocyanin content was highest in S1, reaching 4.79 µmol g⁻¹ FW, and then decreased gradually in other stages, reaching 0.43 µmol g⁻¹ FW in S4 (Figure 1C). Anthocyanin constituents and contents in the four leaf stages were determined using HPLC. Cyanidin 3-galactoside chloride and cyanidin-3-O-glucoside chloride were both found in S1 and S2, but cyanidin-3-O-glucoside chloride was not detected in S3 and S4. Changes in contents of the two compounds were consistent with those of total anthocyanin (Table 1). Nine other types of phenolic compounds were also identified, including five flavonols, two flavanols, and one flavone (Table 1). Because anthocyanin contents were significantly different (P < 0.05), stages S1 and S4 were selected for RNA-sequencing.

Six RNA libraries from M. ‘Radiant’ leaves (S1 and S4, each with three biological replicates) were sequenced. After removing adapter sequences and low-quality reads, raw reads of each sample were equal to or greater than 48,141,832 (Supplementary Table 2). Clean reads with a Q-score of 30 or higher (i.e., base call accuracy ≥ 99.9%) accounted for more than 94% of the total. The GC content (ratio of guanine and cytosine content to total nucleobases) ranged from 47 to 49%. Total mapping of each library was greater than 89%, and average unique mapping was greater than 85%. These results indicated transcriptomic data were suitable for further analysis.

Differentially expressed genes between the two leaf stages were identified. The overall distribution of DEGs is shown in a volcano plot (Figure 2A). A total of 14,660 DEGs were detected (Figure 2A). In the comparison between S1 and S4, 8,790 DEGs
Twenty-two DEGs were involved in anthocyanin biosynthesis
Anthocyanin Metabolites
Analysis of Genes Associated With Change in Leaf Pigmentation
Most enzyme-encoding genes had higher FPKM (fragment per kilobase of transcript per million mapped reads) values in S4 than in other stages, except one CHS gene and one UFGT gene (Figure 3A). Consistent with low anthocyanin content in S4, expression of most genes was also very low (Supplementary Table 3).

Genes with FPKM values less than one were excluded in the study. The remaining targets included 18 laccase genes, 17 peroxidase genes, and 12 β-glucosidase genes (Figure 3A). Among them, 10 laccase, 10 peroxidase, and 6 β-glucosidase genes had relatively low expression in S1 but relatively high expression in S4. Those genes were negatively correlated with anthocyanin content (Supplementary Table 3), indicating they promoted anthocyanin degradation.

Results from quantitative real-time PCR (qRT-PCR) were used to validate whether differences in RNA-seq levels of DEGs involved in the anthocyanin metabolism pathway reflected actual transcription levels in M. 'Radiant' leaves at different stages of development (Figure 3B). Expression patterns of six anthocyanin biosynthesis genes [MrPAL (HF01560), MrCHS (HF35684), MrCHI (HF23114), MrDFR (HF31029), MrANS (HF39612)] and three anthocyanin degradation genes [MrLAC (HF27740), MrPER4 (HF19260), and MrBGLU (HF06072)] obtained by qRT-PCR were consistent with RNA-Seq data, confirming expression patterns in the four stages of development (Figure 3B). Levels of expression of anthocyanin biosynthesis genes gradually decreased as red faded from leaves. By contrast, the anthocyanin degradation genes MrLAC, MrPER4 and MrBGLU were most highly expressed in S4.

To screen potential regulators of anthocyanin biosynthesis, 12 TF families were identified. After removal of genes with extremely low expression (FPKM < 1), 44 MYBs, 37 bHLHs, 1 WD40, 34 WRKYs, 8 SPLs, 2 HY5s, 25 LBDs, 22 HSPs, 11 T3, 38 ERFs, 94 NACs, and 4 bZIPs were identified. In MYB, WRKY, ERF and bZIP families, more genes had higher expression in S4 than in S1. By contrast, in bHLH, WD40, SPL, HY5, LBD, TCP, and NAC families, more genes had lower expression in S4 than in S1 (Table 2). All TF families had complex expression patterns (Supplementary Table 4). Gene expression in the same family was either positively or negatively correlated with anthocyanin content, which indicated a complex network regulated change in leaf coloration.

Analysis of MYB Transcription Factors Identified as Differentially Expressed Genes
In RNA-Seq data, 44 MYBs were identified as DEGs (both R2R3 MYBs and R3 MYBs). Heat map and motif analysis are shown in Supplementary Figure 1. Notably, expression of three MYBs (HF13071, HF21590, and HF27377) increased 2.76 to 3.68-log-fold in S4, with FPKM values ranging from 91.07 to 257.49, which were higher than those of
most of other MYB DEGs (Supplementary Table 3). Results from qRT-PCR showed that expression of all three genes increased as leaves changed from red to green in S1–S4, consistent with RNA-seq expression patterns (Figure 4). These results suggested the three MYBs negatively regulated anthocyanin biosynthesis.

Phylogenetic analysis was performed on the differentially expressed MYB TFs and 126 Arabidopsis MYBs from the TAIR\(^4\) (Supplementary Figure 2). The 44 MYB DEGs were divided into 24 subgroups. The three MYBs (HF13071, HF21590, and HF27377) were in subgroup 22 which including AtMYB44 (AT5G67300), AtMYB70 (AT2G23290), AtMYB73 (AT4G37260), and AtMYB77 (AT3G50060) (Supplementary Figure 2). Then, all of these three MYBs were used for

\(^4\)https://www.arabidopsis.org/
FIGURE 3 | Expression of differentially expressed genes involved in anthocyanin pathways. (A) Expression analysis of flavonoid pathway genes at S1 and S4 stages in fruit. Expression was evaluated via RNA-seq with three biological replicates. Log2 fold change represents expression. (B) Validation of RNA-seq expression profiles in stages S1–S4 via real-time quantitative RT-PCR. Values were calculated relative to the transcription level in S4 (set to one). Error bars represent the standard errors. Different lowercase letters indicate significant differences among treatments according to one-way ANOVA test (P < 0.05).

Cloning and Subcellular Localization of MrMYB44-Likes

Full-length sequences of MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 were obtained from RNA-seq, and cDNA prepared from M. ’Radiant’ S4 leaf RNA was used as templates for PCR amplification of gene sequences (Supplementary Table 1). Amino acid sequence alignment indicated the deduced proteins of MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 shared 41.1, 39.6, and 48.45% sequence identity, respectively, with AtMYB44. As shown in Supplementary Figure 4, MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 all contained the conserved motifs of subgroup 22, including 22.1 (TGLYMSPxSP), 22.2 (D/EPP/MTxLxLSLP), and 22.3 (GxFMxVVQEMIxxEVRSYM) (Stracke et al., 2010; Zhou et al., 2017). Motif 22.2 is partially conserved with the EAR motif found in subgroup 4 R2R3 MYB repressors. Additionally, the conserved bHLH-interacting motif amino acid ([D/E]Lx[L/K]x3Lx6Lx3R) was not found in MrMYB44-like and other amino acid sequences except in AtMYB4 and FuMYB1. In addition, genes MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 were on chromosomes 15, 8, and 2, respectively (Supplementary Figure 3).
TABLE 2 | Candidate anthocyanin regulatory genes in Malus ‘Radiant.’

| Gene family | NO. allb | S1 vs. S4 |  |
|-------------|---------|---------|---|
|             | NO. upc | NO. downc |  |
| MYB         | 44      | 24      | 20 |
| bHLH        | 37      | 12      | 25 |
| WD40        | 1       | 0       | 1  |
| WRKY        | 34      | 30      | 4  |
| SPL         | 8       | 1       | 7  |
| HY5         | 2       | 0       | 2  |
| LBD         | 7       | 1       | 6  |
| HSP         | 22      | 13      | 9  |
| TCP         | 11      | 5       | 6  |
| ERF         | 38      | 23      | 15 |
| NAC         | 94      | 38      | 56 |
| bZIP        | 4       | 4       | 0  |

aIndicates the total number of regulatory genes in differentially expressed genes. 
bIndicates the number of upregulated genes in each comparison. 
cIndicates the number of downregulated genes in each comparison. All candidate anthocyanin regulatory genes in this table are screened from differentially expressed genes.

To examine subcellular localization of MrMYB44-like1/2/3, recombinant (35S:MrMYB44-like1/2/3: GFP) and control (pCambia2300-35S: GFP) vectors were introduced into tobacco leaves by agroinfiltration. The GFP fluorescence of the control vector was clearly distributed throughout the entire cell (Supplementary Figure 4A), whereas 35S:MrMYB44-like1/2/3: GFP vectors displayed strong fluorescence signals in the nuclei of tobacco cells (Supplementary Figures 4B–D). Therefore, the three R2R3-MYB TFs, MrMYB44-like1/2/3, were likely localized and functioned in the nucleus.

MrMYB44-Like Negatively Regulates Peel Coloration in Malus domestica ‘Fuji’

To verify MrMYB44-like down-regulated anthocyanin biosynthesis, overexpression vectors (pCambia2300) of MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 were constructed. Overexpression was initiated by an agrobacterium-mediated transformation method. Gene expression was suppressed by virus-induced gene silencing (VIGS), using a TRV vector. Vectors contained a conserved MrMYB44-like region, and in principle, could silence the expression of MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3.

Overexpression of MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 inhibited fruit peel coloration around injection sites, whereas there was obvious red coloration in sites without injection and with injection of empty pCambia2300 vectors (Figure 5A). Subsequently, RT-qPCR revealed that MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 transcript levels in overexpression peel sites increased by approximately three- to six-fold (Figure 5B), whereas expression of anthocyanin biosynthesis genes, such as MrPAL, MrCHS, MrCHI, MrDFR, and MrANS, decreased (Figure 5B). By contrast, with silencing of MrMYB44-like, red pigmentation increased around injection sites, compared with sites without injection and with injection of empty TRV2 vectors (Figure 6A). Expression levels of the three MrMYB44-like decreased by approximately three- to four-fold, whereas those of the anthocyanin biosynthesis genes greatly increased by approximately two- to twelve-fold (Figure 6B). The abundance of those gene transcripts was consistent with pigmentation.

MrMYB44-Like Negatively Regulates Anthocyanin Biosynthesis in Leaf Disks of Malus ‘Radiant’

To verify MrMYB44-like repressed anthocyanin biosynthesis in crabapple leaves, red, young leaf disks collected in spring were infected with overexpression and suppression recombinant vectors. There was inevitable passive anthocyanin degradation because the leaves were cultured in vitro, and as a result, empty vector (both pCambia2300 and pTRV2)-infected leaf disks also faded to some degree at 3 days post-infection. However, fading of red in the overexpression group was greater than that in those untreated and treated with empty vectors as leaves turned green, whereas the silenced group maintained the red phenotype (Figure 7A).

Total anthocyanin concentration in overexpression leaf disks (1.60, 1.61, and 1.92 μmol g⁻¹ in pC2300-MYB44-like1, pC2300-MYB44-like2, and pC2300-MYB44-like3, respectively) was approximately twofold lower than that in leaf disks with empty pC2300 vectors (3.25 μmol g⁻¹). In addition, anthocyanin concentration in silenced leaf disks (6.98 μmol g⁻¹, with pTRV2-MYB44-like) was approximately twofold higher than that in leaf disks of the control group (3.25 μmol g⁻¹, with pTRV2) (Figure 7B). According to HPLC analyses, concentrations of both cyanidin-3-galactoside chloride and cyanidin-3,5-O-diglucoside with overexpression or suppression of MrMYB44-like were approximately twofold lower or higher, respectively, than the concentrations in the corresponding control groups. Those results were consistent with total anthocyanin concentrations and phenotype coloration (Supplementary Table 5). Moreover, levels of rutin, hyperoside, and naringenin in silenced leaf disks were
higher than those in control leaves, whereas with overexpression, levels were lower than those in control leaves.

According to qRT-PCR results, expression of MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 increased by five-, eight-, and nine-fold, respectively, in overexpression leaf disks. By contrast, expression of anthocyanin biosynthesis genes, including MrPAL, MrCHS, MrCHI, MrDFR, and MrANS, decreased significantly (Figure 7C). Furthermore, MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 transcript levels in MrMYB44-like-silenced leaf disks decreased by approximately 50%, compared with levels in leaf disks with empty vectors. Transcript levels of anthocyanin biosynthesis genes were significantly higher in silenced leaf disks than in control leaf disks (Figure 7D). Overall, expression of these genes was consistent with anthocyanin levels and the pigmentation observed in overexpression, silenced, and control groups.

**MrMYB44-Likes Interact With MrWRKY6 but Not With MrbHLH3**

According to the previous studies, combinatorial interactions between MYB and bHLH TFs are crucial in regulating anthocyanin biosynthesis (Albert et al., 2011; Xu et al., 2018). However, the three MrMYB44-likes had no bHLH-interacting
sites according to amino acid sequence alignment. To further investigate whether there were interactions between MrMYB44-like and MrbHLH, BiFC assays were performed. The gene MrbHLH3 (HF28271) was screened because of its close genetic relationship with MdbHLH3, which was previously characterized as a regulator of the flavonoid pathway in Malus (Xie et al., 2012). Three construct combinations, that is, MrMYB44-like1-YFPN plus MrbHLH3-YFP C, MrMYB44-like2-YFPN plus MrbHLH3-YFP C, and MrMYB44-like3-YFPN plus MrbHLH3-YFP C, were cotransformed into tobacco epidermal cells. Yellow fluorescent signals were not observed in epidermis cells transformed with any of the three combinations (Figure 8). The results suggested that MrMYB44-like1/2/3 did not interact with MrbHLH3 to suppress anthocyanin biosynthesis.

According to Zhou et al. (2017), StMYB44, in MYB subgroup 22, physically interacts with AtWRKY6 and StWRKY6 in vivo. Therefore, BiFCs were performed to investigate whether MrWRKY6 could interact with MrMYB44-like1/2/3 to form protein complexes. MrWRKY6 was fused to the C-terminal fragment of YFP as MrWRKY6-YFP C, and then, three combinations including MrMYB44-like1-YFP N plus MrWRKY6-YFP C, MrMYB44-like2-YFP N plus MrWRKY6-YFP C, and MrMYB44-like3-YFP N plus MrWRKY6-YFP C were cotransformed into tobacco epidermal cells. As shown in Figure 8, yellow fluorescence signals were observed in nuclei of tobacco cells that coexpressed one of the three groups. Thus, MrWRKY6 physically interacted with MrMYB44-like1/2/3 in vivo.

DISCUSSION

In spring, leaf color changes from red to green during growth in M. ‘Radiant.’ The value of a*, which is a standard for...
FIGURE 8 | Bimolecular fluorescence complementation assays showing that MrMYB44-like1/2/3 did not interact with MrbHLH3 but did interact with MrWRKY6 in nuclei of epidermal cells in Nicotiana benthamiana. Bars, 20 µm.

red and green, gradually decreased from S1 to S4. Consistent with color measurements, HPLC results showed contents of cyanidin 3-galactoside chloride and cyanidin-3-O-glucoside chloride decreased with development. Therefore, decreasing anthocyanin content led to fading red color in M. 'Radiant' leaves.

The MYB TFs positively or negatively regulate anthocyanin biosynthesis (Yan et al., 2021). In this study, 44 MYBs, including 38 R2R3-MYBs and 6 R3-MYBs, were screened in a phylogenetic tree analysis, which indicated that R2R3-MYBs have crucial roles as transcriptional regulators affecting changes in leaf color. Among the MYBs, MrMYB44-like1 (HF13071), MrMYB44-like2 (HF21590), and MrMYB44-like3 (HF27377) had notably high FPKM and fold change values, and expression of the three genes was negatively correlated with anthocyanin content. MrMYB44-like1/2/3 are in subgroup 22, which has a partial EAR motif (Liu et al., 2019). EAR motif-mediated transcriptional repression is the main form of transcriptional repression in plants (Jiao et al., 2018). Most importantly, transient overexpression of MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 in M. domestica ‘Fuji’ fruit peels and M. 'Radiant’ leaves repressed expression of structural genes related to anthocyanin biosynthesis and reduced anthocyanin concentrations. By contrast, structural gene expression and red pigmentation increased significantly when MrMYB44-likes were transiently silenced in peels and leaves. Therefore, MrMYB44-likes are likely functional MYB TFs that negatively regulate anthocyanin biosynthesis. The three MYB44-like genes all showed strong inhibition ability when transiently overexpressed, although their amino acid sequences were not identical. The genes were also on different chromosomes. Those results indicated they were functionally redundant genes in the same subgroup of MYBs rather than different copies of one gene or allele genes. The three gene sequences were similar to MdMYB44 which was reported to negatively regulate fruit malate accumulation (Jia et al., 2018, 2021). It was indicated that the MYB44 or MYB44-like may play as important repressors in secondary metabolism in Malus.

In plants, R2R3-MYB repressors can passively compete with activator complexes by interacting with bHLH proteins to reduce their activation capability and thereby repress anthocyanin biosynthesis (Albert et al., 2011; Chen L. et al., 2019;
Yan et al., 2021). However, in this study, according to amino acid sequence alignment, MrMYB44-like1/2/3 did not contain the conserved amino acid signature of the bHLH-interacting motif [(D/E)Rx2[R/K]3Lx6Lx3R], which is used to predict new MYB–bHLH interactions (Zimmermann et al., 2010). Further, in BiFC assays, MrMYB44-like did not interact with MrbHLH3, indicating the repressive function is independent of bHLH. Thus, MrMYB44-like could not repress anthocyanin biosynthesis by interacting with bHLH proteins to compete with MYB activators in the MYB–bHLH complex.

In addition to bHLH, many other TFs interact with MYBs, such as AtWRKY6 and StWRKY6, which physically interact with StMYB44 in vivo (Zhou et al., 2017). Amino acid sequence alignment indicated MrMYB44-like1/2/3 shared high amino acid identity with StMYB44 in DNA-binding domains. In addition, MrWRKY6 (HF12290) had affinities with AtWRKY6 and StWRKY6, and according to RNA-seq, expression of MrWRKY6 was consistent with that of MrMYB44-like1/2/3. Therefore, interactions were predicted between MrWRKY6 and MrMYB44-like1/2/3, and BiFC assays confirmed that MrMYB44-like1/2/3 physically interacted with MrWRKY6 in nuclei of tobacco epidermal cells. Those results suggested that MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 could form a protein complex with MrWRKY6 to regulate transcript levels of anthocyanin biosynthesis genes. In mature leaves, high abundance of transcriptional complexes likely strongly inhibited anthocyanin biosynthesis genes, which led to fading color. In previous studies, WRKY6 responded to plant senescence, in pathogen defense, and to low phosphorus stress in different plants (Robatzek and Somssich, 2002; Wang et al., 2016; Zhou et al., 2017; Chen Z. et al., 2019). In this study, a different potential motif ([D/E]Lx2[R/K]x3Lx6Lx3R), which is used to predict new MYB–bHLH interactions (Zimmermann et al., 2010). Further, in BiFC assays, MrMYB44-like did not interact with MrbHLH3, indicating the repressive function is independent of bHLH. Thus, MrMYB44-like could not repress anthocyanin biosynthesis by interacting with bHLH proteins to compete with MYB activators in the MYB–bHLH complex.

In addition to bHLH, many other TFs interact with MYBs, such as AtWRKY6 and StWRKY6, which physically interact with StMYB44 in vivo (Zhou et al., 2017). Amino acid sequence alignment indicated MrMYB44-like1/2/3 shared high amino acid identity with StMYB44 in DNA-binding domains. In addition, MrWRKY6 (HF12290) had affinities with AtWRKY6 and StWRKY6, and according to RNA-seq, expression of MrWRKY6 was consistent with that of MrMYB44-like1/2/3. Therefore, interactions were predicted between MrWRKY6 and MrMYB44-like1/2/3, and BiFC assays confirmed that MrMYB44-like1/2/3 physically interacted with MrWRKY6 in nuclei of tobacco epidermal cells. Those results suggested that MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 could form a protein complex with MrWRKY6 to regulate transcript levels of anthocyanin biosynthesis genes. In mature leaves, high abundance of transcriptional complexes likely strongly inhibited anthocyanin biosynthesis genes, which led to fading color. In previous studies, WRKY6 responded to plant senescence, in pathogen defense, and to low phosphorus stress in different plants (Robatzek and Somssich, 2002; Wang et al., 2016; Zhou et al., 2017; Chen Z. et al., 2019). In this study, a different potential motif of WRKY6 was identified that indicated it might be a key regulator of the anthocyanin biosynthesis pathway. However, further studies are needed.

CONCLUSION

This study confirms that in spring, anthocyanin content decreases with leaf development in M. ’Radiant.’ During development, transcription of most anthocyanin biosynthesis genes gradually decreases and that of key anthocyanin degradation genes gradually increases. The MYB repressors MrMYB44-like1, MrMYB44-like2, and MrMYB44-like3 are members of subgroup 22 and are important negative regulators that inhibit anthocyanin biosynthesis. The three MrMYB44-like interact with MrWRKY6 but not with MrbHLH3. This study further validates and supplements known functions of MYB subgroup 22, while also providing new insights into the mechanism of leaf color change in crabapple.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive (SRA) with bioproject No. PRJNA784337, and under GenBank accession numbers of SAMN23483827, SAMN23483828, SAMN23483829, SAMN23483830, SAMN23483831, and SAMN23483832.

AUTHOR CONTRIBUTIONS

J-XM performed most of the experiments and data analysis. JW and R-FC carried out material collection and pigment extraction. Y-HQ, R-FC, and HW conducted pigment analysis. JZ conducted a part of RNA extraction and qRT-PCR experiment. JW and Y-LW constructed a part of vectors. J-XM, JW, Y-HQ, and R-FC participated in the preparation of the manuscript. H-HL conceived, designed, and coordinated the studies. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.822340/full#supplementary-material

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