

Two MYB activators of anthocyanin biosynthesis exhibit specialized activities in petiole and fruit of diploid strawberry

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Highlight

Strawberry plants accumulate anthocyanin pigments in petioles through the action of *FveMYB10-Like*. Unlike *FveMYB10*, *FveMYB10-Like* expression is repressed in shade and *FveMYB10-Like* shows different preference for downstream target genes.

Abstract

The R2R3-MYB transcription factor FveMYB10 is a major regulator of anthocyanin pigmentation in the red strawberry fruits. *fvemyb10* loss-of-function mutants form yellow fruits but still accumulate purple-colored anthocyanins in the petioles, suggesting that anthocyanin biosynthesis is under distinct regulation in fruits and petioles. We identified a *green petioles (gp)-1* mutant from chemical mutagenesis in the diploid wild strawberry *Fragaria vesca* that lacks anthocyanins in petioles. Using mapping-by-sequencing and transient functional assays, we confirmed that the causative mutation resides in a *FveMYB10-Like (MYB10L)* gene and that *FveMYB10* and *FveMYB10L* function independently in the fruit and petiole respectively. In addition to their tissue-specific regulation, *FveMYB10* and *FveMYB10L* respond differently to changes in light quality, produce distinct anthocyanin compositions, and preferentially activate different downstream anthocyanin biosynthesis genes in their respective tissues. This work identifies a new regulator of anthocyanin synthesis and demonstrates that two paralogous MYB genes with specialized functions enable tissue-specific regulation of anthocyanin biosynthesis in fruit and petiole tissues.

Keywords: anthocyanin, diploid wild strawberry, *Fragaria vesca*, fruit, *MYB10*, *MYB10-Like*, petiole, tissue-specific regulation,

Introduction

Anthocyanins are a class of flavonoid pigments widely found in plants (Tanaka *et al.*, 2008). Abundantly present in flowers and fruits, anthocyanins help attract pollinators and seed dispersers (Lomascolo *et al.*, 2010; Landi *et al.*, 2015). In vegetative tissues, anthocyanins are produced to enhance plant tolerance of a variety of abiotic and biotic stresses (Chalker-Scott, 1999; Li *et al.*, 2017; Nevo *et al.*, 2018). When consumed, anthocyanins offer significant benefit to human health, ranging from protection against cardiovascular diseases and cancer to suppressors of inflammatory pathways (Kong *et al.*, 2003; Zhang *et al.*, 2014). Plant products that are rich in anthocyanins are more attractive to consumers due to the bright and beautiful colors as well as high antioxidant levels (King and Cliff, 2002; Pojer *et al.*, 2013). Therefore, increasing anthocyanin content is a major goal for crop improvement (Zhu *et al.*, 2017; Sun *et al.*, 2020).

Anthocyanin biosynthesis occurs through the well-characterized flavonoid pathway, which begins with the sequential action of chalcone synthase (CHS), chalcone isomerase (CHI), and flavanone 3 β -hydroxylase (F3H) to synthesize dihydroflavonols. The enzymes F3'H and F3'5'H produce dihydroflavonols with different hydroxylation states, which will affect the hue of the anthocyanins produced (Berardi *et al.*, 2021). Dihydroflavonol 4-reductase (DFR) catalyzes the first committed step for producing anthocyanidins instead of flavonols. Anthocyanidin reductase (ANR) and UDP-glucosyltransferases (UGTs) then act sequentially to synthesize anthocyanins (Xu *et al.*, 2014). Anthocyanin biosynthesis genes are activated by the MBW transcriptional activation complex, composed of an R2R3-MYB transcription factor, a basic-helix-loop-helix (bHLH), and a WD-repeat protein (Xu *et al.*, 2015). MBW complexes regulate many processes in plants, with the R2R3-MYB protein often specifying the regulatory targets of the complex (Heppel *et al.*, 2013).

Strawberry (*Fragaria* $\times$ *ananassa*, octoploid) is an economically important fruit crop worldwide. The wild strawberry *Fragaria vesca*, the diploid progenitor for the dominant subgenome in *F.* $\times$ *ananassa*, has been used as a model system for strawberry and the Rosaceae family, due to its diploidy, high quality genome, ever-flowering accessions, small stature, and available molecular genetic tools (Zhou *et al.*, 2018; Edger *et al.*, 2018; Liu *et al.*, 2020; Gaston *et al.*, 2020). The attractive red color of strawberry fruit has led to extensive research, which revealed *FveMYB10*, an R2R3-MYB, playing a critical role in controlling the biosynthesis of anthocyanins in strawberry fruit. Multiple natural variations in the *MYB10* coding or regulatory sequences underlie white fruit skin or

cortex of both *F. × ananassa* and *F. vesca* (Castillejo *et al.*, 2020). *FaMYB10* and *FveMYB10* expressions increase as fruit ripens (Lin-Wang *et al.*, 2010). Furthermore, RNAi down-regulation of *MYB10* in *F. × ananassa* and *F. vesca* respectively reduces or eliminates anthocyanins in fruits (Lin-Wang *et al.*, 2014; Medina-Puche *et al.*, 2014).

Strawberry also accumulates anthocyanins in the vegetative tissues, most visibly in the petioles. Most white-fruited *F. vesca* accessions, such as ‘Yellow Wonder (YW)’ and ‘Hawaii 4 (H4)’, carry *myb10* mutations (Hawkins *et al.*, 2016; Castillejo *et al.*, 2020), yet they still develop purple-colored petioles, indicating that anthocyanins are produced normally in the petioles and regulated independently of *FveMYB10*. Hence, anthocyanin biosynthesis in fruit and petiole must be regulated by different genetic factors, prompting the hypothesis that a different R2R3-MYB in strawberry might regulate anthocyanin biosynthesis in the petioles. However, the identity of such an R2R3-MYB remains elusive. Previously, a *reduced anthocyanins in petioles (rap)* mutant of *F. vesca* was identified; the causal mutation resides in a GST gene coding for the glutathione S-transferase needed to transport anthocyanins from the cytosol to the vacuole in petioles and fruits. As a result, *rap* mutants exhibit reduced pigmentation in fruit as well as in petiole (Luo *et al.*, 2018). Hence, it is unlikely that *RAP* encodes the unknown factor specific for the petioles.

Here, we report the isolation and characterization of a *green petioles (gp)*-1 mutant in the wild strawberry *F. vesca*. Mapping-by-sequencing identified the causal mutation in an R2R3-MYB gene that is similar in sequence to *FveMYB10*. This new gene is named *FveMYB10-Like (FveMYB10L)*. Phylogenetic and functional analyses showed that, like *FveMYB10*, *FveMYB10L* is also an activator of anthocyanin biosynthesis, but unlike *FveMYB10*, *FveMYB10L* is specifically required for anthocyanin synthesis in the petiole, hence fulfilling the role of the elusive R2R3-MYB for petiole-specific anthocyanin synthesis. Further, *FveMYB10* and *FveMYB10L* expression was shown to respond differently to light quality, and ectopic activation of *FveMYB10L* in fruits induces a distinct anthocyanin profile resembling that of wild-type petioles and shows different activation capability of anthocyanin biosynthesis genes from *FveMYB10*. Our work demonstrates the existence of separate genetic controls for fruit and petiole anthocyanin biosynthesis and identifies the transcription factors that exert such separate controls. The *FveMYB10* and *FveMYB10L* genes offer flexible regulation of anthocyanin accumulation under different developmental and environmental conditions.

Materials and Methods

Plant material and growth conditions

The seventh-generation inbred lines of *F. vesca* accessions Rügen (Rü, red fruits and purplish petioles), Yellow Wonder 5AF7 (YW, white fruits and purplish petioles), and Hawaii 4 (H4, white fruits and purplish petioles) (Hawkins *et al.*, 2016; Joldersma *et al.*, 2022) were used in this study. The natural accession FIN12 lacks anthocyanins in all tissues and was collected from southern Finland (WGS84 N60.15, E21.59). The *rap-1* is from Dr. Chunying Kang (Luo *et al.*, 2018). *gp-1* was obtained from chemical mutagenesis by N-ethyl-N-nitrosourea (ENU) in a YW genetic background. ENU mutagenesis produces point mutations, mainly affecting A/T base pairs, and can produce both transitions and transversions (Justice *et al.*, 1999). The mutagenesis was previously described (Caruana *et al.*, 2018). Briefly, YW seeds were treated with 4mM ENU for four hours. Seeds were then rinsed with water, plated on MS media, and cold-treated for 5 weeks to induce germination. The *gp-1* mutant was identified in the M2 generation. The plants were cultivated in a growth chamber under white light, provided by Phillips F32T8/TL841 linear fluorescent bulbs, with light intensity of 110 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 13/11 h light/dark photoperiod at 23 °C.

Complementation tests

Reciprocal crosses between *gp-1* and *rap-1* were performed and F1 individuals with purple petioles were observed, indicating complementation between *gp-1* and *rap-1*.

Pollen from *gp-1* was used to pollinate the stigma of FIN12. To prevent self-fertilization, the flowers were emasculated before the dehiscence of anthers. The plants were covered and new open flowers removed until fruit development. The seeds were germinated and all of the 46 F1 seedlings were examined at the four-leaf stage and shown to develop green petioles, indicating that *gp-1* and FIN12 mutations are allelic.

Mapping the *gp-1* mutation through bulk-segregant analysis

The *gp-1* mutant was crossed with H4 that possesses wild-type purplish petioles, and a single F1 plant was self-fertilized to produce the F2 mapping population. Petiole color was scored in the F2 population, and genomic DNA was pooled from 25 green petiole individuals and from

25 purple petiole individuals to produce the mutant and wild-type pools respectively. Genomic DNA was extracted with DNAEasy Power Plant Pro kit (Qiagen, USA) or NucleoSpin Plant II kit (Macherey-Nagel, USA), followed by cleanup with the NucleoSpin Genomic DNA Cleanup kit (Macherey-Nagel, USA). The two DNA pools were sequenced using the Illumina NovaSeq 6000 System (Novogene, Sacramento, USA).

A total of 106.5 and 107.9 million paired-end 150 bp reads respectively from *gp-1* and wild-type were filtered using fastp (Chen *et al.*, 2018) to remove low quality reads. Afterwards, 105.9 high-quality mutant and 107.3 high-quality wild-type reads were retained for downstream analysis. Candidate *gp-1* mutations were identified using the SIMPLE pipeline (Wachsman *et al.*, 2017). Briefly, SIMPLE maps sequencing reads to a reference genome, calls SNPs between the mutant and wild type bulks, and identifies candidate SNPs expected to affect gene function. Those variants for which no reference reads occur in the mutant pool and the wild-type pool has about 2/3 reference reads are identified as candidates. Since ENU mutagenesis cannot cause indels, indels were removed from the list of candidates. Known variants occurring in the accessions YW, H4, and Rü, all of which have purplish petioles, were identified by mapping their sequencing reads derived from a previous study (Hawkins *et al.*, 2016) against the *F. vesca* reference genome (Edger *et al.*, 2018; Li *et al.*, 2019). Variants were called using the mapping and variant calling approaches in the SIMPLE pipeline. SNPs that are expected to affect gene function as a missense, nonsense, or splicing variant not known to occur in YW, H4, or Rü genome, as well as any SNPs residing in genes expected to affect anthocyanin biosynthesis, were selected as candidates.

Phylogenetic analysis of *FveMYB10L*

Protein sequences in *Arabidopsis* and tomato from R2R3-MYB subgroup 6, which regulates anthocyanin biosynthesis, and related subgroups 5, 7, and 15 were identified based on a recent phylogenetic analysis that placed these subgroups in a clade with subgroup 6 (Rodrigues *et al.*, 2021) and downloaded from TAIR and Sol Genomics Network (Fernandez-Pozo *et al.*, 2015; Berardini *et al.*, 2015). Their gene accession numbers are summarized in Table S1. Multiple sequence alignment containing these sequences, *FveMYB10*, and *FveMYB10L*, as well as *OsGAMYB* as an outgroup, was created using the MUSCLE algorithm implemented in MEGA X software (Edgar, 2004; Stecher *et al.*, 2020). The rooted maximum likelihood tree was constructed using the James Taylor Thornton matrix-based model with 1000 bootstrap replicates (Jones *et al.*, 1992). The pairwise sequence alignment of the *FveMYB10* and *FveMY10L* protein sequences was made using the Needleman-

Wunsch algorithm implemented in EMBL-EBI (Madeira *et al.*, 2022). The gene IDs of the genes used in this study are listed in the Supplementary Table S1.

Construction of *FveMYB10L* and *FveMYB10* overexpression plasmids

The JH23 vector was previously constructed from a gateway cloning vector pMDC99 (Curtis and Grossniklaus, 2003) by adding OCS terminator and the Arabidopsis Ubiquitin 10 (UBQ) promoter (Zhou *et al.*, 2021). To assemble *pUBQ::GFP*, the GFP coding sequence was Gibson assembled into JH23 following KpnI and PacI vector digestion so that GFP is driven by the UBQ promoter. To assemble *pUBQ::FveMYB10L* and *pUBQ::FveMYB10L^{R90K}* plasmids, total RNA was extracted from YW and *gp-1* mutant petioles respectively using the CTAB RNA extraction method (Gambino *et al.*, 2008) and then treated with DNase I (catalog number M303S, New England Biolabs) to remove genomic DNA contamination. cDNAs were synthesized from total RNA using the RevertAid First Strand cDNA Synthesis Kit (catalog number K1622, Thermo Scientific). The mutant and wild-type *FveMYB10L* coding sequences were amplified from the cDNA and respectively cloned into the *pUBQ::GFP* vector following KpnI digestion to produce *pUBQ::FveMYB10L-GFP* or *pUBQ::FveMYB10L^{R90K}-GFP*. All primers used are listed in the Supplementary Table S2.

The *p35S::FveMYB10* overexpression construct was previously described (Hawkins *et al.*, 2016), in which the *FveMYB10* cDNA is driven by the 35S promoter in the pMDC32 vector (Curtis and Grossniklaus, 2003). An empty pMDC32 vector was used as the control for *p35S::FveMYB10* in the fruit transient expression assay.

Transient expression assay using strawberry fruit

Transient expression assays in wild diploid strawberry fruit were performed as described by Hawkins *et al.* (2016) with minor modifications. *Agrobacterium tumefaciens* strain GV3101 containing an overexpression or control construct was grown in 3 ml LB liquid medium with 50 mg/ml kanamycin overnight at 28 °C incubator. The culture was resuspended in Murashige and Skoog medium with 2% (w/v) sucrose to an exact OD₆₀₀ of 1.0. White stage fruits were identified for injection based on morphological cues, including visible spaces between achenes, the white color of the achenes and receptacle, and approximate age since flower anthesis (Hawkins *et al.*, 2017). A 1 ml insulin syringe was used in injection until the fruit appeared saturated with the infiltration solution (~50 µl). At least 12 fruits were infiltrated for each construct. Fruits were removed from the plants at maturity (~5-10 days after the injection) and photographed.

Anthocyanin analyses

Mature fruits transiently expressing *pUBQ::FveMYB10L*, *pUBQ::FveMYB10L^{R90K}*, or control vector *pUBQ::GFP* were frozen in liquid nitrogen. Total anthocyanin extraction and measurement was performed in three replicates according to Luo *et al.* (2018). Briefly, fresh tissue was ground in liquid nitrogen and combined with 5 ml of extraction solution (70 parts methanol: 27 parts water: 2 parts formic acid: 1 part trifluoroacetic acid). After incubating in the dark at 4°C for 12h, the supernatant was transferred to a new tube and absorbance measured with Nanodrop 2000c at 530 nm and 657 nm to quantify total anthocyanins. Anthocyanin content was calculated as $[A_{530} - (0.25 \times A_{657})]/M$, where A_{530} and A_{657} are the absorbance at the indicated wavelengths and M is the fresh weight of the plant material used for anthocyanin extraction.

Anthocyanin composition was profiled based on a previously published HPLC method (Gao *et al.*, 2020) with minor modifications. Briefly, fruits and petioles from wild-type (Rü), as well as *gp-1* (YW) fruits transiently expressing *pUBQ::FveMYB10L* or *35S::FveMYB10*, were frozen at -80 °C and freeze-dried. Samples were homogenized in a solvent solution consisting of 70% methanol, 28% water, and 2% formic acid followed by centrifugation at 15,000 *g* for 3 min and filtration via a 0.2 µm syringe filter (MicroLiter WHEATON, Millville, NJ, USA). Anthocyanin separation and analyses were done via an Agilent 1200 series HPLC system equipped with a ZORBAX Rapid Resolution StableBond C18 column and a diode-array detector (Agilent, Santa Clara, CA, USA). The binary mobile phase consisted of 5% formic acid in water (solvent A) and 100% methanol (solvent B). Other testing conditions include injection temperature set at 30 °C, flow rate at 1 mL/min, and detection and reference wavelengths at 525 and 650 nm, respectively. The mobile phase composition was 10% B at 0 min, 20% B at 10 min (linear change, same hereinafter), 24% B at 35 min, 60% B between 50 and 60 min, and 10% B at 70 min. Peaks were identified by comparing the chromatograms of the samples to those of known standards including cyanidin-3-glucoside, cyanidin-3,5-diglucoside, pelargonidin-3-glucoside, pelargonidin-3,5-diglucoside, and peonidin-3-glucoside. The fruit and petiole anthocyanin profiling was conducted in triplicate.

Light quality treatments

To test the effect of shade on anthocyanin pigment and gene expression, shade was simulated by supplementing the white light growth condition with constant far-red light (Hydrofarm powerPAR 15-watt LED Bulb, far-red) per 60 x 60 cm² to reduce the red/far-red light ratio (Ballaré *et al.*, 1987). WT (Rü) plants were grown in white light (control) and white light supplemented with far-red (shade

treatment). Five replicate plants were grown in each treatment and observed for anthocyanin phenotypes in the fruits and petioles. Petioles and fruits were sampled for anthocyanin content measurement and gene expression analysis with RT-qPCR.

RNA extraction, cDNA synthesis, and RT-qPCR

For RT-qPCR of endogenous *FveMYB10*, *FveMYB10L*, and anthocyanin biosynthesis genes, total RNA was extracted from white stage fruits or petioles of newly unfolded leaves. White stage fruit was chosen as *FveMYB10* begins expression at this stage (Hawkins *et al.*, 2017), which is also a stage when the fruit metabolites that impede RNA extraction are not yet at a high level. Three fruits or petioles from three individuals were pooled to form one biological sample, and three biological replicates were used. For agrobacterium-infiltrated fruits, total RNA was extracted from the entire fruit at the ripe stage and each infiltrated fruit forms one biological sample.

Tissues were flash-frozen in liquid nitrogen immediately after collection and stored at -80°C until RNA extraction. RNA was extracted following the Cetyl Trimethyl Ammonium Bromide (CTAB) RNA extraction method (Gambino *et al.*, 2008). RNA was reverse transcribed to cDNA using LunaScript RT SuperMix Kit (catalog number E3010, New England Biolabs Inc.), and RT-qPCR was performed using BioRad CFX96 Real-time system and PowerUp SYBR Green Master Mix (catalog number A25742, Applied Biosystems), using a 3-step PCR program (step 1 50°C 2 min, step 2 95°C 2 min, step 3 95°C 15 sec, step 4 55°C 15 sec, step 5 72°C 30 sec, repeat steps 3-5 40 cycles). The relative expression level was analyzed using a modified $2^{-\Delta\Delta CT}$ method normalized to the geometric mean of Ct value of the two internal controls (Vandesompele *et al.*, 2002). For genes whose transcripts were undetectable by RT-qPCR, the cycle number was set to 40 for calculation as only 40 cycles were performed for all PCR reactions. For all RT-qPCRs, *F. vesca Protein Phosphatase 2A* (*FvePP2a*; *FvH4_4g27700*) and *Ubiquitin-conjugating enzyme E2 10* (*FveUBC10*; *FvH4_7g30920*) were used as the internal controls (Lin-Wang *et al.*, 2014; Caruana *et al.*, 2018). Primers used are listed in Supplementary Table S2.

Student's t test was performed for RT-qPCR of two groups (Fig. 3D). One-way ANOVA followed by Turkey's HSD test was performed for RT-qPCR of multiple groups (Fig. 4C-D and Fig. 5C-D) using the Real Statistics Resource Pack software (Release 7.6).

***In silico* analysis of *FveMYB10* and *FveMYB10L* promoters**

The promoter sequences (-2000 bp upstream of ATG) of *FveMYB10* and *FveMYB10L* were retrieved from GDR's *F. vesca* Whole Genome v4.0.a1, (<https://www.rosaceae.org/>), and analyzed in PLANTPAN3.0 (<http://plantpan.itps.ncku.edu.tw/>) (Chow *et al.*, 2019; Jung *et al.*, 2019) using default parameters in the Multiple Promoter Analysis function.

Results

The *green petioles-1* locus in *F. vesca* controls anthocyanin pigment in petioles

A *green petioles* (*gp*)-1 mutant was identified from an N-ethyl-N-nitrosourea (ENU) mutagenesis screen performed previously in the YW background (Caruana *et al.*, 2018) (Materials and Methods). The YW genetic background carries a *myb10*^{W12S} mutation that causes white fruit (Hawkins *et al.*, 2016); however, YW still develops normal purple-pigmented petioles (Fig. 1A-B), suggesting that the effect of *FveMYB10* is fruit-specific. In addition to an absence of red fruit pigmentation, the newly isolated *gp*-1 *myb10*^{W12S} mutant also lacks purple pigment in the petiole (Fig. 1C). Since *gp*-1 resembles the *reduced anthocyanins in petioles* (*rap*) mutant as well as a natural *F. vesca* accession FIN12, both of which develop green petioles (Luo *et al.*, 2018; Castillejo *et al.*, 2020), complementation tests were conducted to determine whether *gp*-1 is allelic with *rap* or FIN12. Reciprocal crosses between *rap* and *gp*-1 yielded F₁ progeny with purple petioles (Supplementary Fig. S1), indicating that *gp*-1 resides in a different gene from *rap*, which is defective in a GST needed for anthocyanin transport. In contrast, F₁ progeny of a cross between *gp*-1 pollen and FIN12 stigma still develop green petioles (Fig. 1D-E), suggesting that *gp*-1 and FIN12 are defective in the same gene that promotes pigmentation in the petiole. However, the identity of the gene affected by *gp*-1 and FIN12 is unknown.

Mapping-by-sequencing maps *gp*-1 to a *FveMYB10*-like transcription factor

To identify the gene defined by *gp*-1, a mapping population was constructed by crossing *gp*-1 *myb10*^{W12S} (YW) with *GP*⁺ *myb10*^{W12S} (H4) that has wild-type petiole pigment. Genomic DNA from 25 F₂ green petiole individuals and 25 F₂ purple petiole individuals were separately pooled and sequenced using the Illumina platform. Analysis using the SIMPLE pipeline (Wachsman *et al.*, 2017)

narrowed it to 5 candidate genes residing within an 8.65 Mb region of chromosome 1 (Supplementary Fig. S2; Supplementary Table S3). Only one of these, *FveMYB10-Like* (*FveMYB10L*), appeared a strong candidate due to its 57.9% sequence similarity to *FveMYB10*, a known regulator of anthocyanin biosynthesis. In addition, *FveMYB10L* resides near *FveMYB10* on chromosome 1 about 30 KB apart (Fig. 2A). Cloning and sequencing the *FveMYB10L* cDNA corrected its annotation (Supplementary Fig. S3) and showed that the SNP found in *gp-1* resides in the third exon causing a missense mutation (R90K) in *FveMYB10L* (Fig. 2A). The fact that FIN12 harbors a ~100 kb deletion on chromosome 1, deleting both *FveMYB10* and *FveMYB10L* (Fig. 2A, Castillejo *et al.*, 2020) is consistent with its inability to complement *gp-1* (Fig. 1E), lending strong support that *FveMYB10L* is *GP*. Therefore, FIN12 possesses a second independent loss-of-function allele of *FveMYB10L*, confirming the role of *FveMYB10L* in petiole anthocyanin synthesis.

***FveMYB10L* falls within a clade of anthocyanin biosynthetic regulators**

To compare *FveMYB10L* with other known anthocyanin biosynthesis regulators, we generated a multiple protein sequence alignment and phylogenetic tree containing *FveMYB10L*, *FveMYB10*, and tomato and *Arabidopsis* MYB transcription factors from R2R3-MYB subgroup 6, which typically regulates anthocyanin biosynthesis (Stracke *et al.*, 2001; Rodrigues *et al.*, 2021) (Fig. 2B). Subgroup 6 MYBs typically have conserved arginine (R), valine (V), and alanine (A) residues and an ANDV motif (indicated by + in Fig. 2C) in the R2-R3 domains which distinguish them from other R2R3 MYBs, although exceptions do occur in the Rosaceae family (Lin-Wang *et al.*, 2010). We also included *Arabidopsis* and tomato protein sequences from related subgroups 5, 7, and 15. The subgroup 6 MYB proteins, including *FveMYB10* and *FveMYB10L*, form a well-supported clade with 99% bootstrap support. The phylogenetic analysis strongly supports that *FveMYB10L* belongs to subgroup 6, and not the other related subgroups, indicating a role in anthocyanin synthesis. Moreover, the multiple sequence alignment used to generate this phylogeny indicates that the R90K mutation in *gp-1* occurs at a highly conserved arginine (R) residue within the R3 domain (Fig. 2C), converting it to lysine (K). To gain a better understanding of the sequence conservation, multiple alignment of full-length protein sequences from subgroup 6 MYBs as well as additional sequences from grape and peach is shown in Supplementary Fig. S4. Like *FveMYB10*, these MYBs show 49-60% similarity at the protein sequence level with *FveMYB10L* with highest similarity at the R2R3 domain.

***FveMYB10L* is not required for fruit coloration**

Since *gp-1 myb10^{W12S}* plants develop yellow fruit due to the *myb10^{W12S}* mutation, it is not known if *FveMYB10L* is also required for the red fruit color. In other words, would a *gp-1* single mutant develop red or yellow fruit? Since *myb10^{W12S}* is tightly linked to *gp-1* (Fig. 2A), we were unable to separate these two mutations via recombination. Hence, we performed a transient expression assay to test if transient expression of wild-type *FveMYB10* in *gp-1 myb10^{W12S}* double mutant fruit could rescue the fruit color in the presence of *gp-1* mutation. The *p35S::FveMYB10* construct from our previous study (Hawkins *et al.*, 2016) was transformed into *Agrobacteria*, which was infiltrated into *gp-1 myb10^{W12S}* double mutant fruit at white stage by injection (see Materials and Methods). After ~5-10 days, all injected fruits with the *p35S::FveMYB10* construct developed red pigmentation in skin and flesh to different extent, a representative image of which is shown in Fig. 3A. The result is similar to prior transient expression experiments showing that *p35S::FveMYB10* was able to rescue *myb10^{W12S}* single mutant fruit color (Hawkins *et al.*, 2016; Luo *et al.*, 2018). In contrast, injection of *Agrobacteria* containing the corresponding empty vector *pMDC32* into *gp-1 myb10^{W12S}* double mutant fruit had no impact on the fruit color (Fig. 3A). Together, the data indicate that *FveMYB10* alone is able to cause red fruit pigment even when *FveMYB10L* is mutated (i.e. in *gp-1* mutant background). Therefore, *FveMYB10L* function is not required for the fruit coloration and its effect appears specific to the petioles.

***FveMYB10L* and *FveMYB10* are tissue-specific activators of anthocyanin biosynthesis**

Taking advantage of this transient functional assay, we tested whether ectopic over-expression of *FveMYB10L* can activate anthocyanin production in fruits. Anthocyanin production in *gp-1 myb10^{W12S}* fruits overexpressing *FveMYB10L* would indicate that *FveMYB10L*, like *FveMYB10*, can activate anthocyanin biosynthesis, while the same assay using the *FveMYB10L^{R90K}* mutant version would test whether this R90K mutation disrupts the ability of *FveMYB10L* to activate anthocyanin biosynthesis. An *Arabidopsis* ubiquitin 10 promoter driven GFP was first constructed in the JH23 vector (Zhou *et al.*, 2021); this *pUBQ::GFP* served as a control construct. Full-length cDNA of wild type *FveMYB10L* as well as mutant *FveMYB10L^{R90K}* were PCR amplified and cloned into JH23. *Agrobacteria* containing *pUBQ::FveMYB10L-GFP*, *pUBQ::FveMYB10L^{R90K}-GFP*, or *pUBQ::GFP* were respectively injected into white-stage *gp-1 myb10^{W12S}* fruits. *pUBQ::FveMYB10L-GFP* injected fruits turned deep red and purple, *pUBQ::FveMYB10L^{R90K}-GFP* injected fruits however showed only slight pink, and the *pUBQ::GFP* injected fruits remained the same yellow fruit color (Fig. 3B). Quantification of total

anthocyanin levels in the injected fruits showed that the anthocyanin level in *pUBQ::FveMYB10L-GFP* injected fruits was more than 50-fold higher than that of fruits injected with *FveMYB10L^{R90K}-GFP* or *pUBQ::GFP*, indicating that the R90K missense mutation greatly reduced the *FveMYB10L* function in anthocyanin biosynthesis (Fig. 3C). The results confirmed that the *gp-1* mutation resides in *FveMYB10L* and that *FveMYB10L*, like *FveMYB10*, can activate anthocyanin biosynthesis.

Since the *myb10^{W12S}* and *myb10^{R90K}* (*gp-1*) mutations each reduce anthocyanin biosynthesis in only one tissue, we hypothesized that each gene may show tissue-specific expression consistent with its mutant phenotype. We sampled the petioles and fruits of wild type (Rügen) plants and examined the expression of *FveMYB10L* and *FveMYB10* using RT-qPCR. *FveMYB10L* was highly expressed in the petiole but lowly expressed in the fruit (Fig. 3D). In contrast, *FveMYB10* was highly expressed in the fruit but undetectable in the petiole (Fig. 3D). Therefore, these two MYB transcription factors are responsible for anthocyanin biosynthesis in petioles and fruits respectively *in vivo*.

Previous transcriptome work in YW sampled 46 different tissues/stages, not including the petioles (Li *et al.*, 2019). Mining this rich dataset revealed that *FveMYB10L* expression is barely detectable (<2 TPM) in all tissues sampled, whereas *FveMYB10* was mainly detected in reproductive tissues and particularly in the fruit (Supplementary Fig. S5). Taken together with our RT-qPCR in the petioles and fruits, *FveMYB10L* and *FveMYB10* appear to represent petiole- and reproductive-tissue-specific regulators of anthocyanin biosynthesis.

FveMYB10 and FveMYB10L induce different anthocyanin molecules by differentially activating anthocyanin biosynthesis genes

When *FveMYB10L* was transiently expressed in the strawberry fruits, we noticed that the color hue appears more purple than the red *FveMYB10* overexpressing fruits (Fig. 3A, B), mirroring the difference in hue between the purple petioles and red fruits in wild-type *F. vesca*. Variation in pigment hue is often caused by different types of anthocyanins. We performed HPLC analysis to test whether the anthocyanin composition differed between *gp-1 myb10^{W12S}* fruits transiently expressing *FveMYB10* and those transiently expressing *FveMYB10L*. We found that *FveMYB10L*-expressing fruits were higher in cyanidin-3,5-diglucoside (peak 1), cyanidin-3-glucoside (peak 3), and peonidin-3-glucoside (peak 5), while *FveMYB10*-expressing fruits are higher in pelargonidin-3-glucoside (peak 4)

(Fig. 4A). We then similarly analyzed wild-type petiole and fruit tissue by HPLC (Fig. 4B). Wild type (Rügen) petioles produced more cyanidin-3,4-diglucoside (peak 1), an unknown peak 2, and peonidin-3-glucoside (peak 5) (Fig. 4B), which resembles the *FveMYB10L*-expressing fruits (Fig. 4A), supporting a role of *FveMYB10L* in petiole. In contrast, pelargonidin (peak 4) that is high in *FveMYB10*-expressing fruit is also high in the Rügen fruits (Fig. 4A, B), supporting the red hue of the fruit. However, peak 3 (cyanidin-3-glucoside) is present at a high level in both petiole and fruit as well as fruits transiently expressing *FveMYB10* or *FveMYB10L* and hence does not appear to be diagnostic between the two MYBs. Taken together, *FveMYB10L* and *FveMYB10* appear to confer distinct anthocyanin compositions characteristic of petiole and fruit respectively.

The above observation suggests that *FveMYB10* and *FveMYB10L* may preferentially activate different anthocyanin biosynthesis genes. To test this hypothesis, we examined the expression of *FveDFR1*, *FveDFR2*, and *FveUFGT*, with *DFRs* acting in the first committed step that produces anthocyanidins instead of flavonols and *UFGT* in the last step of anthocyanin biosynthesis (Xu *et al.*, 2014). RNAs were extracted from individual *gp-1 myb10^{w125}* fruits infiltrated with agrobacteria containing *p35S::FveMYB10*, *pUBQ::FveMYB10L-GFP*, or *pUBQ::GFP* plasmid. RT-qPCR was conducted to first examine transgene expression (Fig. 4C). while the transcripts of GFP or *FveMYB10L* were only detected in infiltrated fruits with respective plasmid, *FveMYB10* transcripts were detected in all fruits but at significantly higher levels in fruits infiltrated with *p35S::FveMYB10* (Fig. 4C). This is because the RT-qPCR cannot distinguish the endogenous nonfunctional *FveMYB10^{w125}* transcripts from *FveMYB10* transcripts derived from the transgene. Next, we examined the expression of anthocyanin biosynthesis genes (Fig. 4D). As expected, fruits infiltrated with the *pUBQ::GFP* containing agrobacterium did not express any anthocyanin biosynthesis genes (Fig. 4D). Interestingly, *FveMYB10* activates all three anthocyanin biosynthesis genes with the fruit (#2) that showed a higher *FveMYB10* expression expressing a higher level of anthocyanin biosynthesis genes (compare Fig. 4C to 4D). In contrast, *FveMYB10L* appeared to activate only *FveUFGT* and *FveDFR2* but not *FveDFR1* (Fig. 4D). Further, while *FveMYB10L* and *FveMYB10* activated *FveDFR2* to similar levels (Fig. 4D), *FveMYB10* activated *FveUFGT* five to six fold higher than *FveMYB10L* did. Together, the data support that *FveMYB10* and *FveMYB10L* have differential activation preference for anthocyanin biosynthesis genes, qualitatively for *FveDFR1* and quantitatively for *FveUFGT*.

Expression of *FveMYB10L* is responsive to change in the red/far-red light ratio

The tissue-specific expression of *FveMYB10* and *FveMYB10L* suggests that these two MYB genes are subject to distinct regulation, responding to tissue-specific or environmental-specific cues. To investigate whether anthocyanin production in fruits and petioles is subject to distinct regulation in response to environmental cues, we tested whether shade could differentially regulate these two MYB genes, since several plant species exhibit reduced anthocyanins in the petiole when under shade (Alokam *et al.*, 2002; Ding *et al.*, 2016). We grew wild type (Rü) plants under white light supplemented with far-red (FR) light, as this reduces the R/FR ratio and mimics shade (Ballaré *et al.*, 1987). After a month, most of the newly formed petioles were completely green, and the plants also exhibited elongated growth (Fig. 5A, B) that is typical of shade avoidance response (Ballaré and Pierik, 2017). Surprisingly, these plants produced red colored fruits (Figure 5A, B), indicating that growth in shade causes reduced anthocyanin production in the petiole but not in the fruit.

To test whether the observed changes in anthocyanin levels may be mediated by *FveMYB10* and *FveMYB10L*, we examined *FveMYB10L* and *FveMYB10* expression in petioles of young leaves and white stage fruits of wild type (Rü) plants. When growing under white light, *FveMYB10L* expression was undetectable in the fruit but highly expressed in the petiole, which is opposite from *FveMYB10* with undetectable expression in the petiole but high expression in the fruit (Fig. 5C). Under simulated shade (white+FR), the expression of *FveMYB10L* in the petiole was about 10-fold lower when compared with that under the white light (Fig. 5C). This shade-mediated repression of *FveMYB10L* expression mirrors the shade-mediated reduction of anthocyanin level in the petiole (Supplementary Fig. S7). In contrast, *FveMYB10* expression level in the fruit does not change much when grown under white light or under the shade (Fig. 5C), suggesting that *FveMYB10* expression is much less sensitive to changes in the light quality. Interestingly, *in silico* analysis of promoter sequences via PlantPAN3.0 (Chow *et al.*, 2019) identified a binding site for the far-red sensing transcription factor *FAR1* in the *FveMYB10L* promoter but not in the *FveMYB10* promoter (Supplementary Fig. S8), supporting that these two MYB genes are regulated differently not only in different tissues but also under different light conditions.

Since the two MYBs differentially regulate downstream anthocyanin biosynthesis genes (Fig. 4D), the anthocyanin biosynthesis genes should also exhibit tissue-specific and light-sensitive expression. Using RT-qPCR, we examined the expression of *FveUGT*, *FveDFR1* and *FveDFR2* in the wild type (Rü)

petiole and fruit. *FveDFR1* is not expressed in the petiole (Fig. 5D), consistent with *FveMYB10L*'s inability to activate *FveDFR1* in transient expression studies (Fig. 4D). Further, while the petiole-expressed *FveUGT* and *FveDFR2* are repressed under white+FR (Fig. 5D), the fruit-expressed *FveUGT* and *FveDFR2* are not repressed under white+FR. Therefore, the tissue- and light- regulated expression pattern of anthocyanin biosynthesis genes mirrors the expression of their respective MYB regulators.

Discussion

In strawberry, loss of *FveMYB10* causes yellow fruit but does not affect the purple color of the petiole (Hawkins *et al.*, 2016; Castillejo *et al.*, 2020), prompting the hypothesis that a petiole-specific MYB transcription factor might exist to regulate anthocyanins in the petiole (Luo *et al.*, 2018). The work reported here suggests that *FveMYB10L* encodes this proposed petiole-specific MYB transcription factor. Interestingly, both *FveMYB10* and *FveMYB10L* reside in nearby locations on chromosome 1 and likely arose by a gene duplication. Phylogenetic analysis and transient over-expression in fruits revealed that *FveMYB10* and *FveMYB10L* both can activate anthocyanin biosynthesis (Fig. 3 and 4), which is consistent with prior reports that ectopic overexpression of *FveMYB10* by the 35S promoter caused elevated anthocyanin pigmentation in leaf tissues (Lin-Wang *et al.*, 2014). Nevertheless, while both MYB genes act to promote anthocyanin biosynthesis, they exhibit somewhat different anthocyanin profiles that are characteristic of petiole and fruit respectively and reflective of their differential regulation of downstream anthocyanin biosynthesis genes. In addition, *FveMYB10* and *FveMYB10L* exhibit distinct tissue-specific expression and mutant phenotypes. *FveMYB10* is expressed and acts specifically in the fruit, while *FveMYB10L* acts specifically in the petiole. The single *myb10*^{W12S} mutant develops yellow fruit but purplish petioles, while a single *gp-1* mutant would develop red fruit but green petioles. Finally, we show that while *FveMYB10* expression is not repressed by shade (low R/FR ratio), *FveMYB10L* expression is dramatically reduced under shade, which corresponds to the formation of green petioles and a reduction of *FveMYB10L* target genes expression under the shade. Together, our study reveals the existence of two paralogous MYB transcription factors that function in different tissues, activate different targets, and respond differently to environmental signals to confer tissue-specific anthocyanin accumulation as summarized in the model (Fig. 6). The evolution of these two paralogous MYB genes likely offers flexibility in tissue-specific anthocyanin regulation compared to a single MYB10 gene.

The *gp-1* mutant harbors an R90K missense mutation in *FveMYB10L*, while FIN12 possesses a ~100kb deletion encompassing both *FveMYB10* and *FveMYB10L* (Fig. 2A) (Castillejo *et al.*, 2020). However, both mutations confer a green petiole phenotype, and a complementation test between *gp-1* and FIN12 confirmed that they contain mutations in the same gene underlying the green petiole phenotype. Interestingly, both *gp-1* and FIN12 eliminate visible pigment in the petioles despite their harboring different types of mutations. In *gp-1* (R90K), R occurs in a highly conserved region of the R3 domain (Fig. 2C) and is in close proximity to the bHLH domain that enables R2R3 MYBs' interaction with the MBW complex (Heppel *et al.*, 2013). As a result, the R90K mutation in *FveMYB10L* may greatly reduce its function with a similar effect as the deleted *FveMYB10L* in FIN12. We noted a slight pink fruit color when *FveMYB10L*^{R90K} was overexpressed in the fruit (Fig. 3B), suggesting some residual activities of *FveMYB10L* only observable when *FveMYB10L*^{R90K} was over-expressed in the transient system.

FveMYB10 and *FveMYB10L* confer distinct anthocyanin composition to the fruits and petioles, likely due to their differential activation of downstream anthocyanin biosynthesis genes including *FveUGT* and *FveDFR1*. While there are only limited differences between *FveMYB10* and *FveMYB10L* in the R2 and R3 DNA binding motifs (Fig. 2C), there are significant differences outside the R2R3 domain (Fig. S6), which could contribute to their differential binding of downstream targets. Moreover, while both MYBs can activate *FveDFR2*, only *FveMYB10* could activate *FveDFR1* in the transient system (Fig. 4D). The altered expression levels of *FveDFR1* and *FveDFR2* were previously shown to cause changes in the hydroxylation patterns of anthocyanins in strawberry fruit due to their different substrate specificities (Miosic *et al.*, 2014). Specifically, only *FveDFR1* can act on dihydrokaempferol, the precursor to pelargonidin-3-glucoside, which is consistent with our detection of higher pelargonidin-3-glucoside levels in *FveMYB10*-overexpressing fruits than in *FveMYB10L*-overexpressing fruits. Therefore, differential activation of *FveDFRs* by tissue-specific MYBs likely underlies the observed differences in hue and anthocyanin composition between fruit and petiole tissues.

In addition to their tissue-specific effects on anthocyanin intensity and composition, *FveMYB10* and *FveMYB10L* differ in their response to environmental cues, specifically light quality. We found that *FveMYB10L*, but not *FveMYB10*, is downregulated under low R/FR ratio, which simulates shade.

Accordingly, petiole looks green in color and petiole anthocyanin level is reduced (Fig. 5B and S7). Consistent with a function in photoprotection, vegetative anthocyanin production is often light-inducible and absorbs light in the range of photosynthetically active radiation (Close and Beadle, 2003; Albert *et al.*, 2009; Gould *et al.*, 2010). In contrast, fruit pigmentation is thought to attract frugivores for seed dispersal, although this function has not been explicitly tested in strawberry (Lomascolo *et al.*, 2010; Nevo *et al.*, 2018). *FveMYB10* expression that is not repressed under our shade condition may allow anthocyanins to be synthesized in fruits even in the shade to attract seed dispersers. We note that previous studies find light-regulation of *FveMYB10*, with dark-grown apple and strawberry fruits failing to accumulate anthocyanins (Tako *et al.*, 2006; Xu *et al.*, 2018; Li *et al.*, 2020); however, these prior studies compare light and dark treatments, while ours compares white light treatments with or without far-red supplementation and is therefore testing the effects of light quality. The ability to separately regulate petiole *FveMYB10L* and fruit *FveMYB10* may provide flexibility in regulating anthocyanin production for different biological functions. The detection of a FAR1-binding site in the promoter of *FveMYB10L* but not *FveMYB10* lays the foundation for future investigations into the mechanism underlying differential regulations of *FveMYB10* and *FveMYB10L*.

Tissue- and environment-specific regulation of anthocyanin biosynthesis by duplicate MYBs was also reported in other plant species, suggesting a common need to decouple the regulation of pigment synthesis between fruit and vegetative tissues. In tomato, two MYBs, *SlANT1* and *SlANT2*, upregulate anthocyanin contents when ectopically overexpressed, but only *SlANT2* is specifically upregulated under cold and high light conditions (Matus *et al.*, 2017). In grape, two clusters of MYBs regulate anthocyanin biosynthesis in fruits and vegetative tissues respectively; when both clusters were ectopically expressed, they caused anthocyanin biosynthesis in hairy roots and affected anthocyanin composition by regulating F3'5'H differentially (Matus *et al.*, 2017). Finally, a vegetative tissue-specific MYB10.4, distinct from the fruit-specific MYB10.2, is involved in the red leaf color of an ornamental peach cultivar (Zhou *et al.*, 2014). These MYBs show 49-60% similarity at the protein sequence level with *FveMYB10L* (Supplementary Fig. S4). Together with our current work in strawberry, it appears that separately regulated paralogous MYBs with different downstream target specificity may have frequently evolved to offer flexibility and advantages to plants under changing environments.

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Author Contributions

M.P., X.L., and Z.L. designed experiments, M.P., X.L., Z.T., K.M., and L.G. performed experiments, T.H., Y.L., and Z. L. supervised and supported the project, M.P., X.L., and Z.L. wrote the manuscript. X.L. and M.P. contributed equally. All authors commented on the manuscript.

Conflict of interest

No conflict of interest declared.

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Data availability

The genome resequencing data used for mapping the *gp-1* mutation are available from the Sequence Read Archive (SRA) at NCBI associated with BioProject PRJNA823731.

Figure Legends

Figure 1. Characterization of *gp-1* mutant in *F. vesca*.

(A-C) Whole plant, fruit, and petioles of WT (Rü) (A), *myb10^{W12S}* (YW) (B), and *gp-1 myb10^{W12S}* (YW) double mutant (C). (D) A FIN12 adult plant. (E) Complementation result between FIN12 and *gp-1*, showing an F1 progeny with green petioles. Scale bars: 8 mm.

Figure 2. Phylogenetic analysis of some MYBs with roles in anthocyanin biosynthesis.

(A) Diagram of a chromosome 1 region corresponding to a large deletion in FIN12 that contains the *FveMYB10* (FvH4_1g22020) and *FveMYB10-like* (FvH4_1g22040) genes. (B) Rooted maximum likelihood phylogeny containing *FveMYB10*, *FveMYB10-like*, and MYBs from subgroup 6 and related subgroups in *Arabidopsis* and tomato. Clades corresponding to subgroups are indicated by labeled arrows. Numbers at each node indicate percent bootstrap support from 1000 replicates. (C) Multiple sequence alignment containing protein sequences of R2R3 domains of *FveMYB10*, *FveMYB10-Like*, and subgroup 6 MYB transcription factors in tomato and *Arabidopsis*. The boxed W indicates a conserved tryptophan that is converted to serine (S) in *myb10^{W12S}*; the boxed R indicates a conserved arginine that is converted to lysine (K) in *gp-1*. Residues conserved among all WT sequences are marked with an asterisk. R2, R3, and bHLH domains are based on a prior study (Heppel *et al.*, 2013). + sign indicates residues diagnostic for subgroup 6 MYBs (Lin-Wang *et al.*, 2010).

Figure 3. Transient over-expression of *FveMYB10* or *FveMYB10L* but not *FveMYB10L^{R90K}* activates anthocyanin biosynthesis in fruit.

(A) *gp-1 myb10^{W12S}* mutant fruits infiltrated with Agrobacteria containing *p35S::FveMYB10* (right) or *pMDC32* empty vector (left). (B) *gp-1 myb10^{W12S}* mutant fruits respectively infiltrated with Agrobacteria containing *pUBQ::FveMYB10L-GFP*, *pUBQ::FveMYB10L^{R90K}-GFP*, or *pUBQ::GFP*. Representative fruit images are shown. (C) Total anthocyanin content quantification of fruits derived from (B) above. Data are means ($\pm$ SD) of three technical replicates. Y-axis is the total anthocyanin content calculated as $[A530 - (0.25 \times A657)]/M$. Two biological replicates were conducted with similar results. (D) RT-qPCR quantification of *FveMYB10* and *FveMYB10L* transcripts in the petiole and fruit of WT (Rü) plants. Y-axis is the relative expression level against two internal controls *FvePP2a* (FvH4_4g27700) and *FveUBC10* (FvH4_7g30920), data are means ($\pm$ SD) of three technical replicates.

Significant difference between petiole and fruit is marked by ** ($P < 0.001$ by two-tailed Student's test). Three biological replicates were conducted with similar results. Scale bars in (A) and (B) are 8 mm.

Figure 4. Anthocyanin profiles and biosynthesis gene expression in fruits transiently expressing *FveMYB10* and *FveMYB10L*

(A) The anthocyanin profiles of fruits transiently over-expressing *FveMYB10L* (red) or *FveMYB10* (blue). Insert is a zoom-in of peaks 4-5. (B) The anthocyanin profile of petiole (black) and fruit (purple). The chemical identity of each peak for (A) and (B) is shown in the insert of (B). Three replicates all show similar profiles and one such replicate is shown here. (C) RT-qPCR results showing over-expressed *FveMYB10*, *FveMYB10L-GFP*, and *GFP* in respective infiltrated fruits. Results of two independently infiltrated fruits (#1 and #2) are shown. (D) RT-qPCR results testing activation of anthocyanin biosynthesis genes by the over-expressed *GFP*, *FveMYB10* and *FveMYB10L-GFP*. Data are from the same two independently infiltrated fruits (#1 and #2) as (C). Statistically significant differences among different samples for the same gene are indicated by different letters (p-value < 0.05 by one-way ANOVA followed by Turkey's HSD test).

Figure 5. Petiole and fruit phenotype and anthocyanin biosynthesis gene expression under different light conditions

(A-B) WT (Rü) plants grown under white light (A) and simulated shade (white + FR) (B). Fruits are indicated with an arrow. One representative plant from 5 replicates is shown. (C) RT-qPCR quantification of *FveMYB10L* and *FveMYB10* transcripts in WT (Rü) petiole and fruit under different light conditions. (D) RT-qPCR quantification of anthocyanin biosynthesis genes in WT (Rü) petiole and fruit under different light conditions. Y-axis indicates the relative expression level against two internal controls *FvePP2a* and *FveUBC10*; data are means ($\pm$ SD) of three technical replicates. Statistically significant differences among different samples/conditions for the same gene are indicated by different letters (p-value < 0.05 by one-way ANOVA followed by Turkey's HSD test). Three white stage fruits or young leaf petioles from three individual plants in each light condition were pooled to form a biological replicate. Three biological replicates were conducted with similar results. Scale bars are 8 mm.

Figure 6. A model summarizing the roles of *FveMYB10* and *FveMYB10L* in fruit and petiole under different light conditions

When the wild strawberry is grown under white light conditions (left) with a high R/FR (red/far-red) ratio, *FveMYB10L* and *FveMYB10* are expressed in petioles and fruits respectively, activating different anthocyanin biosynthesis genes and contributing to different color hues in the petiole and fruit. When the plant is growing under the shade (right) with a low the R/RF ratio, *FveMYB10L* expression in the petiole is reduced, causing the green color petiole due to a lack of anthocyanin biosynthesis. However, *FveMYB10* expression in the fruit is not repressed and red color fruit is still formed.

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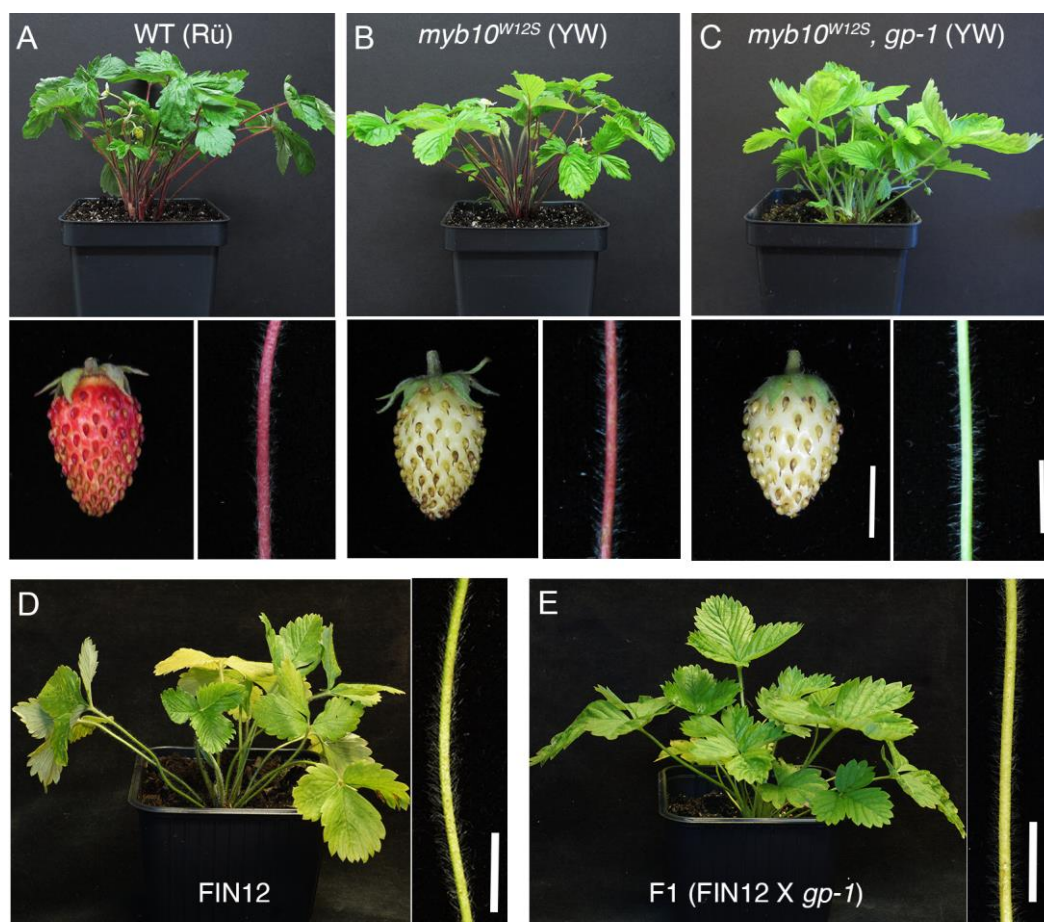
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Figure 1



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Figure 3

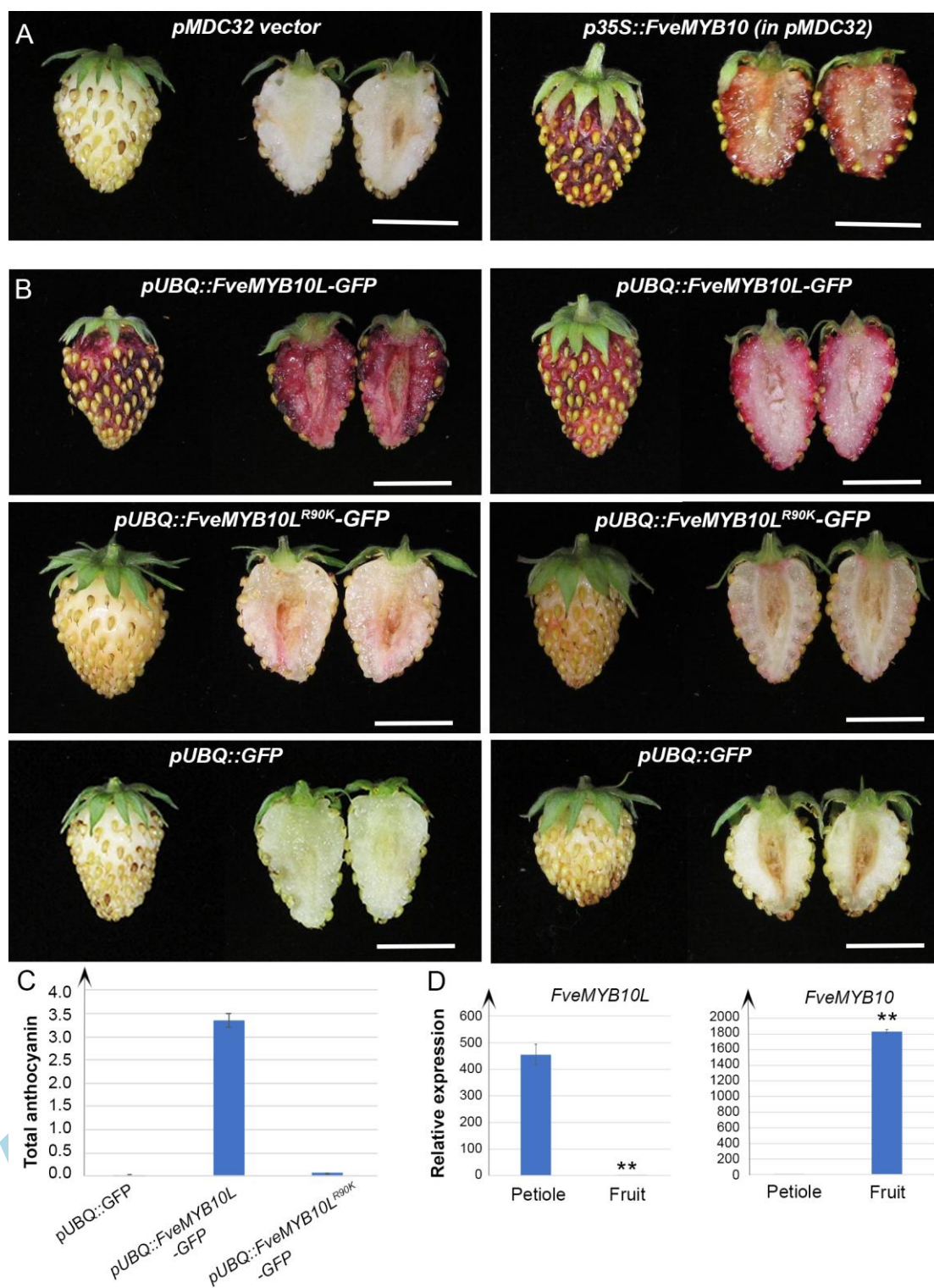


Figure 4

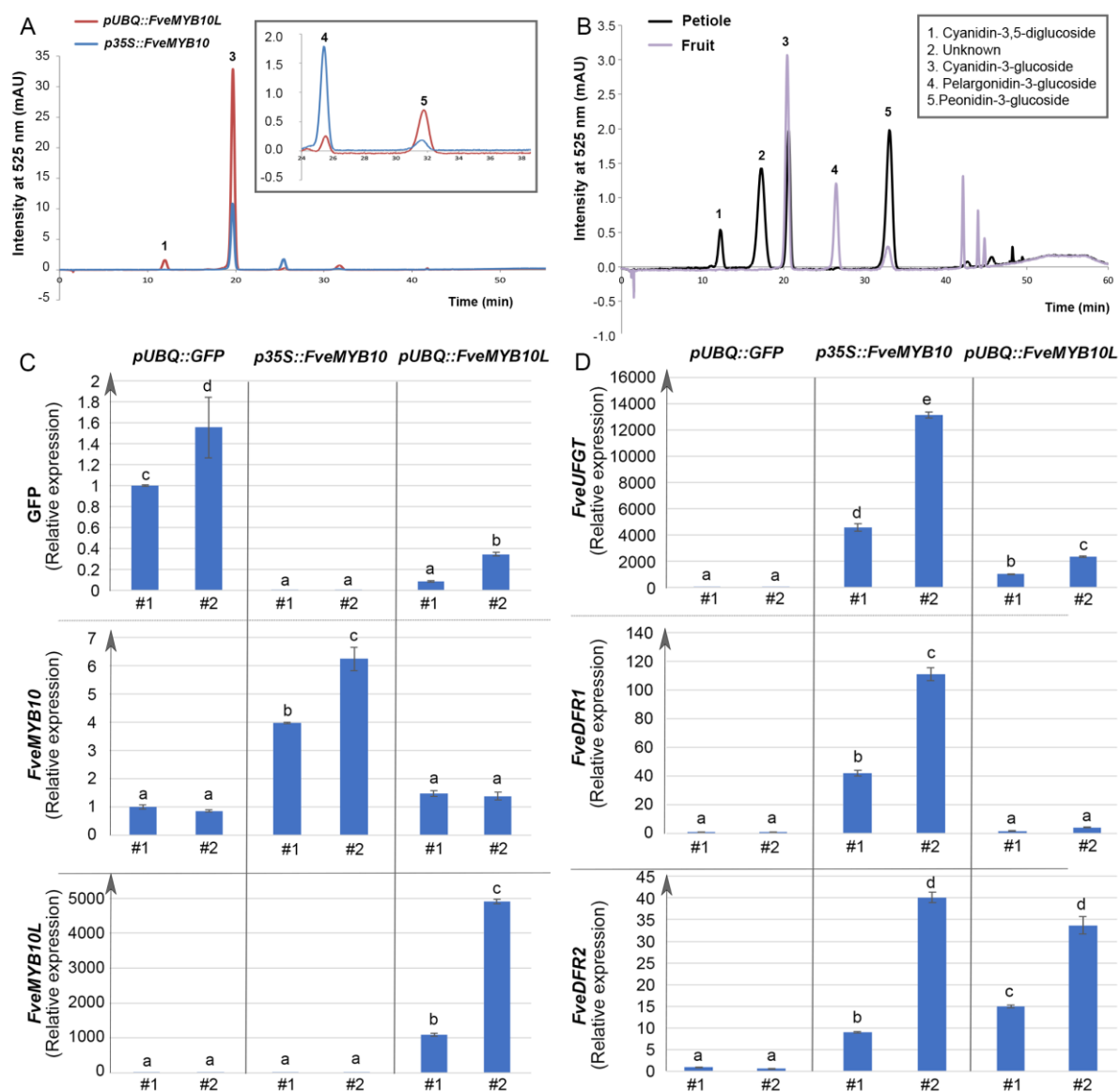


Figure 5

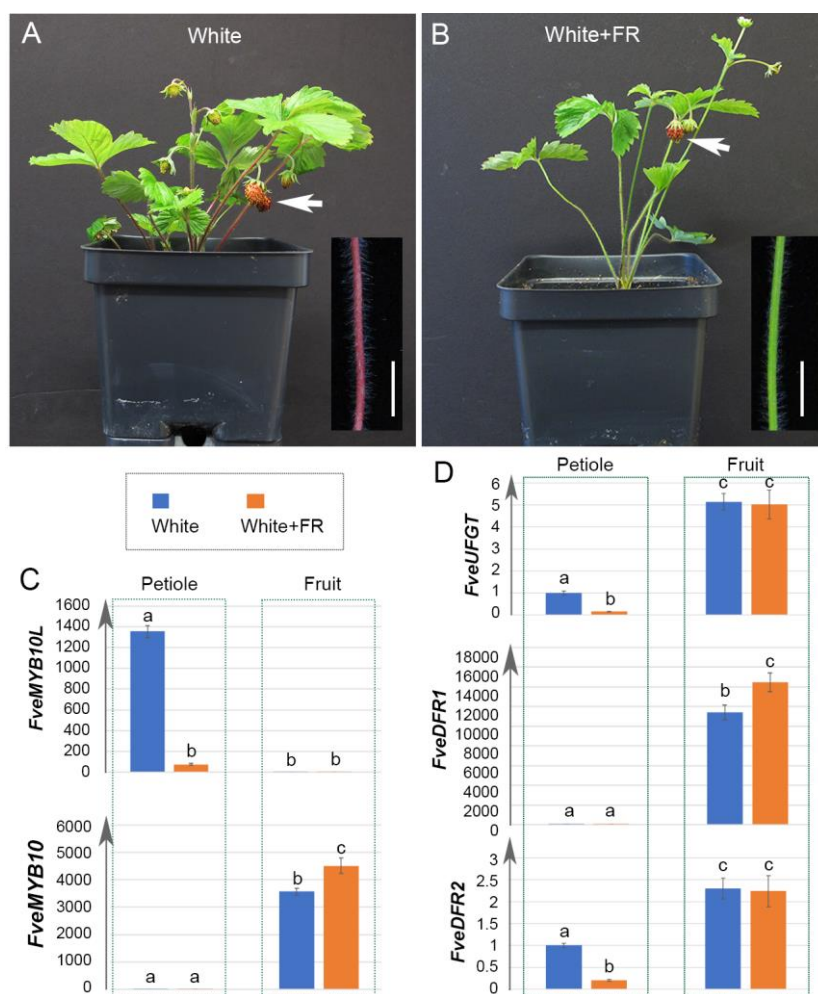
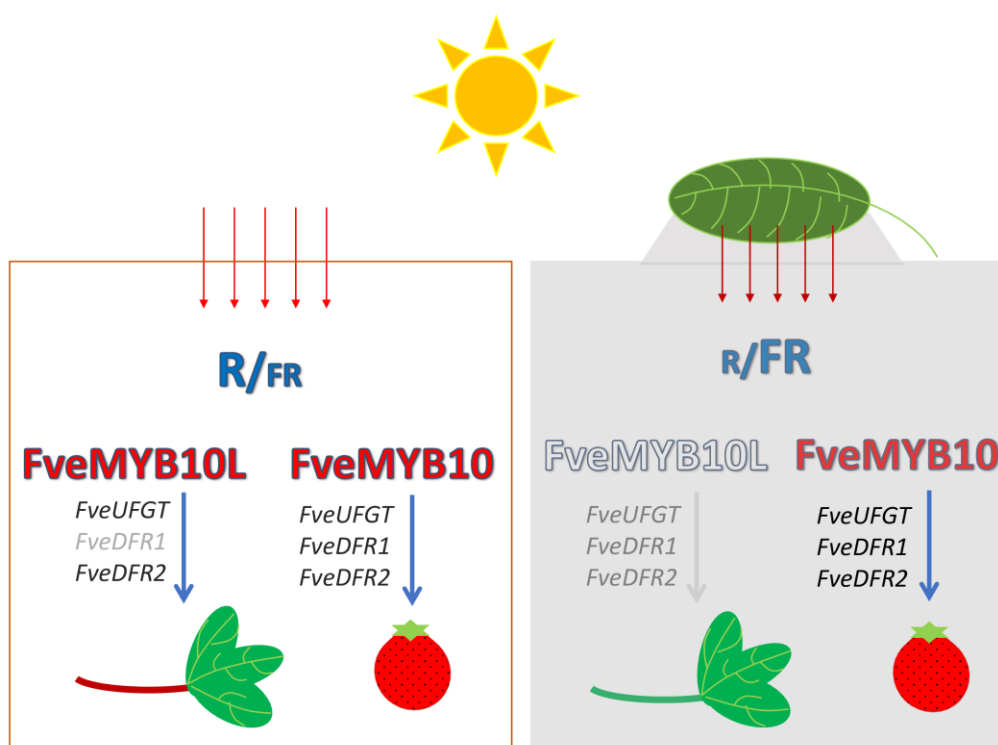


Figure 6



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