Anthocyanin regulatory and structural genes associated with violet flower color of *Matthiola incana*

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Abstract

Main conclusion *MiMYB1* and *MibHLH2* play key roles in anthocyanin biosynthesis in *Matthiola incana* flowers. We established a transient expression system using Turnip mosaic virus vector in *M. incana*.

Abstract Garden stock (*Matthiola incana* (L.) R. Br.) is a popular flowering plant observed from winter to spring in Japan. Here we observed that anthocyanin accumulation in ‘Vintage Lavender’ increased with flower development, whereas flavonol accumulation remained constant throughout flower development. We obtained five transcription factor genes, *MiMYB1*, *MibHLH1*, *MibHLH2*, *MiWDR1*, and *MiWDR2*, from *M. incana* floral cDNA contigs. Yeast two-hybrid analyses revealed that *MiMYB1* interacted with *MibHLH1*, *MibHLH2*, and *MiWDR1*, but *MiWDR2* did not interact with any transcription factor. Expression levels of *MiMYB1* and *MibHLH2* increased in petals during floral bud development. Their expression profiles correlated well with the temporal profiles of *MiF3'H*, *MiDFR*, *MiANS*, and *Mi3GT* transcripts and anthocyanin accumulation profile. On the other hand, *MibHLH1* was expressed weakly in all organs of ‘Vintage Lavender’. However, high expression levels of *MibHLH1* were detected in petals of other cultivars with higher levels of anthocyanin accumulation than ‘Vintage Lavender’. *MiWDR1* and *MiWDR2* maintained constant expression levels in petals during flower development and vegetative organs. Transient *MiMYB1* expression in 1-month-old *M. incana* seedlings using a Turnip mosaic virus vector activated transcription of the endogenous anthocyanin biosynthetic genes *MiF3'H*, *MiDFR*, and *MiANS* and induced ectopic anthocyanin accumulation in leaves. Therefore, *MiMYB1* possibly interacts with *MibHLH2* and *MiWDR1*, and this trimeric protein complex activates the transcription of anthocyanin biosynthetic genes in *M. incana* flowers. Moreover, *MibHLH1* acts as an enhancer of anthocyanin biosynthesis with the *MiMYB1*–*MibHLH2*–*MiWDR1* complex. This study revealed the molecular mechanism involved in the regulation of anthocyanin accumulation levels in *M. incana* flowers.

Keywords Basic helix–loop–helix · Garden stock · R2R3MYB · Turnip mosaic virus · WD40 repeat

Latifa Nuraini, Yukiko Ando, Kentaro Kawai contributed equally to this work.

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Introduction

Garden stock (*Matthiola incana* (L.) R. Br.), which belongs to the *Brassicaceae* family, is an important annual ornamental plant worldwide (Dole and Wilkins 2005). Other members of *Brassicaceae* include *Lunaria* in addition to some of the crucial vegetable crops worldwide such as broccoli, brussels sprouts, cabbage, horseradish, kale, kohlrabi, mustard, radish, rutabaga, and turnip (Dole and Wilkins 2005). The earliest record of *M. incana* describes it as a hoary biennial with purple and white flowers with blackish green seeds (Saunders 1928). Genetic variation is observed at flowering time, which could be attributed to differential sensitivity to low temperature and day length in addition to varying lengths of the juvenile phase (Hisamatsu et al. 2000). Most currently known cultivars are annual, and their low temperature requirement for flowering is generally more stringent for late-flowering cultivars rather than for early-flowering cultivars. *M. incana* cultivars are used as cut and pot flowers, and they bloom in winter and spring in Japan.

Up to 14 commercial colors of *M. incana* are marketed; these range from pure white to lavender, purple, blue, red, and pale yellow (Dole and Wilkins 2005). Anthocyanins, particularly pelargonidin and cyanidin derivatives, are responsible for the flower color, but delphinidin derivatives are not detected in the investigated commercial cultivars of *M. incana* (Tatsuzawa et al. 2012). Wild *M. incana* has petal color ranging from purple to violet (Saunders 1928), with cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside] as the major pigment (Saito et al. 1995). The flower color diversity in *M. incana* cultivars is thought to be caused by a slightly different modification of the major anthocyanins derived from pelargonidin or cyanidin and differences in anthocyanin levels (Tatsuzawa et al. 2012).

The study by Saunders contributed substantially to the understanding of the genetics of *M. incana*, and it was speculated that the flower color of *M. incana* was under the control of four genes (*C*, *R*, *B*, and *V*). *C* and *R* are both required for anthocyanin production in flowers (Saunders 1928). *B* converts red anthocyanins to blue, and *V* is linked to the difference between the pure color *V* and impure color *v* (Crane and Lawrence 1947; Saunders 1928). The colors resulting from the combination of the four genes are as follows: purple, *CRBV*; plum, *CRbv*; red, *CRbV*; copper, *CRbv*; and white, other genotypes. Dilute flower colors (e.g., rose, pale marine, and azure) are dominant over deep colors (e.g., carmine, marine, and purple). In addition to the abovementioned genes, *W* is responsible for the difference between the white color *W* and cream color *w* (Crane and Lawrence 1947; Saunders 1928). Because *W* is not entirely dominant, the heterozygote has an intermediate sulfur color.

M. incana has been applied as a model system for studies on anthocyanin biosynthesis (Forkmann 1993; Seyffert 1971). Studies have explored three loci, *B*, *L*, and *U*, which are involved in the enzyme step involving hydroxylation at the 3' position (Forkmann 1980), glucosylation at the 5' position (Teusch et al. 1986), and acylation of anthocyanin (Teusch et al. 1987), respectively. Hemleben et al. (2004) also reported on three white-flowered mutant lines, which were influenced by *e*, *f*, and *g*. *F* encodes a chalcone synthase (CHS) (Hemleben et al. 2004; Rail and Hemleben 1984; Spribille and Forkmann 1981). The white-flowered and glabrous *e* mutant exhibits one nucleotide change, leading to a direct amino acid substitution in the WD motif (W158R) of *TRANSPARENT TESTA GLABRA1* (*TTG1*, Dressel and Hemleben 2009). Transient expression of two basic helix–loop–helix (*bHLH*) genes, i.e., *MYC146* and *GLABRA 3* (*GL3*), derived from *Arabidopsis thaliana*, can activate anthocyanin biosynthesis in white-flowered *g* mutant (Ramsay et al. 2003). Therefore, both *G* and *E* have been demonstrated to exert a pleiotropic action on the formation of leaf hairs (Forkmann 1993).

Although the genetic analysis of *M. incana* flower colors has been performed extensively, molecular genetics-based information on the flowers is yet to be reported. Recently, Nakatsuka and Koishi (2018) identified the deletion mutation alleles of *M. incana* *AGAMOUS* (*MiAG*), which have been known as doubled flower *s* locus in genetic analysis (Saunders 1928). Numerous molecular biological studies on anthocyanin biosynthesis have been conducted using model plants such as *A. thaliana* (Saito et al. 2013), cabbage (*Brassica oleracea* var. capitata; Yuan et al. 2009), kale (*B. oleracea* var. acephala; Zhang et al. 2012), cauliflower (*B. oleracea* var. botrytis; Chiu et al. 2010), *B. rapa* (Li et al. 2012), and radish (*Raphanus sativus*; Lim et al. 2016, 2017), which also belong to *Brassicaceae*. The MYB–bHLH–WDR (MBW) complex, which is composed of three proteins [i.e., R2R3–MYB, bHLH, and WD40 repeat (WDR)] regulates anthocyanin biosynthesis (Allan and Espley 2018; Nakatsuka et al. 2014; Xu et al. 2015). In *M. incana* flower pigmentation, *bHLH* and *WDR* could be correlated with *G* and *E*, respectively (Dressel and Hemleben 2009; Ramsay et al. 2003). RNA-seq based on next-generation sequencing (NGS) has been used to identify several genes associated with key floral traits in horticultural plants in cases wherein genome information is lacking (Nakatsuka et al. 2019; Sasaki et al. 2017; Suzuki et al. 2016; Zhao et al. 2014). Chen et al. (2018) reported on differentially expressed genes (DEGs) among three flower color varieties: white, light purple, rose bengal *M. incana* flowers. They successfully identified *CHS*, *chalcone isomerase* (*CHI*), *flavanone 3-hydroxylase* (*F3H*), *anthocyanidin synthase* (*ANS*), *UGT78D2*, and *flavonol synthase 1* (*FLS1*) as DEGs of flavonoid biosynthetic

genes, but they could not determine regulatory genes of anthocyanin biosynthesis in *M. incana* flowers.

In the present study, we identified enzymatic and regulatory genes of anthocyanin biosynthesis in *M. incana* flowers, and we revealed genes corresponding to each locus reported previously. Moreover, we investigated the expression profiles of anthocyanin biosynthetic genes in the course of flower development in ‘Vintage Lavender’, which has native flower colors. We attempted to reveal the molecular regulatory mechanism underlying anthocyanin biosynthesis in *M. incana* flowers.

Materials and methods

Plant materials

The seeds of ‘Vintage Lavender’, which is a potted cultivar with purple and doubled flowers, were purchased from M&B flora (Hokuto, Yamanashi, Japan). They were grown in a greenhouse located at Shizuoka University (Shizuoka, Japan) under natural conditions from September 2017 to April 2018. Petal samples were collected at four different

flower developmental stages: S1, flower developmental stage 1, floral bud length of 5–10 mm, and petal not yet emerged from sepal; S2, floral bud length of 10–15 mm and pigmentation beginning at the top of petals; S3, flower length of 15–20 mm; and S4, flower length of > 20 mm and anthesis (Fig. 1a). All samples were frozen immediately using liquid nitrogen and stored at -80°C until use.

Another seven different cultivars, i.e., ‘Vintage Burgundy’, ‘Vintage Lilac’, ‘Vintage Red’, ‘Vintage Rose’, ‘Vintage Copper’, ‘Vintage Peach’, and ‘Vintage Yellow’, were also grown under the same condition. For real-time quantitative PCR (RT-qPCR) and metabolic analyses, petal samples were collected at stages S3 and S4, respectively, and stored at -80°C until use.

Flavonoid analysis

One gram of petal samples collected at different flower developmental stages as well as leaf and stem samples were ground to powder using liquid nitrogen and extracted with 5 mL of MAW (40% methanol and 10% acetic acid) for 24 h at 4°C . Then, the extract was filtered by passage through a 0.22- μm PTFE syringe filter (Osaka Chemical, Osaka,

Fig. 1 Temporal and spatial accumulation patterns of flavonoids in *Matthiola incana* ‘Vintage Lavender’. **a** Flower developmental stages (S1–S4) as described in the “Materials and methods” section. Bar=1 cm. **b** Anthocyanin accumulation in petals at four flower developmental stages (S1–S4), leaves (Le), and stems (St). Anthocyanins extracted from each sample were converted to aglycones through hydrolysis and quantified using cyanidin or pelargonidin as a stand-

ard. Values are expressed as mean $\pm$ standard error ($n=5$). **c** Flavonoid accumulation in petals at four flower developmental stages (S1–S4), leaves (Le), and stems (St). Flavonoids extracted from each sample were converted to aglycones by hydrolysis and quantified using kaempferol as a standard. Values are expressed as mean $\pm$ standard error ($n=5$)

Japan). Anthocyanin and flavonol analyses were conducted using the Agilent Infinity 1290 High Performance Liquid Chromatography system (Agilent Technologies, Santa Clara, CA, USA), Agilent 1200 Diode-Array Detector SL (Agilent Technologies), and the reverse-phase column YMC-Pack Pro C18 RS (4.6 × 150 mm, 5 µm; YMC, Kyoto, Japan). Solvent A [1.5% (v/v) phosphoric acid] and solvent B [1.5% (v/v) phosphoric acid containing 20% (v/v) acetic acid and 25% (v/v) acetonitrile] were used for elution at 40 °C at a flow rate of 0.8 mL min⁻¹. Elution was performed with 20–85% of solvent B for 40 min under gradient elution conditions. Anthocyanidins and flavonols were monitored based on absorbance measured at wavelengths of 520 and 360 nm, respectively.

To identify the types of anthocyanidins and flavonols accumulating in different organs, the extracted flavonoids were converted to aglycone by hydrolysis. Flavonoids were extracted using 5 mL of methanol with 1% hydrochloric acid for 24 h at 4 °C. To convert flavonoids to aglycone by hydrolysis, 4.5 mL of 3 M hydrochloric acid was added to 500 µL of flavonoid extracts, and the mixture was boiled for 1 h. Anthocyanidins extracted using isoamyl alcohol were dried and then resuspended in 500 µL of methanol with 0.1% hydrochloric acid. On the other hand, flavonol aglycones extracted using ethyl acetate were dried and then resuspended in 500 µL of 80% methanol. The flavonol extracts were filtered as mentioned above. Anthocyanidin and flavonol aglycone analyses were conducted using Agilent Infinity 1290 HPLC with the reverse-phase column YMC-Triart C18 (2.0 × 50 mm, 1.9 µm; YMC, Kyoto, Japan), and they were used for elution at 40 °C at a flow rate of 1.0 mL min⁻¹. Elution was performed with 10% solvent A for 1 min and then 60% solvent A for 8 min under gradient elution conditions. Anthocyanidins and flavonols were monitored based on absorbance measured at wavelengths of 530 and 360 nm, respectively. Anthocyanidin and flavonol levels were determined using authentic standards (anthocyanidins: pelargonidin, cyanidin, and delphinidin; flavonols: kaempferol, quercetin, myricetin; Extrasynthese, Lyon, France). In flavonoid analyses, five biological replicates were used.

RNA-seq of *M. incana* floral buds

Total RNA was isolated from petal, leaf, and stem samples using RNAiso Plus (Takara Bio, Otsu, Shiga, Japan) and purified using lithium acetate precipitation. Total RNAs isolated from floral buds of ‘Vintage Lavender’ at stage S3 were treated using the DNA-free DNA Removal kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) to eliminate genomic DNA contamination. RNA-seq analysis was performed by Eurofins Genomics (Tokyo, Japan) using 125-base pair (bp) paired-ends on Illumina HiSeq2500. Adaptors and low-quality reads were discarded from raw sequences;

then, data were assembled using Trinity v.2.4.0 (Grabherr et al. 2011) in DDBJ read annotation pipeline. The obtained contigs were defined as unigenes after the removal of any redundancy. The unigenes were submitted to *A. thaliana* protein database TAIR10 from The Arabidopsis Information Resource for homolog and annotation comparison using the BLASTX algorithm.

Isolation of open reading frames (ORFs) of candidate genes

To confirm the sequences of candidate anthocyanin biosynthetic genes in *M. incana*, we attempted to amplify their ORFs using ‘Vintage Lavender’ petal cDNA as a template. In brief, 25 µL of PCR comprised 1 × *Ex* buffer, 0.2 mM dNTPs, 0.4 µM of each primer, 1.25 U *Ex Taq* polymerase (Takara Bio), and 1 µL of cDNA template. The primers used in the present study are listed in Supplementary Table S1. The reaction conditions were as follows: preheating at 94 °C for 2 min; 35 cycles at 95 °C for 20 s, 60 °C for 40 s, and 72 °C for 2 min; and then extension at 72 °C for 10 min. The amplified fragments were subcloned into a pGEM-T Easy vector system (Promega, Madison, WI, USA). The sequences of all constructs were confirmed by DNA sequencing (Fasmac, Atsugi, Kanagawa, Japan).

Gene expression analysis of anthocyanin biosynthetic genes

To obtain the temporal and spatial expression profiles of anthocyanin biosynthetic genes in ‘Vintage Lavender’, total RNAs were isolated from petals of each floral organ at different developmental stages, leaves and stems as described above. To compare the gene expression levels of *MibHLH1*, total RNA was isolated from petals of another seven different cultivars at stage S3. The elimination of genomic DNA and cDNA synthesis were performed using PrimeScript RT Reagent Kit with gDNA Eraser (Takara Bio). Real-time qPCR of each gene was performed using Thermal Cycler Dice Real Time System (TP850; Takara Bio) using the KAPA SYBR FAST qPCR Master Mix kit (KAPA Biosystems, Wilmington, MA, USA). Briefly, the reaction mixture (10 µL) comprised 1 × Master Mix, 0.2 µM of each primer, and 1 µL of template cDNA, and the cycling conditions were as follows: 95 °C for 20 s, followed by 40 cycles of 95 °C for 1 s and 60 °C for 20 s. The specificity of each amplification reaction was checked by the addition of a dissociation analysis step after the cycle reaction. Data were analyzed by second derivative maximum methods using Thermal Cycler Dice Real Time System II v5.0 (Takara Bio). The transcript level of each gene was calculated relative to that of *M. incana* actin-encoding *MiACT2* (LC496063) as a reference gene. qPCR was performed using five biological replicates. The

sequences of all primers used in qPCR are listed in Supplementary Table S1.

Yeast two-hybrid analysis of anthocyanin biosynthetic transcription factors

To investigate protein-to-protein interactions among MiMYB1, MibHLH1, MibHLH2, MiWDR1, and MiWDR2 proteins, we employed yeast two-hybrid analysis using Matchmaker Two-Hybrid System 3 (Clontech, Takara Bio). The coding regions of each transcription factor were cloned into either the pGAD-T7 (GAL4 activation domain) or pGBK-T7 (GAL4 DNA-binding domain) vector. All constructs were transformed into *Saccharomyces cerevisiae* AH109 (Clontech, Takara Bio). The transformed yeast cells were grown on SD selective medium without leucine (–Leu) and tryptophan (–Trp) at 30 °C for 3 days. The survival test for each transformed yeast culture was performed on selective quadruple dropout medium (–Leu, –Trp, histidine [–His], and adenine [–Ade]) supplemented with 15 mM 3-amino-1,2,4-triazole (3-AT) at 30 °C for 3 days.

Turnip mosaic virus (TuMV) inoculation into *N. benthamiana* and *M. incana* seedlings

pGTuMVUK1 is a binary vector constructed from full length TuMV UK1 isolate under the control of CaMV35S promoter (Sánchez et al. 1998; Llorente et al. 2019). pBTuMVUK1DAE-EZE is an intermediate cloning vector partly containing nucleotide sequences of both nuclear inclusion (NIB) and coat protein (CP) from TuMV UK1 isolate. Green fluorescent protein (GFP) and *MiMYB1* ORF without stop codon were inserted into pBTuMVUK1DAE-EZE digested by *Esp3* I using NEBuilder HiFi assembly master mix (New England BioLabs Japan, Tokyo, Japan), and the constructed plasmids were designated as pBTuMVUK1:GFP and pBTuMVUK1:MiMYB1, respectively. pBTuMVUK1:GFP and pBTuMVUK1:MiMYB1 were double-digested using *Mlu* I and *Apa* I, and then the digested fragments were ligated with pGTuMVUK1 treated with the same restriction enzymes. They were designated as pGTuMVUK1:GFP and pGTuMVUK1:MiMYB1 (Fig. 4a). *Agrobacterium tumefaciens* GV3101 harboring pCLEAN-S48, which is the helper plasmid for pGreen (Thole et al. 2007), was transformed with pGTuMVUK1:GFP or pGTuMVUK1:MiMYB1, and selected on YEB solid medium supplemented with 30 mg L^{−1} kanamycin, 7.5 mg L^{−1} tetracycline, and 25 mg L^{−1} rifampicin, at 28 °C for 3 days. A single colony was cultured overnight in YEB liquid medium containing 30 mg L^{−1} kanamycin and 7.5 mg L^{−1} tetracycline at 28 °C. *A. tumefaciens* cells were harvested and suspended in infiltration buffer (10 mM MgCl₂, 200 mM acetosyringone, and 10 mM MES, pH 5.6), and the suspensions were adjusted

to an optical density of 1.0 at 600 nm (OD₆₀₀). *A. tumefaciens* suspension was infiltrated into *Nicotiana benthamiana* leaves using a needleless 1 mL syringe (Terumo, Tokyo, Japan). At 7 day after *Agrobacterium* infiltration, upper symptomatic leaves of *N. benthamiana* infected with each virus construct were used as inoculum for 1-month-old seedlings of *M. incana* ‘Kiss me Violet’ (Takii-seed, Kyoto, Japan). Crude *N. benthamiana* infectious extract was prepared by grinding the leaves with an infiltration buffer at a four-fold volume relative to leaf fresh weight. The crude sap was rub-inoculated onto mature leaves dusted with carborundum. Inoculated garden stock seedlings were grown at 25 °C under 16 photoperiod of fluorescent lamp.

Anthocyanins were extracted from 100-mg leaves of TuMV-inoculated plants using 1 mL of 80% methanol with 0.1% hydrochloric acid, and they were measured using HPLC as described above. Anthocyanin concentration was calculated on the basis of a standard curve of cyanidin-3-glucoside. The expression levels of transgene and endogenous anthocyanin biosynthetic genes of TuMV:GFP-inoculated or TuMV:MiMYB1-inoculated plants were investigated using RT-qPCR as described above. As an internal reference gene in RT-qPCR for *N. benthamiana* plants, we used *ubiquitin C* (AB026056.1, Yang et al. 2019).

Results

Flavonoid profiles of ‘Vintage Lavender’

The extract derived from the petals of ‘Vintage Lavender’ showed two major peaks at 520 nm on chromatograph, which were identified as cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(*p*-coumaroyl)-glucoside]-5-[6-(malonyl)-glucoside] and cyanidin 3-[2-(2-(sinapoyl)-xylosyl)-6-(feruloyl)-glucoside]-5-[6-(malonyl)-glucoside] (Supplementary Fig. S1a; Tatsuzawa et al. 2012). On the other hand, three major peaks detected at 360 nm were identified as 1-(sinapoyl)-glucopyranoside, kaempferol 3-(2-rhamnopyranosyl)- β -glucopyranoside-7-rhamnopyranoside, and kaempferol 3-(2-rhamnopyranosyl)-arabinopyranoside-7-rhamnopyranoside (Supplementary Fig. S1b; Tatsuzawa et al. 2014). After hydrolyzed flavonoids were extracted from ‘Vintage Lavender’ petal, cyanidin and kaempferol were detected as aglycones (Supplementary Fig. S1c–f; Tatsuzawa et al. 2014). Therefore, cyanidin and kaempferol derivatives were thought to be responsible for purple flower color of ‘Vintage Lavender’.

To investigate the temporal and spatial accumulation profiles of anthocyanins and flavonols, we hydrolyzed flavonoid extracts and then determined cyanidin and kaempferol levels. The accumulation levels of cyanidin derivatives in petals increased from stage S2 and then peaked [263 μ g g^{−1} fresh weight (FM)] at stage S4, which is the anthesis stage

Fig. 2 Relative expression levels of anthocyanin biosynthetic genes in petals, leaves, and stems of *Matthiola incana* ‘Vintage Lavender’. The expression levels of chalcone synthase (*MiCHS*), chalcone isomerase (*MiCHI*), flavanone 3-hydroxylase (*MiF3H*), flavonoid 3'-hydroxylase (*MiF3'H*), dihydroflavonol 4-reductase (*MiDFR*), anthocyanidin synthase (*MiANS*), anthocyanidin 3-O-glucosyltransferase (*Mi3GT*), flavonol synthase 1 (*MiFLS1*), *MiFLS2*, and *MiFLS3*, *MiMYB1*, *MibHLH1*, *MibHLH2*, *MiWDR1*, and *MiWDR2* were normalized using that of *MICT2*. Stages S1–S4 indicate petal samples at flower developmental stages S1–S4. Le and St indicate leaf and stem samples, respectively. Values are expressed as mean $\pm$ standard error ($n = 5$)

(Fig. 1b). However, no anthocyanin accumulation was detected in their leaves and stems. In contrast, kaempferol derivatives as flavonols were detected in the petals, leaves, and stems of ‘Vintage Lavender’ (Fig. 1c). The highest kaempferol level ($1,631 \mu\text{g g}^{-1}$ FW) was detected in petals at the early developmental stages (stages S1 and S2), which then reduced with petal development. In leaves, $273 \mu\text{g g}^{-1}$ FW of kaempferol was accumulated, whereas $5.5 \mu\text{g g}^{-1}$ FW of kaempferol accumulation was detected in stems. Consequently, the accumulation profile of flavonols was different from that of anthocyanins in *M. incana*.

Identification of anthocyanin biosynthetic genes using NGS

To construct a unigene catalog of ‘Vintage Lavender’ flowers, we performed RNA-seq analysis on floral buds at stage S3 using 125-bp paired-end reads on Illumina HiSeq2500. After quality filtering and read cleaning, we obtained approximately 16 million raw sequencing reads, from which 130,224 contigs were assembled with an average sequence length of 1,508 bp. In addition, 73,286 contigs assembled from 32 million raw sequencing reads (SRR5195384 and SRR5195385) deposited by Chen et al. (2018) were used to identify orthologs. Based on a BLASTX search using the amino acid sequences of anthocyanin biosynthetic enzymatic genes in *A. thaliana* as queries, single orthologs for *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, and *3GT* were obtained from *M. incana* contigs. However, we could not find *F3'5'H* orthologs from these floral contigs, suggesting that *M. incana* does not synthesize any delphinidin derivatives. The deduced amino acid sequences of *M. incana* orthologs exhibited 82.5–95.0% identities with those of corresponding proteins (Supplementary Table S2). On the other hand, three *FLS* orthologs were obtained from *M. incana* contigs, which were termed *MiFLS1*, *MiFLS2*, and *MiFLS3*. The deduced amino acid sequences of *MiFLS1*, *MiFLS2*, and *MiFLS3* exhibited 60.7%, 64.0%, and 52.7% identities, respectively, with those of *A. thaliana FLS1*. The deduced amino acid sequence of *MiFLS1* exhibited 57.0% and 47.5% identities with those of *MiFLS2* and *MiFLS3*, respectively.

Transcription factor genes associated with anthocyanin biosynthesis were obtained from *M. incana* contigs, particularly one *MYB* (*MiMYB1*), two *bHLH* (*MibHLH1* and *MibHLH2*), and two *WDR* orthologs (*MiWDR1* and *MiWDR2*). The deduced amino acid sequence of *MiMYB1* exhibited 72.2% and 67.6% identities with those of *B. oleracea* var. botrytis *MYB2* and *A. thaliana PAPI*, respectively (Supplementary Fig. S2 and Table S2). *MibHLH1* and *MibHLH2* were classified into different clades belonging to the TT8 and EGL3 families, respectively (Supplementary Fig. S3 and Table S2). The deduced amino acid sequence of *MibHLH1* exhibited 78.4% and 72.5% identities with those of *A. thaliana TT8* and *B. oleracea* var. botrytis *TT8* (ADP76654), respectively. On the other hand, the deduced amino acid sequences of *MibHLH2* exhibited 84.6% and 80.0% identities with those of *A. thaliana EGL3* and *B. rapa* subsp. *rapa* EGL3 (ADK11703), respectively. *MibHLH1* exhibited 31.3% identity with *MibHLH2* in terms of amino acid sequences.

MiWDR1 and *MiWDR2* were classified into different clades, including TTG1 and AN11 families, respectively (Supplementary Fig. S4 and Table S2). Their partial sequences have been deposited in GenBank (accession numbers CAE53274 and CAE76645, respectively; Dressel and Hemleben 2009). The deduced amino acid sequence of *MibWDR1* exhibited 96.5% and 93.2% identities with those of *A. thaliana TTG1* and *B. oleracea* var. botrytis *TTG1*, respectively. The deduced amino acid sequence of *MibWDR2* displayed 99.1% identity with that of *A. thaliana AN11*, whereas *MiWDR1* exhibited 62.6% identity with *MiWDR2*.

Temporal and spatial expression profiles of flavonoid biosynthetic genes

To investigate temporal and spatial expression profiles of candidate anthocyanin biosynthetic genes, we performed RT-qPCR (Fig. 2). High expression levels of *MiCHS*, *MiCHI*, and *MiF3H* were detected in petals at all floral developmental stages and in leaves. The expression levels of *MiF3'H*, *MiDFR*, *MiANS*, and *Mi3GT* increased markedly at stage S2 and then peaked at stage S4. However, no or slight expression level of these genes was detected in leaves and stems. Therefore, the temporal and spatial profiles of *MiF3'H*, *MiDFR*, *MiANS*, and *Mi3GT* transcripts were correlated well with those of anthocyanin accumulation (Figs. 1b and 2).

Among the three *MiFLS* genes, *MiFLS1* was expressed at high levels in petals compared with *MiFLS2* and *MiFLS3* (Fig. 2). *MiFLS1* constantly exhibited high expression levels in the course of petal development; however, it was not observed in leaves and stems. However, the expression levels of *MiFLS2* and *MiFLS3* were higher in leaves and stems than in petals.

We also investigated the temporal and spatial expression profiles of five regulatory genes involved in anthocyanin biosynthesis. *MiMYB1* expression in petals decreased from stages S1 to S3 and then increased remarkably at stage S4 (Fig. 2). *MiMYB1* was expressed below detectable levels in leaves and stems. In addition, *MibHLH1* expression was the highest at stage S2, but it was considerably lower than those of other regulatory genes of anthocyanin biosynthesis in the petals of ‘Vintage Lavender’. In contrast, *MibHLH2* expression increased with flower development and was detected in leaves. Both *MiWDR1* and *MiWDR2* were expressed constantly in petals at different flower developmental stages as well as in leaves and stems (Fig. 2). Therefore, the *MibHLH2* expression profile was highly correlated with anthocyanin accumulation profile in *M. incana* (Figs. 1b and 2).

Protein-to-protein interactions among *MiMYB1*, *MibHLHs*, and *MiWDRs*

To confirm protein-to-protein interaction among the five transcription factors of *M. incana*, we employed the GAL4 yeast two-hybrid system (Fig. 3). The yeasts harboring *MiMYB1*–*MibHLH1*, *MiMYB1*–*MibHLH2*, *MiMYB1*–*MiWDR1*, *MibHLH1*–*MiWDR1*, and *MibHLH2*–*MiWDR1* combinations grew on quadruple dropout medium, indicating protein-to-protein interactions (Fig. 3). However, the *MibMYB1*–*MiWDR1* combination grew only on yeasts harboring AD:MYB1 and BK:MiWDR1 on quadruple dropout medium. On the other hand, *MiWDR2* could not form homodimers or heterodimers with any other transcription factor. Taken together, these results suggest that

MiMYB1 can form heterodimers with *MibHLH1* or *MibHLH2* and that they can form either trimeric or tetrameric complex with *MiWDR1*.

Ectopic expression of *MiMYB1* using TuMV in seedlings of *N. benthamiana* and *M. incana*

To investigate whether *MiMYB1* actually plays the role of a regulatory gene in anthocyanin biosynthesis, we performed a transient expression analysis of this transcription factor using a TuMV vector in *M. incana* leaves. High expression levels of other transcription factor genes, including *MibHLH1*, *MibHLH2*, *MiWDR1*, and *MiWDR2*, were measured in leaves, but no expression level of *MiMYB1* was detected in leaves compared with those in petals (Fig. 2). Therefore, we attempted to transiently express *MiMYB1* in mature leaves of ‘Kiss me Violet’ seedlings. ‘Kiss me Violet’ is a cultivar with glabrous leaves, which has a higher efficiency for sap inoculation from *N. benthamiana* than ‘Vintage Lavender’ with haired leaves.

To produce virus particles, *A. tumefaciens* GV3101 (containing the helper plasmid pCLEAN-S48) harboring pGTuMVUK1:MiMYB1 or pGTuMVUK1:GFP was infiltrated into *N. benthamiana* leaves. Six days after inoculation, RT-qPCR showed TuMV proliferation and transgene (*MiMYB1* and *GFP*) expression in the leaves inoculated with TuMVUK1:MiMYB1 or TuMVUK1:GFP (Supplementary Fig. S5a). Anthocyanin accumulation in the form of red pigmentation was observed in TuMVUK1:MiMYB1-infected leaves (Supplementary Fig. S5b and c). On the other hand, TuMVUK1:GFP-infected leaves displayed GFP

Fig. 3 Protein-to-protein interactions among *MiMYB1*, *MibHLHs*, and *MiWDRs*. *MiMYB1*, *MibHLH1*, *MibHLH2*, *MiWDR1*, and *MiWDR2* proteins were fused to the GAL4 DNA-binding domain or GAL4 activation domain. pGBKT7 and pGADT7 were used as the negative controls for bait and prey, respectively. Yeast was grown on double-selection medium (– Leu, and – Trp; right) and quadruple dropout medium (– Leu, – Trp, – His, and – Ade) supplemented with 15 mM 3-AT (left) at 30 °C for 2–3 days

fluorescence but not anthocyanin pigmentation (Supplementary Fig. S5b, d, and e).

Extracts derived from TuMVUK1:MiMYB1-infected or TuMVUK1:GFP-infected *N. benthamiana* leaves were used to inoculate 1-month-old ‘Kiss me Violet’ seedlings. Seven days after inoculation, ectopic anthocyanin accumulation was initiated in the upper leaves of TuMVUK1:MiMYB1-infected plants; this accumulation increased up to 1 month after inoculation (Fig. 4b, Supplementary Fig. S6). On the other hand, no pigmentation was observed in any leaves of the control TuMVUK1:GFP-infected plants (Fig. 4b, Supplementary Fig. S6). RT-qPCR revealed the propagation of recombinant TuMV to the upper leaves of independent infected plants (Fig. 4c). The upper leaves of TuMVUK1:MiMYB1-infected plants showed 5500-fold higher *MiMYB1* expression levels than those of TuMVUK1:GFP-infected plants. In addition, the expression levels of endogenous anthocyanin biosynthetic genes, i.e., *MiCHS*, *MiF3'H*, *MiDFR*, and *MiANS*, enhanced by 6.6, 137, 2059, and 3.8 folds, respectively, in TuMVUK1:MiMYB1-infected plants (Fig. 4c). However, no difference in expression levels was observed for other regulatory genes of anthocyanin biosynthesis, i.e., *MibHLH2* and *MiWDR1*, and flavonol biosynthesis, i.e., *MiFLS1*, between TuMVUK1:MiMYB1-infected and TuMVUK1:GFP-infected plants. These results strongly suggested that *MiMYB1* activates the transcription of anthocyanin biosynthetic genes and induces anthocyanin accumulation in *M. incana*.

Expression of *MibHLH1* among different cultivars with colored flowers

To determine the function of *MibHLH1*, we also investigated anthocyanin accumulation and *MibHLH1* expression using eight cultivars of ‘Vintage series’ with different flower colors. In the petals of ‘Vintage Lavender’ and ‘Vintage Burgundy’, the accumulation of cyanidin derivatives as anthocyanins was detected, whereas in other cultivars, only pelargonidin derivatives were detected (Fig. 5a). Among the eight cultivars, ‘Vintage Burgundy’ had the highest anthocyanin accumulation ($1,662 \mu\text{g g}^{-1}$ FW), which was 6.3-fold higher than that detected in ‘Vintage Lavender’ (Fig. 5a). The petals of ‘Vintage Red’, ‘Vintage Rose’, and ‘Vintage Copper’ also had higher accumulation levels of pelargonidin derivatives (917 , 463 , and $301 \mu\text{g g}^{-1}$ FW, respectively) than those of ‘Vintage Lavender’. Thus, different accumulation levels of anthocyanin pigments were observed among the eight cultivars.

We compared the expression levels of *MibHLH1* among the eight cultivars using the petals at stage S3, at which we detected strong expression levels of almost all anthocyanin biosynthetic genes in ‘Vintage Lavender’ (Fig. 2). Higher

expression levels of *MibHLH1* were detected in ‘Vintage Burgundy’, ‘Vintage Red’, ‘Vintage Rose’, and ‘Vintage Copper’ than in ‘Vintage Lavender’ (Fig. 5b). The weak expression levels of *MibHLH1* in the petals of other cultivars were similar to that observed in ‘Vintage Lavender’. Thus, the expression levels of *MibHLH1* correlated well with anthocyanin accumulation levels depending on each cultivar of ‘Vintage series’ (Fig. 5a, b). It is possible that *MibHLH1* plays an important role in controlling the amount of anthocyanin accumulation in *M. incana* petals.

Discussion

M. incana was one of the classic materials for genetic study in the early twentieth century, and it was used to identify loci related to flower pigments, flower shape, and leaf color (Forkmann 1993; Saunders 1928). However, only few recent studies have investigated anthocyanin biosynthetic genes in *M. incana* (Dressel and Hemleben 2009; Hemleben et al. 2004; Chen et al. 2018). Here, we identified anthocyanin biosynthetic genes associated with flower coloration in *M. incana* using NGS. The construction of cDNA libraries and use of NGS-based technologies are critical for performing functional analyses, particularly on horticultural plants lacking genome information. NGS-based technologies have been used to identify numerous genes associated with key floral traits in *Chrysanthemum morifolium* (Sasaki et al. 2017), *Lilium* hybrid (Suzuki et al. 2016), *Paeonia lactiflora* (Zhao et al. 2014), and *Cymbidium* hybrid (Nakatsuka et al. 2019). We obtained a total of 130,224 contigs with an average sequence length of 1,508 bp from the floral organs of ‘Vintage Lavender’. Chen et al. (2018) reported 52,789 transcripts of *M. incana* and DEGs between five colored petals including white, light purple, rose bengal, dark purple, and pink petals. Twelve anthocyanin biosynthetic genes, including *CHS*, *CHI*, *F3H*, *ANS*, and *UGT78D2*, have been identified in each comparative analysis of DEG. Nevertheless, anthocyanin regulatory genes involved in the MBW complex, except *MiTTG1* (Dressel and Hemleben, 2009), have not been identified yet.

M. incana belongs to *Brassicaceae*, which contains the model plant *A. thaliana* and important vegetative crops (e.g., cabbage, cauliflower, kale, and radish) (Dole and Wilkins 2005). Using known sequences of anthocyanin biosynthetic genes from *A. thaliana* as queries, we found their orthologs in the *M. incana* floral contigs constructed in the present study. The deduced amino acid sequences of *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *ANS*, and *3GT* in *M. incana* displayed 82.5–95.0% identities with those of the corresponding proteins in *A. thaliana* (Supplementary Table S2). Although we also attempted to identify regulatory genes of anthocyanin biosynthesis from the *M. incana* cDNA library, similarities

Fig. 4 Ectopic expression of *MiMYB1* in *Matthiola incana* seedling. **a** pGTuMVUK1:GFP and pGTuMVUK1:MiMYB1 vectors. **b** TuMVUK1:GFP-infected seedling (left), TuMVUK1:MiMYB1-infected seedling (right) at 28 days after inoculation. The upper leaf that induced anthocyanin accumulation is showed by an arrow. **c** RT-qPCR of anthocyanin biosynthetic genes in TuMV-infected plants. Total RNA was isolated from the first leaf above the inoculated portion at 6 days after inoculation. TuMVUK1:GFP and TuMVUK1:MiMYB1 infection were performed to three and five independent 1-month-old seedlings, respectively, of ‘Kiss me Violet’. Values are expressed as mean $\pm$ standard error ($n=3-5$). Asterisks indicate significant difference between TuMV:GFP and TuMV:MYB1 using Student’s *t*-test ($P<0.05$). ns indicates no significant difference

in amino acid sequences of regulatory genes of anthocyanin biosynthesis were lower than those of anthocyanin structural genes (Supplementary Table S2). Therefore, we performed more functional analyses to characterize these genes. In plants, the regulation of anthocyanin biosynthesis is controlled by the MBW complex, which is composed of three proteins, namely, R2R3-MYB, bHLH, and WDR (Xu et al. 2015). In *A. thaliana*, *PAP1/2* and *TT2* have been reported to be anthocyanin and proanthocyanidin biosynthetic R2R3-MYB genes, respectively (Bernhardt et al. 2003; Nesi et al. 2001). Only *MiMYB1* was found in *M. incana* floral contigs and was categorized into a clade together with *PAP1/2* (Supplementary Fig. S1). However, a *TT2* ortholog was not found within the *M. incana* floral contigs constructed in the present study. *A. thaliana* *TT2* and *B. napus* *BnTT2* are expressed in seeds and regulate proanthocyanidin biosynthesis (Nesi et al. 2001; Wei et al. 2007). *M. incana* *TT2* ortholog may also be expressed only in seeds, which may have precluded identification in the present study. We identified two bHLH transcription factors, i.e., *MibHLH1* and *MibHLH2*, from *M. incana* floral contigs. According to phylogenetic tree analysis, *MibHLH1* and *MibHLH2* were classified into *TT8* and *EGL3* clades, respectively (Supplementary Fig. S3). In *A. thaliana*, *TT8* and *EGL3* participate in proanthocyanidin biosynthesis in seed coats and anthocyanins in leaves, respectively (Bernhardt et al. 2003; Nesi et al. 2001; Zhang et al. 2003). *MibHLH2* expression increased with petal development, and it was highly correlated with the anthocyanin accumulation profiles (Figs. 1b and 2). On the other hand, *MibHLH1* is an ortholog of *A. thaliana* *TT8*, and weak *MibHLH1* expression levels were observed in the petals of ‘Vintage Lavender’ (Fig. 2). However, cultivars with abundant anthocyanin accumulation such as ‘Vintage Burgundy’, ‘Vintage Red’, ‘Vintage Rose’, and ‘Vintage Copper’ had higher *MibHLH1* expression levels than ‘Vintage Lavender’ (Fig. 5). In ‘Vintage burgundy’, the temporal expression of *MibHLH1* in petals increased with flower development, and it was similar to that of *MibHLH2* (data not shown). Ramsay et al. (2003) also reported that the transient expression of two *A. thaliana* bHLH genes, i.e., *GL3* and *MYC146* (= *EGL3*), in petals of a white-flowered *M. incana* mutant

were associated with a lack of anthocyanin. Consequently, *MibHLH2*, which belongs to the GL3/EGL3 clade, might play a key role as a component of the MBW complex with *MiMYB1* and *MiWDR1* in anthocyanin biosynthesis in *M. incana*. Moreover, *MibHLH1* might enhance anthocyanin accumulation when it coexists with the MBW complex. The nucleotide sequences of *MiWDR1* and *MiWDR2* exhibited high similarity with *TTG1* and *TTG1*-like (*AN11*) genes, respectively, as reported previously (Dressel and Hemleben 2009).

Based on the expression profiles of anthocyanin biosynthetic genes in ‘Vintage Lavender’, they could be classified into three groups. Group 1 included *MiF3'H*, *MiDFR*, *MiANS*, and *Mi3GT*, and temporal expression levels of these genes increased with petal development (Fig. 2). The expression profiles of genes belonging to group 1 were highly correlated with the anthocyanin accumulation profiles (Figs. 1b and 2). Group 2 included *MiFLS1*, which exhibited constant expression levels in petals throughout flower development (Fig. 2). In addition, the expression profiles of genes belonging to group 2 tended to resemble flavonol accumulation profiles (Figs. 1c and 2). Finally, group 3 contained *MiCHS*, *MiCHI*, and *MiF3H*, whose temporal expression profiles seem to be consistent with those of combined groups 1 and 2. The expression of group 3 genes was observed not only in petals but also in leaves (Fig. 2). *MiCHS*, *MiCHI*, and *MiF3H* were essential enzymes for anthocyanin and flavonol biosynthesis. In petals, flavonol derivatives were predominantly produced during the early floral developmental stage (S1); then, the biosynthesis of anthocyanin derivatives increased according to the transcription activation of group 1 genes during the late floral developmental stages (S2–S4). The targeted genes of the anthocyanin biosynthetic pathway under the control of a regulatory gene have been reported to be conserved in plant species such as petunia (Quattrocchio et al. 1993) and Japanese gentian (Nakatsuka et al. 2005, 2008).

Yeast two-hybrid analysis revealed that *MiMYB1* formed a heterodimer with *MibHLH1* and *MibHLH2* (Fig. 3). In addition, *MiWDR1* formed a heterodimer with *MiMYB1*, *MibHLH1*, and *MibHLH2* (Fig. 4). These protein-to-protein interaction data support the fact that trimeric complexes *MiMYB1*–*MibHLH1*–*MiWDR1* or *MiMYB1*–*MibHLH2*–*MiWDR1* could form and regulate anthocyanin biosynthesis. Although a constant level of *MiWDR1* expression was observed in the whole plant, the *MiMYB1* and *MibHLH2* expression levels in petals were higher than those in leaves and stems (Fig. 2). Therefore, the coexpressions of *MiMYB1* and *MibHLH2* in petals would activate the transcriptions of group 1 enzymatic genes including *MiDFR*, *MiANS*, and *Mi3GT*, resulting in enhanced anthocyanin accumulation with flower development.

Fig. 5 Anthocyanin accumulation and expression level of *MibHLH1* in eight different cultivars with colored flowers of *Matthiola incana*. **a** Anthocyanidin accumulation levels in petals of each cultivar at stage S4. **b** Relative expression levels of *MibHLH1* in petals of each cultivar at stage S3. 1, 'Vintage Lavender'; 2, 'Vintage Burgundy'; 3, 'Vintage Lilac'; 4, 'Vintage Red'; 5, 'Vintage Rose'; 6, 'Vintage Copper'; 7, 'Vintage Peach'; 8, 'Vintage Yellow'. Values are expressed as mean $\pm$ standard error ($n = 5$)

To investigate whether *MiMYB1* can activate anthocyanin biosynthesis, we developed a transient expression system using a TuMV vector in *M. incana* (Fig. 4). It is recalcitrant to develop stable transgenic plants in *M. incana* for the investigation of gene function, and no transformant has been reported yet. As an alternative to stable transformants, the overexpression or suppression of genes using virus vectors is a useful tool for investigating gene function (Mortimer et al. 2015). *Tobacco rattle virus* (TRV, Tian et al. 2014) and *Apple latent spherical virus* (ALSV, Kamada et al. 2018) vectors have been used for the overexpression of foreign genes in several horticultural plants. We attempted to transiently overexpress foreign genes in *M. incana* using TRV and ALSV vectors, but no reproducible results were obtained (data not shown). In contrast, the transient expression of *MiMYB1* using

TuMV induced anthocyanin accumulation in infected leaves 6 days after infection (Fig. 4b). The upper leaves of TuMVUK1:*MiMYB1*-infected *M. incana* plants had 5,500-fold higher expression levels of *MiMYB1* than those of TuMVUK1:GFP-infected plants (Fig. 4c). Similarly, the expression levels of endogenous *MiCHS*, *MiF3'H*, *MiDFR*, and *MiANS* in TuMVUK1:*MiMYB1*-infected plants were significantly higher than those in control plants. However, the expression levels of *MiCHI*, *MiF3H*, *Mi3GT*, *MiFLS1*, *MibHLH1*, *MiWDR1*, and *MiWDR2* were not activated by the ectopic expression of *MiMYB1* in leaves (Fig. 4c and data not shown). High expression levels of *MibHLH2* and *MiWDR1*, which are other regulatory factors of the MBW complex, were observed in petals and leaves of *M. incana* (Fig. 2). These results indicated that *MiMYB1* was a key gene strictly regulating anthocyanin biosynthesis in the petals of *M. incana* and that the *MiMYB1*–*MibHLH2*–*MiWDR1* complex activates the transcription of endogenous enzymatic genes such as *MiF3'H*, *MiDFR*, and *MiANS* (Fig. 6). *Antirrhinum majus ROSEA1* (*Ros1*), an anthocyanin-related R2R3–MYB transcription factor, has been used for the visual tracking of plant viral infection and movement (Bedoya et al. 2012). *Tobacco etch virus*:*Ros1*-inoculated tobacco plants and TuMV:*Ros1*-inoculated *A. thaliana* plants turned purple and had induced anthocyanin accumulation (Bedoya et al. 2012). In the present study on *M. incana*, TuMVUK1:*MiMYB1* could also track plant viral infection and movement (Fig. 4b). Therefore, the transient expression system developed in the present study using a TuMV vector could facilitate not only the functional analysis of candidate genes derived from *M. incana* but also the characterization of TuMV.

In radish, the activation of *RsDFR* expression was controlled by *RsMYB1* and enhanced by the presence of *RsTT8* (Lim et al. 2017). In red cabbage, the transcriptional upregulation of *BoMYB2* and *BoTT8* appears to be essential for the activation of anthocyanin structural genes for anthocyanin production during different developmental stages of vegetative growth (Yuan et al. 2009). In *M. incana*, the *MiMYB1*–*MibHLH2*–*MiWDR1* complex activates the transcriptions of *MiF3'H*, *MiDFR*, and *MiANS*. Moreover, *MibHLH1* probably enhances anthocyanin biosynthesis regulation using this complex (Fig. 6). The flower color diversity among *M. incana* cultivars involves differences not only in the anthocyanin component but also in anthocyanin accumulation levels (Dole and Wilkins 2005; Tatsuzawa et al. 2012). However, determining the function of *MibHLH1* would require further research. In future studies, this finding would also be useful to identify the mutated genes responsible for each flower color in *M. incana*.

Fig. 6 Regulation of anthocyanin biosynthesis in *Matthiola incana* flower. The MiMYB1–MibHLH2–MiWDR1 complex activates the transcriptions of *MiF3'H*, *MiDFR*, and *MiANS* as well as induces anthocyanin accumulation in *M. incana* petals. Moreover, MibHLH1 might function as an enhancer for anthocyanin biosynthesis when it coexists with the MiMYB1–MibHLH2–MiWDR1 complex. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:anthocyanidin 3-O-glucosyltransferase

Author contribution statement TN conceived and designed research. LN and TN conducted quantitative expression analysis. LN, FT, and TN conducted flavonoid analysis. KK and TN conducted next-generation sequencing data analysis. YA, KK, KT, VA, and JAD conducted transient expression analysis using virus vectors. MO and KS discussed and revised the manuscript. LN and TN wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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