



## Research article

# The R2R3-MYB transcription factor MdMYB24-like is involved in methyl jasmonate-induced anthocyanin biosynthesis in apple

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## ABSTRACT

Anthocyanins in apple species are important secondary metabolites that are beneficial for human health. Previous studies revealed that methyl jasmonate (MeJA) promotes anthocyanin accumulation by up-regulating the transcription of related genes. In this study, we isolated a jasmonate (JA)-induced apple MYB gene, *MdMYB24-like* (*MdMYB24L*). The encoded nuclear protein contains a conserved R2R3 domain and is homologous to *Arabidopsis thaliana* AtMYB24. Additionally, MdMYB24L was observed to interact with JA signaling factors (MdJAZ8, MdJAZ11, and MdMYC2) in yeast and in planta. The MdMYC2 protein was also targeted by MdJAZ8 and MdJAZ11, which are rapidly degraded under MeJA treatment. The overexpression of *MdMYB24L* resulted in higher anthocyanin contents in the transgenic apple ‘Orin’ calli than in the wild-type control calli. Moreover, the expression levels of the anthocyanin biosynthesis structural genes *MdUFGT* and *MdDFR* were up-regulated in the transgenic calli. Furthermore, MdMYB24L positively regulated the transcription of *MdDFR* and *MdUFGT* by binding to the MYB-binding site motifs in their promoters. Interestingly, the interaction between MdMYC2 and MdMYB24L further enhanced the transcription of *MdUFGT*, whereas MdJAZ8 and MdJAZ11 attenuated this effect. We herein provide new details regarding the molecular mechanism by which MYB transcription factors help regulate anthocyanin biosynthesis via JA signaling pathways.

## 1. Introduction

Anthocyanins are naturally occurring water-soluble pigments that are responsible for the coloration of flowers and fruits (Schaefer et al., 2004), while also increasing plant resistance to stresses (Pourcel et al., 2005). In humans, anthocyanins function as natural antioxidants that eliminate free radicals and other harmful substances from the body, which is beneficial for human health (Cos et al., 2004; Dixon et al., 2005).

In all studied species, the following two major gene types are reportedly involved in anthocyanin biosynthesis: structural genes encoding anthocyanin biosynthesis enzymes [e.g., *CHS*, *CHI*, *F3H*, *DFR*, *LDOX*, and *UFGT* (Honda et al., 2002; Telias et al., 2011)] and transcription factor (TF) genes [e.g., members of at least three TF families, including the MYB, bHLH, and WD40/WDR families, regulate anthocyanin biosynthesis (Zhang et al., 2003; Koes et al., 2005)]. The formation of the MYB-bHLH-WD40/WDR complex by these TFs to induce the expression of structural genes has been reported for many species such as *Arabidopsis thaliana*, petunia, and apple (Baudry et al., 2004;

Quattrocchio et al., 2006; An et al., 2012).

Accumulating evidence indicates that the R2R3-MYB TFs are crucial for many plant processes, including growth, development, and secondary metabolism (Dubos et al., 2010; Martin and Paz-Ares, 1997). Several R2R3-MYB TFs function as essential components of the MYB-bHLH-WD40/WDR complex to control flavonoid biosynthesis (Stracke et al., 2001). The MYB TF ZmC1, which is associated with anthocyanin metabolism, was first detected in maize, and was subsequently characterized in petunia and *A. thaliana* (Paz-Ares et al., 1988; Chandler et al., 1989; Quattrocchio et al., 1993; Borevitz et al., 2000). In apple, the three genes encoding R2R3-MYB TFs (*MdMYB10*, *MdMYB1*, and *MdMYBA*) involved in anthocyanin biosynthesis are likely alleles (Lin-Wang et al., 2010). Additionally, environmental factors, including temperature, light, nutrient availability, and exogenous hormones, also regulate anthocyanin biosynthesis by affecting the transcription of the MYB genes (Takos et al., 2006; Cominelli et al., 2008; Rowan et al., 2009; Azuma et al., 2012). In *A. thaliana*, the sugar-induced up-regulation of *AtMYB75/PAP1* expression increases anthocyanin levels (Teng and Smeekens, 2005). Moreover, the low temperature-induced

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MdMYB15L negatively regulates the cold tolerance and anthocyanin accumulation in the apple callus (Xu et al., 2018). A recent study indicated that in *A. thaliana*, *AtMYB24* encodes an R2R3 TF responsive to methyl jasmonate (MeJA), and the effects of this TF on jasmonate (JA) signaling influences plant floral organ development (Song et al., 2011).

MeJA regulates multiple biological processes, including defense against biotic and abiotic stresses, organ development, and secondary metabolite biosynthesis (Sasaki et al., 2001; Cheng et al., 2009; Chua et al., 2010). A previous study revealed that COI1/JAZs/MYC2 is the core module of the JA signaling pathway (Kazan and Manners, 2012). Jasmonate ZIM-domain (JAZ) proteins are recruited by the F-box protein COI1 (Kazan and Manners, 2012). These proteins directly interact with various TFs, such as MYC, MYB, and WRKY, and impair their activities to repress diverse JA responses (Qi et al., 2011; Zhang et al., 2015; Yan et al., 2018). JA induces the degradation of JAZ proteins in *A. thaliana*, resulting in the release of bHLH and MYB TFs, which promote anthocyanin accumulation (Qi et al., 2011). In apple, the application of JA eliminates the interaction between MdJAZ2 and MdbHLH3, which induces the activity of the *MdMYB9* and *MdMYB11* promoters to increase the anthocyanin content (An et al., 2015).

In this study, we confirmed the interaction between MdMYB24L and MdJAZ8, MdJAZ11, and MdMYC2 in yeast and in planta. The over-expression of *MdMYB24L* increases anthocyanin accumulation via the regulation of putative downstream genes. These results suggest that MdMYB24L is directly targeted by JAZs to specifically mediate JA-regulated anthocyanin biosynthesis.

## 2. Materials and methods

### 2.1. Plant materials and growth conditions

The ‘Orin’ callus induction medium contains 4  $\mu$ M 2,4-dichlorophenoxyacetic acid and 7  $\mu$ M 6-benzyl-aminopurine. The calli were incubated at 25 °C in Murashige and Skoog (MS) medium in darkness. Cultured calli were used to study the accumulation of anthocyanins in response to 100  $\mu$ M MeJA as previously described (An et al., 2012).

### 2.2. Cloning of *MdMYB24L*

The *A. thaliana* *AtMYB24* amino acid sequence was used as a query to screen the National Center for Biotechnology Information (NCBI) database with the Basic Local Alignment Search Tool (BLAST) program, which resulted in the identification of a MYB protein (XM\_008343218.2) as the most homologous protein in apple. Further analysis revealed that this gene was not the same as *MdMYB24* (AAZ20440.1) in apple. Thus, we named it *MdMYB24-like* (*MdMYB24L*). Primers specific for the *MdMYB24* sequence were designed with the DNAMAN program, and then used for a PCR amplification with the cDNA of the ‘Orin’ tissue as the template.

### 2.3. Measurement of anthocyanin content

The harvested calli (0.5 g each) were ground to a powder in liquid nitrogen and then treated with 10 mL 1% (v/v) HCl-methanol at 4 °C for 24 h in darkness. The solution was then centrifuged at 8000  $\times$  g for 10 min. The absorbance of the supernatant was measured at 530 nm with a UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan).

### 2.4. Total RNA extraction and quantitative real-time PCR analysis

Total RNA was extracted with an RNAPrep Pure Plant kit (Tiangen, Beijing, China), and then used as the template for a reverse transcription into cDNA with the RevertAid™ First Strand cDNA Synthesis kit (TransGen, Beijing, China). A quantitative real-time PCR (qRT-PCR) assay was completed with the CFX96 Real-Time PCR Detection system

(Bio-Rad, Hercules, CA, USA) and the SYBR Green PCR Master Mix (TransGen). Each sample was analyzed in triplicate, and *MdActin* was used as an internal control. Relative expression levels were calculated according to the  $2^{-\Delta\Delta Ct}$  method. The qRT-PCR primers were prepared by Sangon Biotechnology Co. Ltd. (Shanghai, China; Supplementary Table S1).

### 2.5. Amino acid sequence analysis and phylogenetic tree construction

The MdMYB24L amino acid sequence was used as the query to search for homologous sequences in the NCBI database. The following identified homologous sequences (NCBI accession number is provided) were included in a phylogenetic tree, which was constructed according to the neighbor-joining method of the MEGA5.0 program: *AtMYB24* (At5g40350), *BrMYB24* (XP\_009151925.1), *CaMYB24* (XP\_004489258.1), *CcMYB24* (XP\_020206840.1), *CmMYB24* (XP\_022983180.1), *CpMYB24* (XP\_021887771.1), *CrMYB24* (XP\_006285780.1), *CsMYB24* (XP\_010435910.1), *EgrMYB24* (XP\_010033288.1), *EsMYB24* (XP\_006405505.1), *FvMYB24* (XP\_011468270.1), *GhMYB24* (AFJ21697.1), *GrMYB24* (XP\_012483815.1), *HuMYB24* (XP\_021299171.1), *MaMYB24* (XP\_021598931.1), *MnMYB24* (XP\_010108787.1), *MdMYB24L* (XM\_008343218.2), *NaMYB24* (XP\_019253730.1), *NtMYB24* (XP\_016500297.1), *PaMYB24* (XP\_021822585.1), *PmMYB24* (XP\_016649474.1), *PpMYB24* (XP\_007217766.1), *PyMYB24* (XP\_009375123.1), *RcMYB24* (XP\_015576153.1), *RsMYB24* (XP\_018463684.1), *SiMYB24* (XP\_011097428.1), *SlMYB24* (XP\_004233330.1), *TcMYB24* (XP\_007031495.1), *ThMYB24* (XP\_010528247.1), *VaMYB24* (XP\_017418182.1), *VvMYB24* (NP\_001268062.1), and *ZjMYB24* (XP\_015879077.1).

### 2.6. Yeast two-hybrid assay

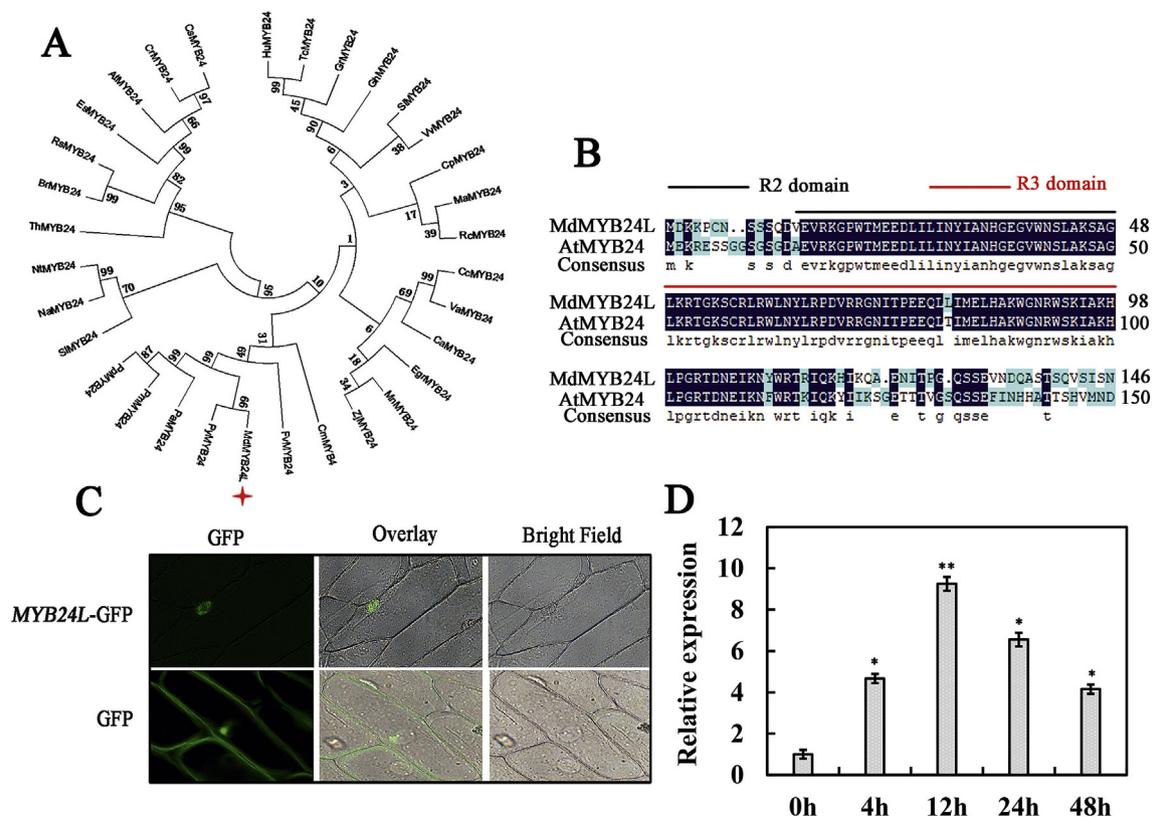
To verify the interaction between MdMYB24L and other TFs, the *MdMYB24L* coding sequence (CDS) was inserted into the pGBKT7 vector, whereas the *MdMYC2*, *MdJAZ8*, and *MdJAZ11* CDSs were inserted into the pGADT7 vector. Details regarding the primers used to prepare the constructs are provided in Supplementary Table S1. The yeast strains carrying the recombinant vectors were first cultured on selective medium lacking Leu and Trp (–Leu/–Trp; Clontech), after which the growing cells were cultured on selective medium lacking Ade, His, Leu, and Trp (–Ade/–His/–Leu/–Trp; Clontech). Finally, X- $\alpha$ -gal was added as a substrate to the selective medium lacking Ade, His, Leu, and Trp to detect  $\beta$ -galactosidase activity.

### 2.7. Bimolecular fluorescence complementation (BiFC) assay

A recombinant MdMYB24L-NYFP plasmid was generated by inserting the *MdMYB24L* CDS into the pSPYNE-YFP vector. Additionally, MdJAZ8-CYFP, MdJAZ11-CYFP, and MdMYC2-CYFP recombinant plasmids were generated by inserting the *MdJAZ8*, *MdJAZ11*, and *MdMYC2* CDSs into the pSPYCE-YFP vector. Different combinations of the two types of recombinant vectors were inserted into *Agrobacterium tumefaciens* LBA4404 competent cells, which were then cultured until the optical density at 600 nm was about 0.8. Onion epidermal cells were immersed in the *A. tumefaciens* culture for 30 min, transferred onto agar-solidified MS medium containing acetosyringone (100  $\mu$ M), and incubated at 28 °C for 1–2 days. The cells were then analyzed and scanned with the LSM 510 Meta laser scanning confocal microscope (Zeiss).

### 2.8. Pull-down assay

The *MdMYB24L* CDS was inserted into the pET-32a vector with the His tag sequence, whereas the *MdJAZ8*, *MdJAZ11*, and *MdMYC2* CDSs were inserted into the pGEX-4T-1 vector with the glutathione S-



**Fig. 1.** Identification of a JA-induced *MYB* gene in apple. (A) Phylogenetic analysis of MdMYB24L and MYB24 protein sequences from 31 other plant species in the NCBI database. (B) Protein alignment of the R2R3 domain of MdMYB24L and its homologs in *Arabidopsis thaliana*. (C) Subcellular localization of MdMYB24L based on the green fluorescent protein signal in onion epidermal cells. Bar = 10  $\mu$ m. (D) 'Orin' calli were treated with 100  $\mu$ M JA, after which *MdMYB24L* expression levels were monitored by qRT-PCR at the indicated time points. Values are provided as the mean  $\pm$  standard deviation of three replicates. Asterisks indicate significant differences with the values for the wild-type calli: \* $P < 0.05$ ; \*\* $P < 0.01$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

transferase (GST) tag sequence. Different combinations of the recombinant plasmids were inserted into *Escherichia coli* BL21 (DE3) cells, after which the production of the fusion proteins was induced. The protein mixtures in binding buffer were purified on a column with a fixed His-tagged bait protein after incubating for 12 h at 4 °C. The eluted protein mixtures were analyzed in a western blot in which proteins were detected with an anti-His or anti-GST antibody (Clontech, Palo Alto, CA, USA).

## 2.9. Yeast one-hybrid (Y1H) assay

The *MdMYB24L* CDS was inserted into the pGADT7 vector to construct the recombinant MdMYB24L-AD plasmid. The promoter sequences of the anthocyanin structural genes *MdLDOX* (–38 bp to –927 bp), *MdDFR* (–12 bp to –798 bp), *MdUGFT* (–8 bp to –882 bp), *MdF3H* (–71 bp to –1084 bp), *MdCHI* (–21 bp to –1582 bp), and *MdCHS* (–15 bp to –779 bp) were inserted into the pHIS2 vector. The promoter sequences are listed in [Supplementary Table S2](#). Different combinations of the recombinant plasmids were inserted into yeast Y187 cells, after which interactions were analyzed on media lacking Trp, Leu, and His (SD/–Trp/–Leu/–His), but supplemented with the optimal 3-amino-1,2,4-triazole (3-AT) concentration.

## 2.10. Electrophoretic mobility shift assay

An electrophoretic mobility shift assay (EMSA) was completed with the EMSA kit (Pierce, Rockford, IL, USA) and biotin-labeled probes. Briefly, biotin-labeled probes were incubated at 24 °C for 25 min in binding buffer (2.5% glycerol, 10 mM EDTA, 5 mM MgCl<sub>2</sub>, and 50 mM

KCl) with or without protein. Unlabeled probes were then added to the reaction solution.

## 2.11. Chromatin immunoprecipitation-PCR analysis

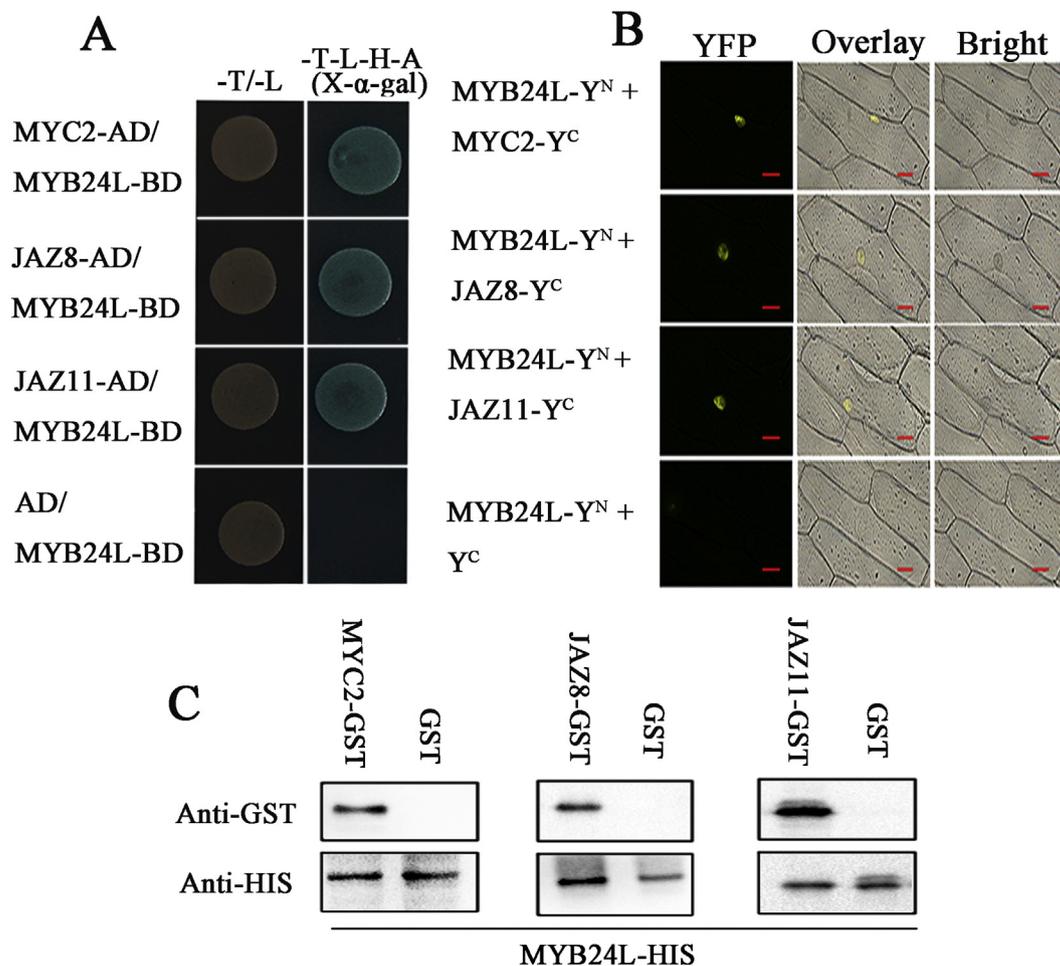
Transgenic calli producing green fluorescent protein (GFP)-tagged MdMYB24L were used for chromatin immunoprecipitation (ChIP)-PCR assays with the ChIP kit (Upstate, Waltham, MA, USA). The amount of immunoprecipitated chromatin was determined by a PCR with the primers listed in [Supplementary Table S1](#).

## 2.12. Luciferase reporter assay

The *MdMYB24L*, *MdJAZ8*, *MdJAZ11*, and *MdMYC2* CDSs were inserted into the pHBT-AvrRpm1 vector (effector) under the control of the 35S promoter. The promoter fragments of the cloned *MdUGFT* (–8 bp to –882 bp) and *MdDFR* (–12 bp to –798 bp) genes were inserted into the pFRK1-LUC-nos vector (reporter). 'Orin' callus protoplasts (100  $\mu$ L) were mixed with 6  $\mu$ L effector, 3  $\mu$ L LUC reporter, and 1  $\mu$ L GUS plasmid (for normalization). The plasmid concentrations were adjusted to 2000 ng/mL. The transiently transfected wild-type callus protoplasts were incubated at 24 °C for 6 h before GUS and LUC activities were detected with the Victor X4 Multimode Plate Reader (PerkinElmer, Waltham, MA, USA). The ratio of LUC to GUS activity was calculated to quantify the promoter activity.

## 2.13. Transformation of the apple callus with *MdMYB24L*

The *MdMYB24L* CDS was inserted into the pRI101 vector carrying



**Fig. 2. Interaction between MYB24L and JA signaling factors.** (A) The interaction between MYB24L and JA signaling factors was verified in a yeast two-hybrid assay. The pGAD empty vector and pGBD-MdMYB24L were used as controls. Yeast cells were grown on SD/-Trp/-Leu and SD/-Trp/-Leu/-His/-Ade media supplemented with X- $\alpha$ -gal. (B) A bimolecular fluorescence complementation assay involving onion epidermal cells was completed to detect the interaction between MdMYB24L and JA signaling factors. Bar = 10  $\mu$ m. (C). Pull-down assay for the in vitro detection of MdMYB24L interactions with JA signaling factors. The GST or GST-tagged JA signaling factors produced in *Escherichia coli* were incubated with a cobalt chelate affinity resin containing the immobilized histidine-tagged MdMYB24L protein. The protein mixtures were purified using a HIS Purification kit.

the GFP tag sequence to generate the 35S:MdMYB24L-GFP construct. The resulting recombinant plasmid was inserted into *A. tumefaciens* LBA4404 cells. Apple calli grown for 14 days were infected with the *A. tumefaciens* cells carrying the vector with the 35S:MdMYB24L-GFP construct, after which the calli were cultured on agar-solidified MS medium for 2 days at 24 °C in darkness. The calli were then transferred to selective medium containing 662  $\mu$ M carbenicillin and 74  $\mu$ M kanamycin to identify transformants. Three independent transgenic lines were tested in subsequent experiments.

#### 2.14. In vitro protein degradation assay

Protein degradation assays were performed as previously described (An et al., 2016) to study the post-translational regulation of JAZ in vitro. Wild-type apple calli were treated with protein extraction solution (25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM MgCl<sub>2</sub>, 5 mM dithiothreitol, 10 mM ATP, and 4 mM phenylmethanesulfonyl fluoride). The purified MdJAZ8-GST and MdJAZ11-GST proteins were separately incubated with protein extract from the callus for specific periods. The relative JAZ protein contents were determined in a western blot involving the anti-GST monoclonal antibody. The resulting bands were quantified with the Quantity One program (Bio-Rad, Hercules, CA, USA). The protein content at 0 h was set as 100%.

#### 2.15. Subcellular localization

The MdMYB24L CDS was inserted into the pHBT-AvrRpm1-GFP vector. The recombinant plasmid was inserted into *A. tumefaciens* cells, which were then used to transform onion epidermal cells as previously described. After culturing for 24 h in darkness, the GFP fluorescence of the onion epidermis was observed with the BX53F fluorescence microscope (Olympus, Tokyo, Japan).

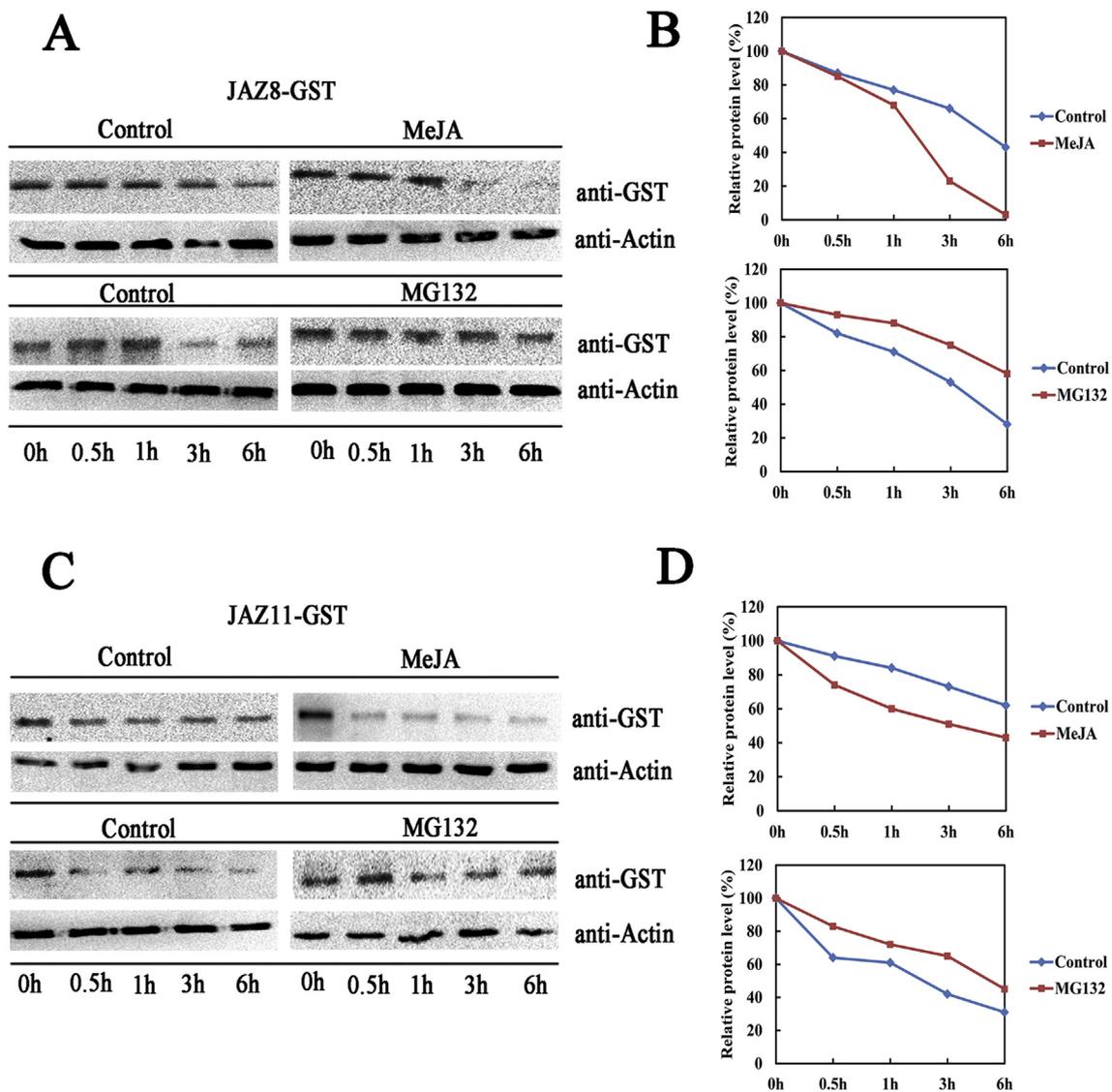
#### 2.16. Data analysis

All of the results presented herein were based on the average of three parallel experiments. The data were analyzed with appropriate methods with the R Commander package of R (3.0.2). Differences were considered significant when  $P < 0.05$  and  $< 0.01$ .

### 3. Results

#### 3.1. Identification of a JA-induced MYB gene in apple

A search of the NCBI database with the BLAST program for apple sequences that are similar to the *A. thaliana* AtMYB24 protein sequence revealed that an R2R3-MYB TF, MdMYB24L (XM\_008343218.2), is highly similar to AtMYB24 (Supplementary Fig. S1). A phylogenetic



**Fig. 3.** Degradation of JAZ proteins by the 26S proteasome in the JA signaling pathway. (A and C) Wild-type calli were treated with DMSO or 100  $\mu$ M MeJA. Callus extracts were mixed with MdJAZ8-GST and MdJAZ11-GST fusion proteins and incubated for the indicated periods. Actin was used as a loading control. The MG132 treatment inhibited the degradation of JAZ proteins. Wild-type apple callus extracts were treated with DMSO or 100  $\mu$ M MG132 for 30 min and then incubated with MdJAZ8-GST or MdJAZ11-GST for the indicated periods. (B and D) Relative protein contents are presented. The SDS-PAGE analysis revealed the residual protein levels.

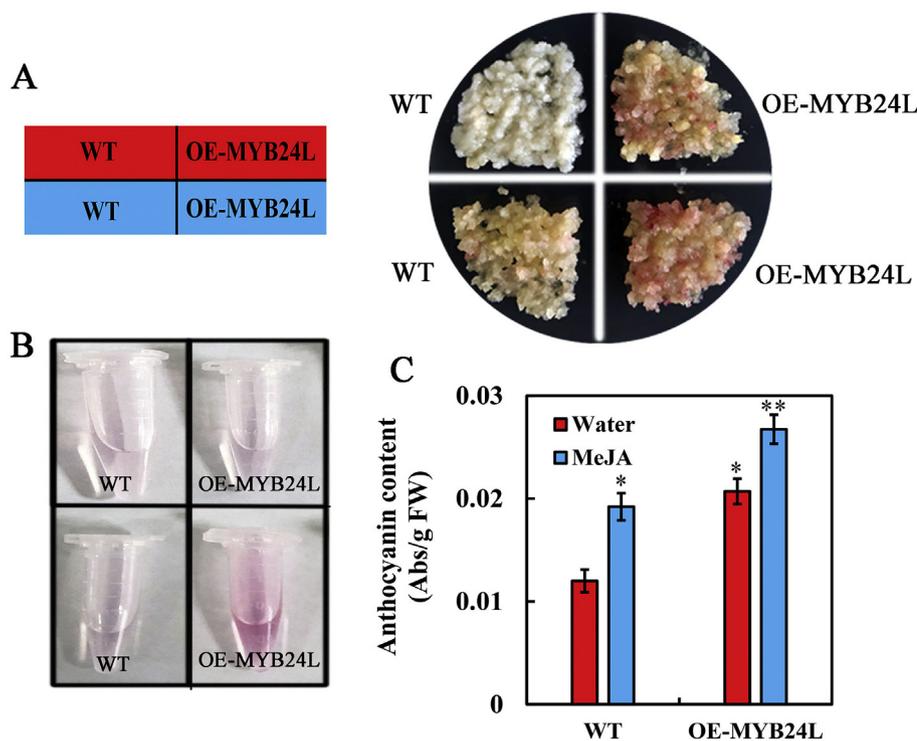
tree was constructed to determine the relationship between apple MdMYB24L and other plant MYB24 protein sequences. The phylogenetic tree indicated that MdMYB24L is the most homologous to PyMYB24 from *Pyrus bretschneideri*, both of which belong to the same clade (Fig. 1A). Furthermore, an R2R3 domain was detected on the N-terminal side of the MdMYB24L protein (Fig. 1B). A subcellular localization analysis proved that MdMYB24L was expressed in the nucleus of onion epidermal cells (Fig. 1C). To examine the effects of JA on MdMYB24L expression, a qRT-PCR assay was conducted with cDNA isolated from 'Orin' calli treated with MeJA for specific periods. The MdMYB24L expression level increased in response to MeJA (Fig. 1D), suggesting MdMYB24L may be important for apple responses to JA.

### 3.2. The MdMYB24L protein interacts with JA signaling factors

Previous studies confirmed that JAZ repressors interact with other proteins to mediate various JA-regulated biological processes (Qi et al., 2011; An et al., 2015). In *A. thaliana*, an R2R3-MYB transcription factor, MYB24, is directly targeted by JAZ8/11 to specifically regulate male

fertility (Song et al., 2011). A yeast two-hybrid assay was performed to investigate whether MdMYB24L interacts with MdJAZ proteins. The results revealed that MdMYB24L can interact with MdJAZ8 and MdJAZ11 (Fig. 2A). Interestingly, MdMYC2 acts as a transcriptional activator of JA signaling, and also binds to MdMYB24L.

We conducted a BiFC assay to examine the interaction between the JA signaling factors and MdMYB24L. We detected YFP fluorescence in the nucleus when MdMYB24L-NYFP was co-expressed with MdJAZ8-CYFP, MdJAZ11-CYFP, and MdMYC2-CYFP (Fig. 2B), indicating that MdJAZ8, MdJAZ11, and MdMYC2 interact with MdMYB24L. Pull-down assays were completed with the recombinant MdMYB24L-His as well as MdJAZ8-GST, MdJAZ11-GST, and MdMYC2-GST fusion proteins. The assays confirmed that MdMYB24L can interact with these proteins in vitro. Specifically, these proteins were pulled down by MdMYB24L-His, whereas GST alone was not co-purified with MdMYB24L-His (Fig. 2C). Our results demonstrate that these JA signaling factors can interact with MdMYB24L to form transcription complexes.



**Fig. 4. Overexpression of *MdMYB24L* increases the anthocyanin content.** (A) Appearance of the wild-type (WT) and *MdMYB24L*-overexpressing (OE-*MdMYB24L*) calli grown on MS medium with a water (red square) or 100  $\mu$ M MeJA (blue square) treatment. (B) Comparison of the color of methanol extracts of calli treated with water or MeJA. (C) Anthocyanin contents in the WT control and the OE-*MdMYB24L* calli grown on MS medium with a water or MeJA treatment. Values are presented as the mean  $\pm$  standard deviation of three replicates. Asterisks indicate significant differences with the values for the wild-type calli: \* $P < 0.05$ ; \*\* $P < 0.01$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

### 3.3. Methyl jasmonate degrades *MdJAZ8* and *MdJAZ11*

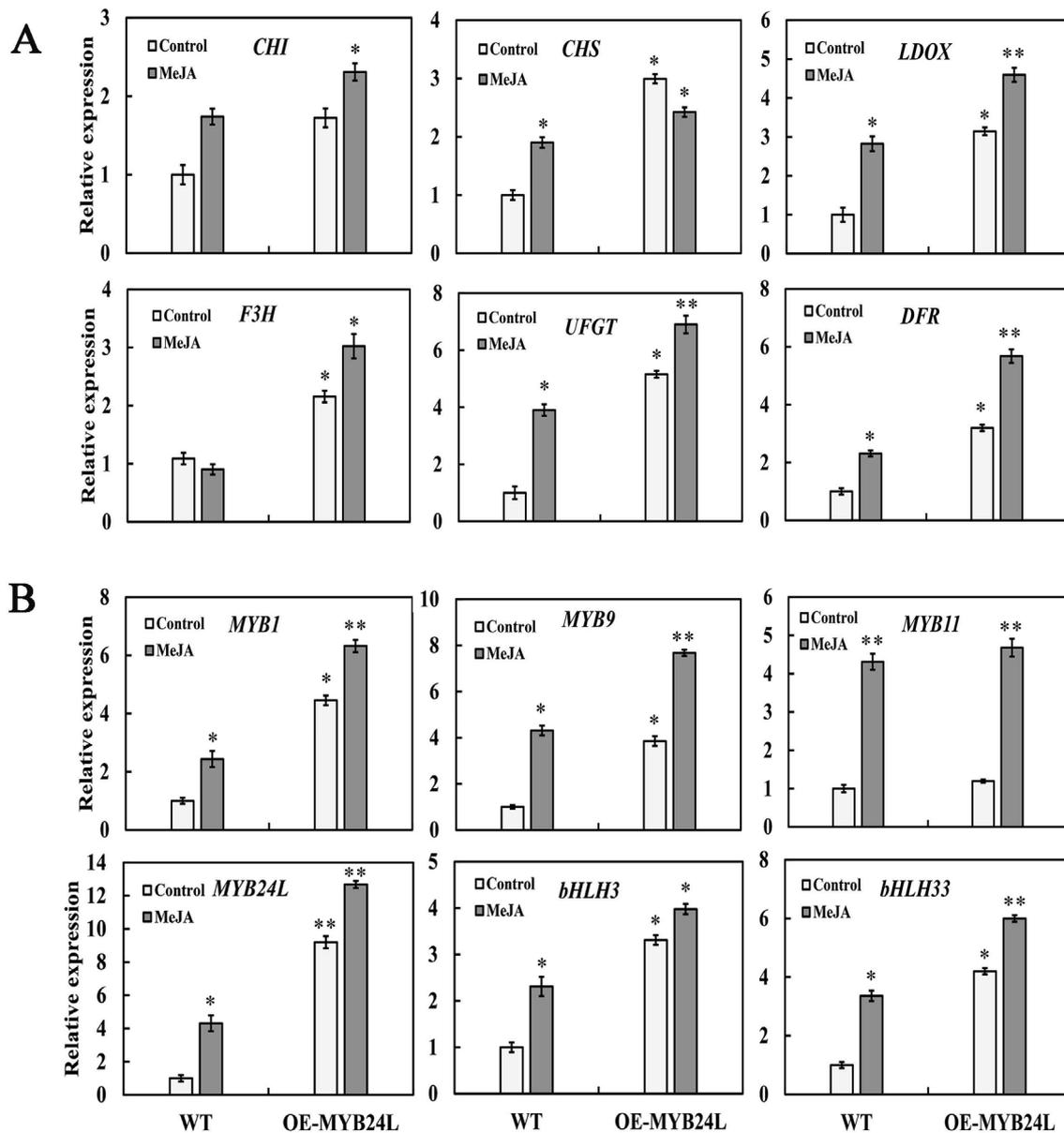
Previous studies indicated that JAZ is an unstable JA-regulated protein that is degraded by the 26S proteasome (Thines et al., 2007; Pauwels and Goossens, 2011). In the current study, a protein degradation assay was carried out to study the post-transcriptional regulation of JAZ in vitro. Upon the addition of JA, *MdJAZ11*-GST was rapidly degraded [i.e., within 30 min (Fig. 3C and D)], whereas *MdJAZ8*-GST was significantly degraded only after 1 h (Fig. 3A and B). Thus, both *MdJAZ8* and *MdJAZ11* are degraded in response to a JA treatment, but *MdJAZ11* likely binds to the 26S proteasome more efficiently than *MdJAZ8*. Treatments with MG132 and DMSO confirmed that MG132 significantly impairs the effect of JA on JAZ stability (Fig. 3). These results indicated that JA contributes to the post-translational regulation of *MdJAZ8* and *MdJAZ11*.

### 3.4. The overexpression of *MdMYB24L* promotes anthocyanin accumulation in 'Orin' calli

To examine whether *MdMYB24L* regulates anthocyanin accumulation, *MdMYB24L* was overexpressed in 'Orin' apple calli. The resulting transgenic calli changed from yellow to pink (Fig. 4A), and the anthocyanin content increased (Fig. 4B and C). Furthermore, a JA treatment of the transgenic calli enhanced the effect of *MdMYB24L* on anthocyanin biosynthesis (Fig. 4C). The anthocyanin biosynthesis pathway is regulated by several enzymes (Fig. S2). A qRT-PCR analysis revealed that the expression of structural genes involved in anthocyanin biosynthesis was higher in the transgenic calli than in the wild-type control (Fig. 5A). Specifically, the *MdUFGT* and *MdDFR* expression levels were 3–5 times higher in the *MdMYB24L*-overexpressing calli than in the wild-type control (Fig. 5A). Moreover, the expression levels of genes encoding anthocyanin biosynthesis-related TFs, namely MYB TFs (*MdMYB1*, *MdMYB9*, and *MdMYB11*), and bHLH TFs (*MdbHLH3* and *MdbHLH33*) were also up-regulated by varying degrees in the transgenic calli, suggesting that *MdMYB24L* may positively regulate their transcription. Consistent with the changes to the anthocyanin content, a JA treatment of transgenic and wild-type calli further increased the transcription of anthocyanin-related genes.

### 3.5. The *MdMYB24L* protein binds specifically to the *MdUFGT* and *MdDFR* promoters

Some R2R3-MYB TFs recognize the MYB cis-element in the promoters of anthocyanin structural genes, thereby influencing transcription (Wang et al., 2017; Xu et al., 2017). A yeast one-hybrid assay was conducted to prove that *MdMYB24L* can bind to the *MdDFR* and *MdUFGT* promoters (Fig. 6A). Using the PlantCARE database (<http://bioinformatics.Psb.ugent.be/webtools/plantcare/html/>), we determined that the *MdUFGT* and *MdDFR* promoters contain putative MYB-binding elements (Supplementary Table S2). According to their different locations, we named these elements U1, U2, and U3 in *MdUFGT* and D1 and D2 in *MdDFR* (Fig. 6B). To verify the specific binding of *MdMYB24L* to the *MdUFGT* and *MdDFR* promoters in vivo, we generated transgenic apple calli containing *MdMYB24L*-GFP as well as control calli carrying the pBIN-GFP vector. The in vivo *MdUFGT* and *MdDFR* promoter binding test involved a ChIP-PCR assay. The results proved that the U2 and D1 promoter regions containing the MYB-binding element were enriched by ChIP more for the *MdMYB24L*-GFP transgenic calli than the pBIN-GFP control calli (Fig. 6B). This observation provided in vivo evidence that *MdMYB24L* binds to the *MdUFGT* and *MdDFR* promoters. To further confirm that *MdMYB24L* interacts with MYB-binding sites (U2 and D1) in the *MdUFGT* and *MdDFR* promoters, probes were designed corresponding to the MYB-binding site sequences at the U2 and D1 positions. The subsequent EMSAs indicated that MYB24L interacts with the *UFGT* promoter fragment (U2) and *DFR* promoter fragment (D1) (Fig. 6C). Additionally, we validated the *MdUFGT* and *MdDFR* promoter activities with a luciferase reporter assay involving *MdMYB24L* alone or in combination with *MdJAZ8*, *MdJAZ11*, and *MdMYC2* (Fig. 6D). The results indicated that *MdMYB24L* alone significantly affects *MdDFR* and *MdUFGT* promoter activities (Fig. 6E). Interestingly, the combination of *MdMYB24L* and *MdMYC2* further enhanced the *MdUFGT* promoter activity (Fig. 6E). However, the presence of *MdJAZ8* and *MdJAZ11* attenuated the *MdMYB24L*-mediated activation of the *MdUFGT* and *MdDFR* promoters (Fig. 6E).



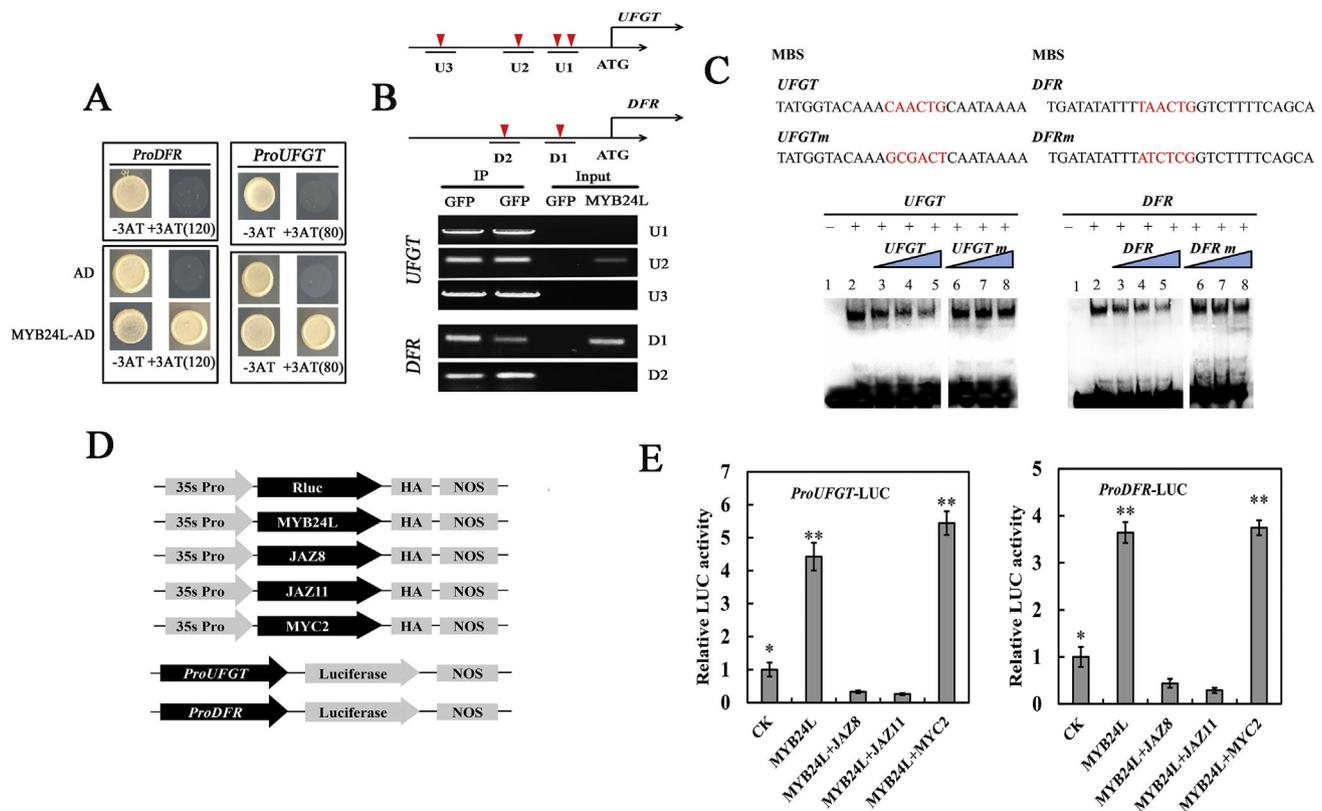
**Fig. 5.** Relative expression of transcription factor genes and anthocyanin biosynthesis structural genes. WT: wild-type calli; OE-MdMYB24L: *MdMYB24L*-overexpressing calli. (A) A qRT-PCR assay revealed the expression levels of anthocyanin biosynthesis structural genes (*MdCHS*, *MdCHI*, *MdF3H*, *MdLDOX*, *MdDFR*, and *MdUFGT*) in WT and OE-MdMYB24L calli treated with water or 100  $\mu$ M MeJA for 14 days. (B) A qRT-PCR assay revealed the expression levels of transcription factor genes (*MdMYB1*, *MdMYB9*, *MdMYB11*, *MdMYB24L*, *MdbHLH3*, and *MdbHLH33*) in WT and OE-MdMYB24L calli treated with water or 100  $\mu$ M MeJA for 14 days. *MdActin* was used as the internal control. Values are presented as the mean  $\pm$  standard deviation of three replicates. Asterisks indicate significant differences with the values for the wild-type calli: \* $P < 0.05$ ; \*\* $P < 0.01$ .

#### 4. Discussion

In eukaryotes, the *MYB* genes form a large family with diverse functions. The *MYB* gene family has been divided into the following five groups based on the number of imperfect repeats in the MYB domain: 4R-MYB, 3R-MYB, R2R3-MYB, single-repeat MYB, and MYB-like genes (Rosinski and Atchley, 1998; Jin and Martin, 1999; Dubos et al., 2010). Of these groups, the R2R3-MYBs, which contain two MYB domains, control many aspects of plant secondary metabolism, including anthocyanin biosynthesis (Stracke et al., 2001). In the current study, we characterized an apple gene that encodes a protein with a highly conserved R2R3 motif at its N-terminus. The encoded protein is highly homologous to the MYB24 proteins from other species, suggesting that *MdMYB24L* may be functionally similar to other MYB24 proteins. A previous study confirmed that *AtMYB24* functions in JA-mediated

stamen development (Song et al., 2011). As we predicted, *MdMYB24L* encodes a nuclear protein and responds rapidly to JA.

MeJA affects many physiological processes, including development, aging, maturation, and secondary metabolism (Creelman and Mullet, 1997). Additionally, JA-induced anthocyanin accumulation has been observed in plants (Rudell and Mattheis, 2008). In apple, two genes (*MdMYB9* and *MdMYB11*) that encode R2R3-MYB TFs are important for JA-induced anthocyanin biosynthesis (An et al., 2015). Furthermore, JAZs and MYC2 are important components of the JA signaling pathway and JAZ proteins interacting with MYB and bHLH TFs to influence anthocyanin biosynthesis (Qi et al., 2011). In the current study, we observed that the protein encoded by a JA-induced MYB gene, *MdMYB24L*, interacts with *MdJAZ8*, *MdJAZ11*, and *MdMYC2*. The JAZ proteins can function as repressors that dimerize with the target *MdMYC2* during JA signaling. An in vitro protein degradation analysis



**Fig. 6. The MdMYB24L protein promotes anthocyanin biosynthesis by interacting directly with the promoters of the structural genes *MdUFGT* and *MdDFR*.** (A) Yeast one-hybrid assays indicated that MdMYB24L binds directly to the promoters of *MdDFR* and *MdUFGT*. Yeast cells co-transformed with pGADT7 + *MdUFGT*-pHIS2 or pGADT7 + *MdDFR*-pHIS2 were used as controls. The numbers in parentheses indicate the optimal 3-AT concentrations for suppressing the background histidine leakiness of the pHIS2 vectors. (B) Schematic diagram of the *UFGT* and *DFR* promoters with potential MYB-binding sites (MBSs). Three and two MBSs were predicted for the *MdUFGT* promoter (U1, U2, and U3) and *MdDFR* promoter (D1 and D2), respectively. The results of a ChIP-PCR analysis implied that MdMYB24L binds to U2 and D1 in the *MdUFGT* and *MdDFR* promoters, respectively. (C) An EMSA was also conducted. The red letters indicate the mutant nucleotides of the MBSs within the *MdUFGT* and *MdDFR* promoters (UFGTm and DFRm). Lane 1 of each blot presents the labeled DNA probes without the MdMYB24L protein. Lane 2 presents the labeled wild-type (WT) DNA probes and the MdMYB24L protein without a competitor. Increasing amounts (25-times, 50-times, and 100-times) of the unlabeled WT DNA fragments (*MdDFR* and *MdUFGT*) in lanes 3, 4, and 5 of each blot or the unlabeled mutant fragments (*MdDFRm* and *MdUFGTm*) in lanes 6, 7, and 8 of each blot were added as cold competitors. (D) Diagram of the pHBT-AvrRpm1-HA effector carrying the transcription factor genes (*JAZ8*, *JAZ11*, *MYC2*, and *MYB24L*). Diagram of the pFRK1-LUC-nos reporter carrying the promoter fragments (*DFR* and *UFGT* promoters). (E) Effect of MdMYB24L alone and in combination with other proteins on the activation of the promoters of anthocyanin structural genes (*MdUFGT* and *MdDFR*). Values are presented as the mean  $\pm$  standard deviation of three replicates. Asterisks indicate significant differences with the values for the wild-type calli: \* $P < 0.05$ ; \*\* $P < 0.01$ . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

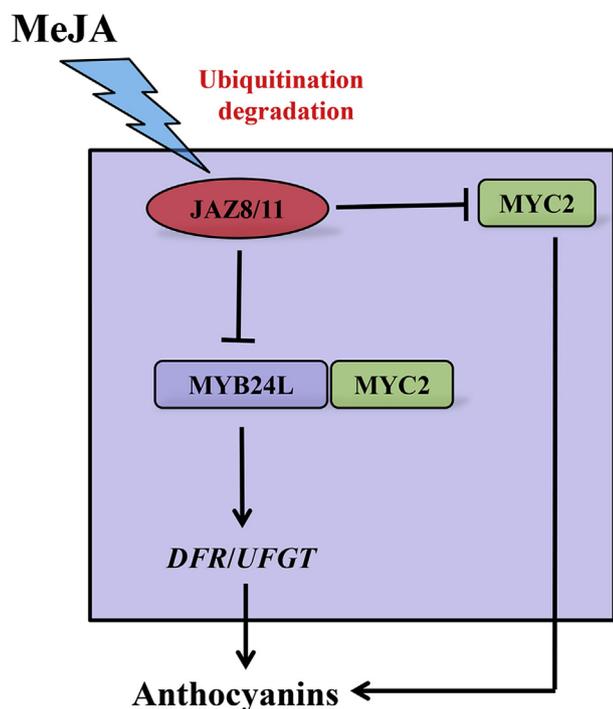
revealed that MdJAZ8 and MdJAZ11 can be degraded following a JA treatment. Thus, in apple, the application of JA can degrade MdJAZ8 and MdJAZ11 to release MdMYC2 and MdMYB24L, both of which are involved in anthocyanin biosynthesis.

To further characterize MdMYB24L functions during anthocyanin biosynthesis, *MdMYB24L* was overexpressed in 'Orin' calli. The *MdMYB24L*-overexpressing apple calli produced more anthocyanin than the wild-type control calli. Additionally, a qRT-PCR analysis indicated that the expression levels of anthocyanin biosynthesis genes, including *MdDFR*, *MdUFGT*, *MdCHI*, and *MdCHS*, were clearly up-regulated in the *MdMYB24L*-overexpressing transgenic lines. Moreover, the expression levels of the genes encoding MYB and bHLH TFs, which help regulate anthocyanin biosynthesis, were differentially up-regulated in transgenic calli, suggesting that the overexpression of *MdMYB24L* also promotes their transcription. However, the specific underlying mechanism will need to be elucidated in future studies.

The MYB TFs contain a DNA-binding motif that allows them to regulate the expression of downstream genes via interactions with MYB-binding sites in promoters (Wang et al., 2017; Xu et al., 2017). Our yeast one-hybrid experiments indicated that MdMYB24L binds only to the promoters of the anthocyanin structural genes *MdUFGT* and *MdDFR*. Additionally, our EMSA and ChIP assay results confirmed that

MdMYB24L specifically binds to the U2 and D1 sequences within the *MdDFR* and *MdUFGT* promoters, respectively. We also proved that MdMYC2 interacts with MdMYB24L to enhance the activation of the *MdUFGT* promoter, while MdJAZ8 and MdJAZ11 interact with and weaken the ability of MdMYB24L to activate the *MdDFR* and *MdUFGT* promoters. The interactions of MdJAZ8 and MdJAZ11 with MdMYB24L may inhibit the transcriptional function of the MYB-MYC2 complex. Therefore, JA promotes the accumulation of anthocyanins in apple, most likely through the JA signaling pathway, in which it helps degrade JAZ proteins. We also speculate that MdMYB24L indirectly up-regulates the expression of other structural genes (i.e., *MdCHI*, *MdCHS*, and *MdLDOX*) by inducing the transcription of *MdMYB1/9/11* and *MdbHLH3/33*.

In summary, the data presented herein may be useful for elucidating the molecular mechanism by which JA signaling regulates anthocyanin biosynthesis in apple (Fig. 7). Additionally, MdJAZ8 and MdJAZ11 can form a complex with MdMYB24L to attenuate the formation and transcriptional activity of the MYB-MYC2 complex and inhibit JA-mediated anthocyanin accumulation. In response to the application of JA, MdJAZ8 and MdJAZ11 are degraded and release MdMYC2 and MdMYB24L. In apple, MdMYC2 reportedly functions as a positive regulator of anthocyanin biosynthesis (An et al., 2016). Moreover,



**Fig. 7.** Proposed model for how MdMYB24L promotes anthocyanin biosynthesis in response to JA signals in apple. JA degrades MdJAZ8 and MdJAZ11 to release MdMYB24L and MdMYC2. In apple, MdMYC2 reportedly functions as a positive regulator of anthocyanin biosynthesis (An et al., 2016). Furthermore, MdMYB24L interacts with MdMYC2 and also directly activates the transcription of downstream genes (*MdDFR* and *MdUGFT*), thereby participating in the regulation of anthocyanin biosynthesis. The arrow indicates activation, whereas the T-bar indicates repression.

MdMYB24L can bind to MdMYC2 to form a bHLH-MYB complex that synergistically promotes JA-mediated anthocyanin accumulation. Furthermore, MdMYB24L can bind directly to the promoters of the anthocyanin structural genes *MdUGFT* and *MdDFR* to up-regulate expression, thereby further enhancing anthocyanin biosynthesis.

#### Conflicts of interest

All authors have no competing financial interests to declare.

#### Author contributions

Conceived and designed the experiments: Yicheng Wang, Wenjun Liu, and Xuesen Chen. Performed the experiments: Yicheng Wang, Huiyan Jiang, Zuolin Mao, Nan Wang, and Shenghui Jiang. Analyzed the data: Yicheng Wang and Zongying Zhang. Wrote the manuscript: Yicheng Wang, Haifeng Xu, and Guanxian Yang. All authors approved the contents of this manuscript.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://>

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