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EIN3-LIKE1, MYB1, and ETHYLENE RESPONSE FACTOR3 Act in a Regulatory Loop That Synergistically Modulates Ethylene Biosynthesis and Anthocyanin Accumulation[1][OPEN]

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Ethylene regulates climacteric fruit ripening, and EIN3-LIKE1 (EIL1) plays an important role in this process. In apple (*Malus domestica*), fruit coloration is accompanied by ethylene release during fruit ripening, but the molecular mechanism that underlies these two physiological processes is unknown. In this study, we found that ethylene treatment markedly induced fruit coloration as well as the expression of *MdMYB1*, a positive regulator of anthocyanin biosynthesis and fruit coloration. In addition, we found that MdEIL1 directly bound to the promoter of *MdMYB1* and transcriptionally activated its expression, which resulted in anthocyanin biosynthesis and fruit coloration. Furthermore, MdMYB1 interacted with the promoter of *ETHYLENE RESPONSE FACTOR3*, a key regulator of ethylene biosynthesis, thereby providing a positive feedback for ethylene biosynthesis regulation. Overall, our findings provide insight into a mechanism involving the synergistic interaction of the ethylene signal with the MdMYB1 transcription factor to regulate ethylene biosynthesis and fruit coloration in apple.

For many fleshy fruits, the fruit-ripening process is accompanied by dramatic changes in fruit characteristics, including color changes (degradation of chlorophylls and accumulation of pigments), degradation of starches, accumulation of soluble sugars and volatile compounds, as well as the release of ethylene (Klee and Giovannoni, 2011). Fruit ripening is crucial for cultivated fruits and determines the quality and storage ability of fruits. Therefore, it is important to elucidate the regulatory mechanisms of fruit ripening, which will provide information for improving fruit quality.

Ethylene is essential for the ripening of climacteric fruits, and a rapid burst of ethylene production and a rise in respiration occur at the transition point of fruit ripening (Theologis, 1992; Giovannoni, 2004). Ethylene biosynthesis and the signal transduction pathway have been studied intensively in many species, including tomato (*Solanum lycopersicum*) and apple (*Malus domestica*; Alexander and Grierson, 2002; Alonso and Stepanova, 2004; Lin et al., 2009; Klee and Giovannoni, 2011; Gapper et al., 2013; Grierson, 2013; Ireland et al., 2013; Li et al., 2016, 2017).

In plants, *S*-adenosyl Met is a precursor in ethylene biosynthesis and is first catalyzed to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS). Then, ACC is oxidized and converted to active ethylene by ACC oxidase (ACO; Yang and Hoffman, 1984; Wang et al., 2002). During the signal transduction process, ethylene activates the downstream signaling pathway through several components, including CONSTITUTIVE TRIPLE RESPONSE1 and ETHYLENE INSENSITIVE2 (EIN2). Subsequently, the primary responsive transcription factors (TFs), EIN3/EIN3-LIKEs (EILs), induce the secondary responsive TFs, ETHYLENE RESPONSE FACTORs (ERFs), which, in turn, activate the expression of downstream ethylene-responsive related genes, ultimately inducing the ethylene response (Alonso and Stepanova, 2004; Lin et al., 2009; Klee and Giovannoni, 2011; Gapper et al., 2013).

ACS and ACO proteins are essential for ethylene biosynthesis as well as fruit ripening and have been characterized extensively in tomato (Barry et al., 2000; Lin et al., 2009; Klee and Giovannoni, 2011; Gapper et al., 2013), apple (Sunako et al., 1999; Harada et al.,

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2000; Dandekari et al., 2004; Oraguzie et al., 2004; Dougherty et al., 2016), and other species. To date, a series of proteins that regulate ethylene biosynthesis and fruit ripening through ACSs and ACOs have been identified. In tomato, the MADS box protein RIPENING INHIBITOR promotes fruit ripening through transcriptionally activating the expression of *LeACS2* (Ito et al., 2008; Fujisawa et al., 2013). In banana (*Musa nana*), MaERF11 recruits HISTONE DEACETYLASE1 to suppress the expression of *MaACO1* (Han et al., 2016). In apple, MdMADS8 and MdMADS9 interact with the promoters of *MdACS1* and *MdACO1* and activate their expression, promoting ethylene production (Ireland et al., 2013). In apple, MdERF3 promotes, whereas MdERF2 represses, the expression of *MdACS1* (Li et al., 2016, 2017). Aside from transcriptional regulation, posttranslational modification also is important in the regulation of ethylene biosynthesis. The Bric-à-brac, Tramtrack, and Broadcomplex ubiquitin ligase ETHYLENE OVERPRODUCER1 targets several ACSs for degradation to negatively regulate ethylene biosynthesis (Wang et al., 2004). In addition, the phosphorylation of ACS6 by MPK6 represses 26S proteasome pathway-mediated degradation (Joo et al., 2008), whereas this phosphorylation is suppressed by PROTEIN PHOSPHATASE2A (Skottke et al., 2011). These findings suggest that both the transcriptional and posttranslational regulations are important cues that modulate ethylene synthesis.

EIN3 and its EIL homologs are key TFs that initiate the ethylene-mediated downstream transcriptional cascade (Chao et al., 1997; Solano et al., 1998). In Arabidopsis (*Arabidopsis thaliana*), the MAP kinase (MAPK/MPK) cascade signaling and Glc signaling pathways interact with EIN3 to regulate its phosphorylation and protein stability (Yanagisawa et al., 2003; Yoo et al., 2008). In addition, EIN3/EIL stability is tightly regulated by the EIN3-BINDING F-BOX PROTEIN1/2-mediated ubiquitin-proteasome degradation pathway (Guo and Ecker, 2003; Potuschak et al., 2003). As a central TF, EIN3 activates the expression of a wide range of downstream genes, including *ERF1*, *HOOKLESS1*, *C-REPEAT BINDING FACTOR*, *EPITHELIUM-SPECIFIC ETS1*, and others, to regulate hypocotyl elongation, apical hook formation, abiotic stress tolerance, and other ethylene response-related processes (Zhang et al., 2011; Shi et al., 2012; Shen et al., 2016). In banana, MaEIL5 interacts physically with NAM, ATAF1/2, CUC22, which may be involved in ethylene-mediated fruit ripening (Shan et al., 2012). In kiwifruit (*Actinidia chinensis*), AdEIL2 and AdEIL3 regulate the fruit-ripening processes by activating the transcription of *AdACO1* and stimulating ethylene production (Yin et al., 2010). Taken together, EIN3/EILs may emerge as key regulators in ethylene signaling transduction.

Anthocyanins are secondary metabolites that are widely distributed in flowers and fruits and attract animals for pollination and seed dispersal (Cipollini and Levey, 1997; Schaefer et al., 2004). They are synthesized via the phenylpropanoid pathway, and many synthesis-related catalytic enzymes have been identified in a variety of plant species, including PHENYLALANINE AMMONIA LYASE (PAL), CHALCONE SYNTHASE (CHS), CHALCONE ISOMERASE (CHI), FLAVANONE 3-HYDROXYLASE (F3H), DIHYDROFLAVONOL 4-REDUCTASE (DFR), ANTHOCYANIDIN SYNTHASE, and UDP-GLUCOSE/FLAVONOID 3-O-GLUCOSYL-TRANSFERASE (UFGT; Koes et al., 2005; Tanaka et al., 2008). The MYB/bHLH/WD40 complex has been recognized as central to the regulation of anthocyanin accumulation (Ramsay and Glover, 2005; Gonzalez et al., 2008; Hichri et al., 2011). MYB TF-regulated anthocyanin accumulation has been well studied in Arabidopsis (Borevitz et al., 2000), apple (Ban et al., 2007; Espley et al., 2007; Allan et al., 2008), pear (*Pyrus bretschneideri*; Yao et al., 2017), peach (*Prunus persica*; Zhou et al., 2016), and other species (Jaakola, 2013; Xu et al., 2015). In apple, the homologs of Arabidopsis PRODUCTION OF ANTHOCYANIN PIGMENT1/2 (PAP1/2), MdMYB1/10/A, are responsible for anthocyanin biosynthesis. They are known as positive regulators for anthocyanin biosynthesis and fruit coloration by directly targeting the downstream anthocyanin-associated genes (Ban et al., 2007; Espley et al., 2007; Allan et al., 2008).

In plants, a series of developmental and environmental factors, including internal hormones and external environments, influence anthocyanin biosynthesis (Lancaster and Dougall, 1992; Jaakola, 2013). Recent studies have shown that MYB TFs play important roles in the internal and external cue-modulated anthocyanin accumulation by both transcriptional and posttranscriptional regulation (Jaakola, 2013; Xu et al., 2015). For example, light regulates anthocyanin accumulation by modulating the expression levels and protein stabilities of the MYB TFs (Takos et al., 2006; Li et al., 2012; An et al., 2017a). Nitrate acts as a negative factor in regulating anthocyanin accumulation mainly by decreasing the expression of *PAP1/2* in Arabidopsis (Rubin et al., 2009). It is also well known that the expression of *MdMYB1* and its alleles is up-regulated in fruit ripening, accompanied by fruit coloration and ethylene release (Faragher and Brohier, 1984; Whale and Singh, 2007; Whale et al., 2008). However, further research is required to clarify how these anthocyanin-related MYB TFs are regulated by ethylene and other stimuli.

Apple is a climacteric fruit that exhibits a burst of respiration and ethylene production during fruit ripening after harvest (Faragher and Brohier, 1984; Song and Bangerth, 1996). Multiple ethylene-regulated proteins that are involved in fruit ripening have been identified by a proteomic approach (Zheng et al., 2013). In apple, it is crucial to clarify the regulatory mechanism of ethylene release and fruit coloration during fruit maturation, which determines the storage life and quality of the fruit. Previous studies have found that ethylene release is increased and fruit pigment is accumulated during fruit ripening (Faragher and Brohier, 1984; Whale and Singh, 2007; Whale et al., 2008), but less is known about whether cross talk exists between these two physiological processes. In this study, we found that ethylene

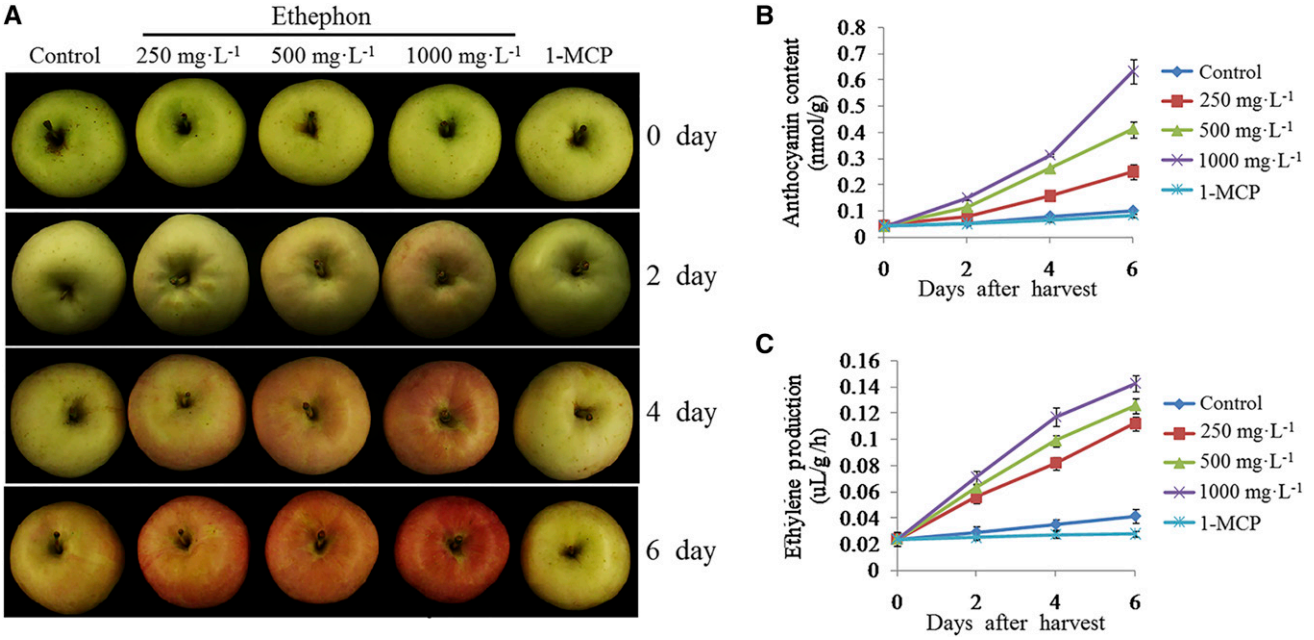

Figure 1. Ethephon induces anthocyanin accumulation in cv Red Delicious apples. A, The cv Red Delicious apples were grown in bags beginning at 30 DAFB. They were debagged at 120 DAFB and treated with the indicated concentrations of ethephon solutions (250, 500, and 1,000 mg L^{-1}) or 1-MCP (1 μL L^{-1}) and then were stored in a phytotron at 24°C with constant high light (70 μmol m^{-2} s^{-1}) for 6 d. Untreated fruits were used as the control. Representative images were taken. The assay was performed in three replicates. B and C, Measurements of anthocyanin content (B) and ethylene production (C) in ethephon- or 1-MCP-treated and control apples. The assay was performed in three replicates. The results represent means of these three replicates. Error bars indicate SD.

treatment markedly induced fruit coloration as well as the expression of *MdEIL1* and *MdMYB1*. MdEIL1 bound directly to the promoter of *MdMYB1* to increase the ethylene-mediated anthocyanin accumulation and fruit coloration. In addition, MdMYB1 also induced ethylene release by transcriptionally activating the expression of *MdERF3*, a positive regulator of ethylene biosynthesis, which accounts for more ethylene release in red-flesh apple fruits. Overall, these results provide new insights into the regulatory mechanism of the synergistic interaction of the ethylene signal with the MdMYB1 TF to regulate anthocyanin accumulation and fruit coloration.

RESULTS

Ethephon Treatment Promotes Anthocyanin Biosynthesis in Apple Fruits

A previous study showed that the onset of anthocyanin accumulation coincided with the start of rapid ethylene release during apple ripening (Faragher and Brohier, 1984). For an in-depth investigation of the role of ethylene in the coloration of apples, cv Red Delicious apples harvested 120 d after full bloom (DAFB) were treated with different concentrations of ethephon solutions (0, 250, 500, and 1,000 mg L^{-1}) or 1-methylcyclopropene (1-MCP, an ethylene inhibitor; 1 μL L^{-1}) and stored in a phytotron at 24°C with constant light (70 μmol m^{-2} s^{-1}) for 6 d. The anthocyanin contents and ethylene production of cv Red Delicious apples were then measured. Anthocyanin accumulation and ethylene production increased significantly with the ethephon treatment but were suppressed by the 1-MCP treatment (Fig. 1). These results indicated a positive association between ethylene production and anthocyanin synthesis and that the ethephon treatment contributed to anthocyanin accumulation in the apples.

In addition, the expression levels of the ethylene- and anthocyanin-associated genes were analyzed. The expression of ethylene synthetic genes (*MdACS1*, *MdACS3a*, *MdACS4*, *MdACS5a*, *MdACS5b*, and *MdACS6*), anthocyanin synthetic genes (*MdDFR* and *MdUFGT*), and the regulatory gene *MdMYB1* was induced by the ethephon treatment but was suppressed by the 1-MCP treatment (Fig. 2). These results suggested that ethylene promoted apple anthocyanin synthesis and fruit coloration through regulating the transcription of *MdMYB1* and anthocyanin synthetic genes.

MdMYB1 Is a Direct Target of MdEIL1

Considering that the transcription of *MdMYB1* was induced by ethylene (Fig. 2) and that MdMYB1 has an essential role in regulating anthocyanin synthesis and

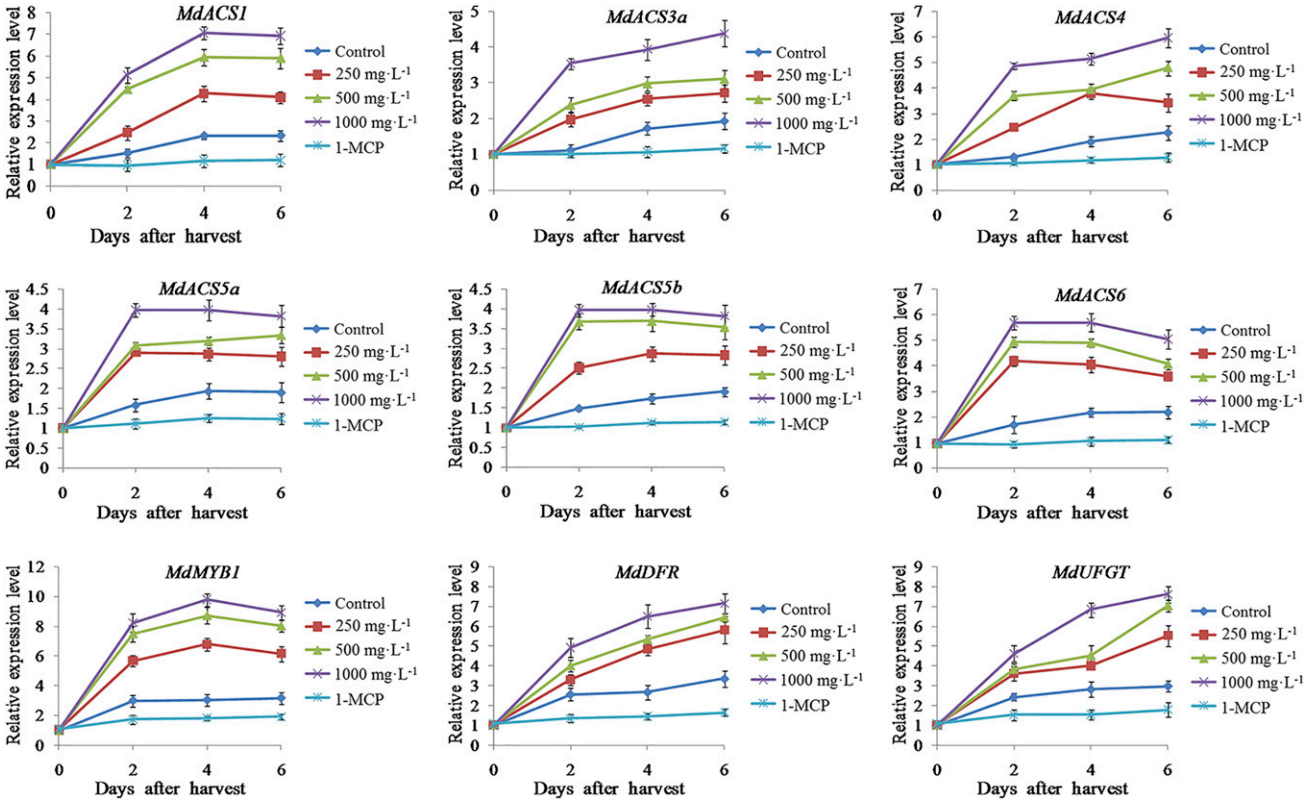

Figure 2. Expression analysis of ethylene and anthocyanin synthetic genes in ethephon- or 1-MCP-treated and control cv Red Delicious apples by reverse transcription-quantitative PCR (RT-qPCR). Ethylene biosynthetic genes are *MdACS1*, *MdACS3a*, *MdACS4*, *MdACS5a*, *MdACS5b*, and *MdACS6*, and anthocyanin biosynthetic genes are *MdMYB1*, *MdDFR*, and *MdUFGT*. The value for day 0 was set to 1. RT-qPCR was performed with three technical replicates and three biological replicates. The results represent means of these three replicates. Error bars indicate SD.

fruit coloration in apple (Takos et al., 2006; Espley et al., 2007), we speculated that ethylene affects anthocyanin biosynthesis by regulating the transcription of *MdMYB1*. To verify this hypothesis, we analyzed the potential binding sequences of ethylene-related genes, such as the dehydration-responsive element, the GCC box, and the ATGTA motif in the *MdMYB1* promoter region (Fujimoto et al., 2000; Liu et al., 2006; Zhang et al., 2011). Three ATGTA motifs (sites MdMYB1-1, MdMYB1-2, and MdMYB1-3) were found (Supplemental Fig. S1; Supplemental Table S1). To screen the potential ethylene-responsive element-binding factors, mixed biotin-labeled probes containing the three different ATGTA sequences were prepared for a DNA-affinity trapping assay. As shown in Figure 3A, when the total protein extracts were incubated with the mixed biotin-labeled probes, a blurry DNA-protein complex was observed. The band became clearer in the sample treated with ethephon, whereas it disappeared when treated with 1-MCP. Subsequently, the binding proteins were analyzed using mass spectrometry, and the candidate protein (GenBank accession no. MDP0000423881) bound to the ATGTA sequence was named MdEIL1 through multiple sequence alignment and phylogeny evolution analysis (Supplemental Fig. S2). *MdEIL1* had a similar expression pattern to *MdMYB1* in response to the ethephon and 1-MCP treatments (Fig. 3B), indicating an association between these two genes.

To verify whether MdEIL1 bound to the *MdMYB1* promoter, EMSAs were performed using an MdEIL1-HIS fusion protein. As shown in Figure 3C, MdEIL1 specifically bound to the MdMYB1-3 site. When unlabeled probes were added as competitors, the binding was reduced, whereas the band disappeared when the mutated probes were added (Fig. 3D). These results indicated that MdEIL1 bound to the ATGTA motif of the *MdMYB1* promoter in vitro.

To further confirm the binding of MdEIL1 to the *MdMYB1* promoter in vivo, a chromatin immunoprecipitation (ChIP)-PCR assay was conducted using *35S:MdEIL1-GFP* transgenic apple calli and the empty vector GFP transgenic apple calli as a control. The fragment containing MdMYB1-1 had higher enrichment in *MdEIL1* transgenic apple calli, suggesting that MdEIL1 bound to the promoter of *MdMYB1* in vivo (Fig. 3E).

In addition, a yeast one-hybrid (Y1H) assay was performed. The MdMYB1-3 sequence was cloned to

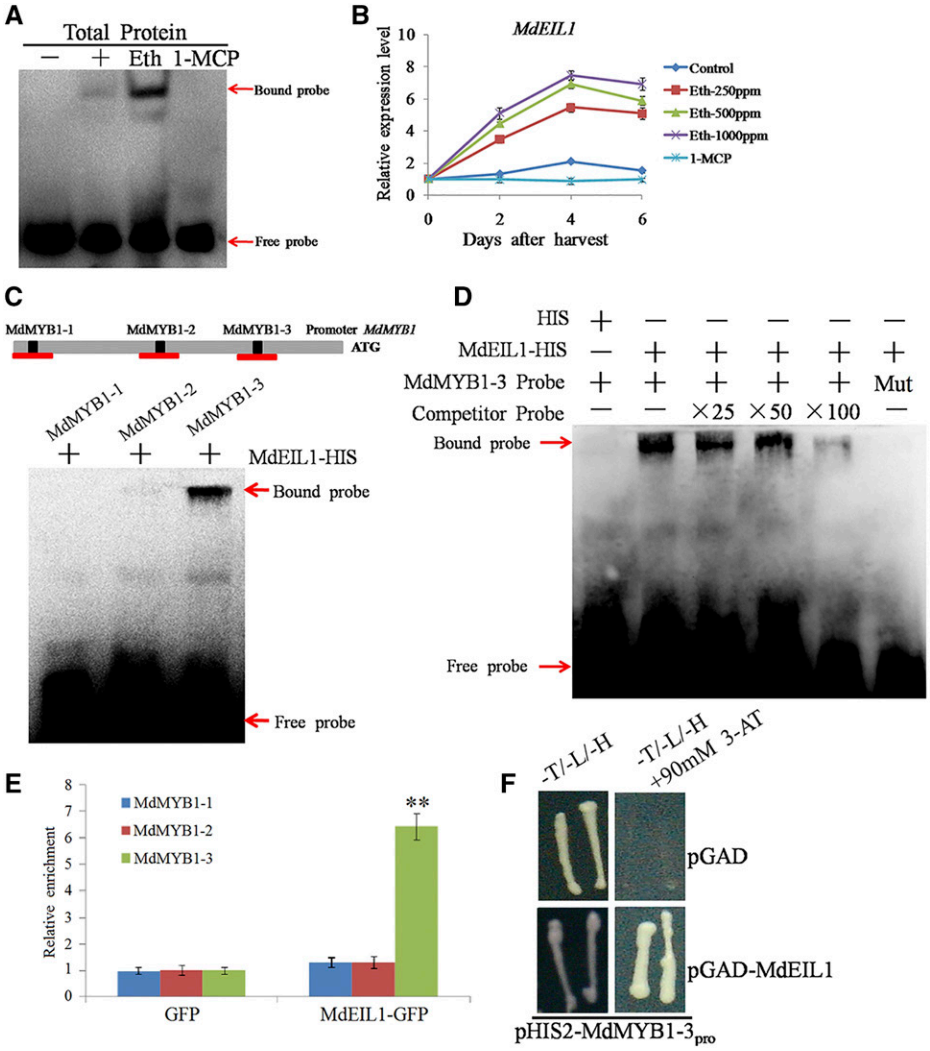

Figure 3. Binding of MdEIL1 to the *MdMYB1* promoter. A, Identification of the MdEIL1 protein that binds the cis-element of the *MdMYB1* promoter with an electrophoretic mobility shift assay (EMSA). The total proteins were extracted from ethephon- or 1-MCP-treated and untreated apple plants. – and + represented samples without or with the addition of total protein extracted from the untreated apple plants, respectively. Eth and 1-MCP represent samples with the addition of total protein extracted from the ethephon- or 1-MCP-treated apple plants, respectively. B, Expression analysis of *MdEIL1* in ethephon- or 1-MCP-treated and control apples by RT-qPCR. The value for day 0 was set to 1. RT-qPCR was performed with three technical replicates and three biological replicates. The results represent means of these three replicates. Error bars indicate SD. C, Top, schematic diagram of the *MdMYB1* promoter showing the potential MdEIL1-binding sites (MdMYB1-1, MdMYB1-2, and MdMYB1-3). The predicted ATGTA sequences are indicated by black boxes. Red lines represent the fragments amplified in the ChIP-PCR assay. Bottom, EMSA showing the MdEIL1-HIS fusion protein bound to the MdMYB1-3 site of the *MdMYB1* promoter and the free and bound DNAs separated on an acrylamide gel. D, EMSA showing that the MdEIL1-HIS fusion protein bound directly to the MdMYB1-3 site of the *MdMYB1* promoter. Unlabeled probes were used as competitors. Mut represents a mutated probe in which the ATGTA motif was replaced by CTTGC. E, ChIP-PCR assay of MdEIL1 binding to the promoter of the *MdMYB1* gene. Chromatin from the empty vector control (GFP) and *35S*:*MdEIL1-GFP* apple calli (MdEIL1-GFP) were immunoprecipitated with anti-GFP antibodies or without antibodies. Three regions (MdMYB1-1, MdMYB1-2, and MdMYB1-3) were examined by RT-qPCR. The enrichment of the wild type was set to 1. RT-qPCR was performed with three technical replicates and three biological replicates to examine the enrichment of *MdMYB1* fragments. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: **, $P < 0.01$. F, Y1H assay showing MdEIL1 interaction with the promoter of *MdMYB1*. The promoter fragment of *MdMYB1* (MdMYB1-3) was fused to the pHIS2 vector, and the *MdEIL1* gene was fused to the pGAD vector. The columns represent the addition of the pHIS2-MdMYB1-3_{pro} vector. The rows represent the addition of the pGAD and pGAD-MdEIL1 vectors.

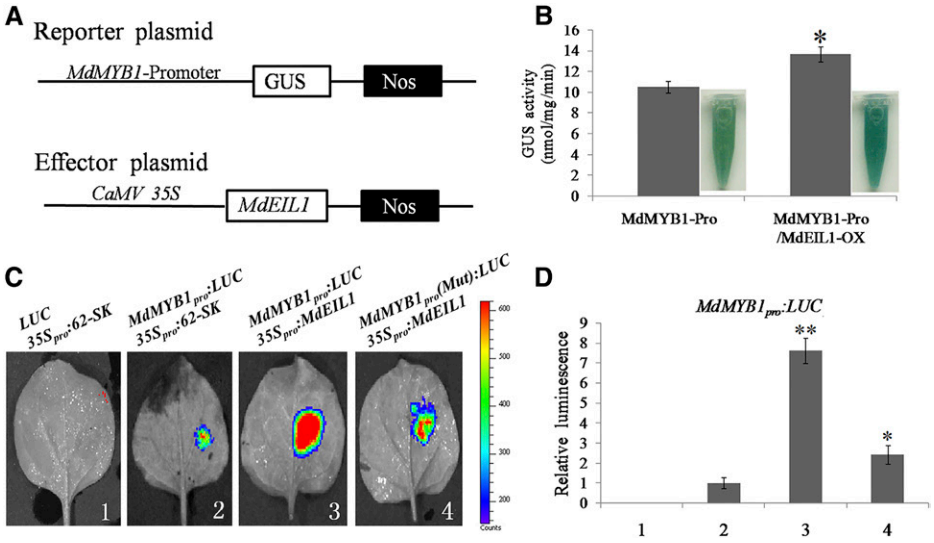

Figure 4. Regulation of the *MdMYB1* promoter by MdEIL1. A, Schematic representation of the GUS reporter vector containing the *MdMYB1* promoter and the effector vector containing MdEIL1. B, GUS staining analysis and GUS activity detection of *pMdMYB1-GUS* and *pMdMYB1-GUS/MdEIL1-OX* transgenic apple calli. *MdMYB1-Pro*, *MdMYB1-Promoter-GUS* transgenic apple calli; *MdMYB1-Pro/MdEIL1-OX*, *MdMYB1-Promoter-GUS* and *35S:MdEIL1-GFP* cotransformed apple calli. The GUS activity of *MdMYB1-Pro* was used as the reference. Transgenic apple calli were stained using GUS buffer. GUS staining analysis and GUS activity detection were performed in three replicates. The results represent means of these three replicates. Error bars indicate SD. The asterisk denotes Student's *t* test significance: *, $P < 0.05$. C, Transient expression assays showing MdEIL1 promotion of *MdMYB1* expression. The promoter fragment of *MdMYB1* was cloned into the pGreenII 0800-LUC vector to generate the reporter construct. The effector (35S$_{pro}$:MdEIL1) was generated by recombining the *MdEIL1* gene into the pGreenII 62-SK vector. In MdMYB1$_{pro}$(Mut), the ATGTA motif was replaced by CTTGC. D, Quantitative analysis of luminescence intensity. The value for column 2 (MdMYB1$_{pro}$:LUC-35S$_{pro}$:62-SK) was set to 1. The transient expression assay was performed in three replicates. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: *, $P < 0.05$ and **, $P < 0.01$.

a pHIS2 vector, and the coding sequence (CDS) of *MdEIL1* was inserted into the pGADT7 vector. The optimal concentration (90 mM) of 3-amino-1,2,4-triazole (3-AT; Supplemental Fig. S3) was used for the experiments. The results showed that the coexpressed yeast strains of pGAD-*MdEIL1* and pHIS2-*MdMYB1-3* were able to grow on a SD/-Trp/-Leu/-His/90 mM 3-AT plate, but no growth was observed on the control plate (Fig. 3F). These results provide evidence that MdEIL1 binds to the *MdMYB1* promoter.

MdEIL1 Positively Regulates the Expression of *MdMYB1*

A GUS transactivation assay was performed in apple calli to determine how MdEIL1 regulates the expression of *MdMYB1*. The 2,000-bp sequence of the *MdMYB1* promoter was cloned into the pBI101-GUS vector as an effector plasmid (MdMYB1-Pro), and the effector plasmid was then transformed into wild-type apple calli. The *35S:MdEIL1* construct was cotransformed into the *MdMYB1-Pro* transgenic apple calli (*MdMYB1-Pro/MdEIL1-OX*; Fig. 4A). GUS activity analysis demonstrated that the *MdMYB1-Pro/MdEIL1-OX* transgenic apple calli had higher GUS activity than MdMYB1-Pro alone (Fig. 4B), suggesting that MdEIL1 activated the transcription of *MdMYB1*.

To verify the transcriptional activation results, a transient transactivation assay was conducted in tobacco (*Nicotiana tabacum*) leaves. The CDS of *MdEIL1* was fused to the pGreenII 62-SK vector as an effector, and the promoter sequences of *MdMYB1* and the mutated *MdMYB1* promoter fragments were inserted into the pGreenII 0800-LUC vectors to generate reporters [*MdMYB1$_{pro}$* and *MdMYB1(Mut)$_{pro}$*, respectively; Supplemental Fig. S4]. *Agrobacterium tumefaciens* containing recombinant plasmids were mixed and coinjected into the tobacco leaves. The luminescence detection indicated that the coexpression of *35S$_{pro}$:MdEIL1* with *MdMYB1$_{Pro}$:LUC* resulted in much stronger luminescence compared with the controls, whereas *35S$_{pro}$:MdEIL1* was unable to induce the expression of *MdMYB1$_{Pro}$(Mut):LUC* (Fig. 4, C and D). These results indicated that MdEIL1 directly activated the expression of *MdMYB1*.

MdEIL1 Promotes Anthocyanin Biosynthesis and Fruit Coloration

Based on the results that MdEIL1 interacted with the *MdMYB1* promoter and activated its transcription (Figs. 3 and 4), we speculated that MdEIL1 may play a crucial role in regulating anthocyanin biosynthesis. Hence, the overexpression vector 35S:MdEIL1-GFP

was constructed and transformed into wild-type apple calli (Supplemental Fig. S5A), and two transgenic lines (MdEIL1#1 and MdEIL1#2) were selected for the following assays. The coloration assays showed that the overexpression of *MdEIL1* significantly increased the anthocyanin accumulation compared with the wild type (Supplemental Fig. S5, B and C). In addition, RT-qPCR was performed to test the expression levels of anthocyanin synthetic genes in wild-type and *MdEIL1* transgenic apple calli. Overexpression of *MdEIL1* up-regulated the expression of anthocyanin synthetic genes (*MdDFR*, *MdUFGT*, *MdF3H*, *MdCHI*, and *MdCHS*) and the regulatory gene *MdMYB1* (Supplemental Fig. S5D). To further characterize its function, 35S:MdEIL1-GFP was transformed into wild-type Arabidopsis, and three lines (MdEIL1-L1, MdEIL1-L2, and MdEIL1-L4) were selected. Coloration assays and RT-qPCR analysis demonstrated that MdEIL1 positively regulated anthocyanin synthesis in Arabidopsis by up-regulating anthocyanin synthesis-related genes, including *AtPAP1*, *AtPAP2*, *AtPAL1*, *AtDFR*, *AtUFGT*, *AtCHI*, and *AtCHS* (Supplemental Fig. S5, E–G).

Moreover, viral vector-mediated overexpression was conducted using 120-DAFB apples. MdEIL1-pIR vectors (IL60-1+MdEIL1-IL60-2) were generated, and pIR vectors (IL60-1+IL60-2) were used as a control. Compared with the empty vector control (pIR), overexpression of *MdEIL1* promoted anthocyanin accumulation in the apple skin around the injection sites (Fig. 5, A and B). The expression levels of *MdMYB1*, *MdDFR*, *MdUFGT*, *MdF3H*, *MdCHI*, and *MdCHS* were elevated in the MdEIL1-pIR injection areas compared with the controls (Fig. 5C). Therefore, these results demonstrated that MdEIL1 played a positive role in anthocyanin synthesis and fruit coloration.

MdEIL1 Promotes Anthocyanin Accumulation in an MdMYB1-Dependent Pathway

To examine whether MdEIL1 regulated MdMYB1-modulated anthocyanin synthesis and fruit coloration, wild-type, transgenic (*MdMYB1-OX*, *MdMYB1-Anti*, and *MdEIL1#1*), and cotransformed (*MdEIL1#1/MdMYB1-Anti*) apple calli were used for coloration assays. *MdMYB1* or *MdEIL1* overexpression promoted, while *MdMYB1* suppression inhibited, the synthesis of anthocyanin (Supplemental Fig. S6, A and B). Moreover, MdEIL1-promoted anthocyanin accumulation was inhibited significantly in the *MdEIL1#1/MdMYB1-Anti* apple calli (Supplemental Fig. S6, A and B). *MdEIL1*-induced up-regulation of anthocyanin synthetic genes was inhibited in the *MdEIL1#1/MdMYB1-Anti* apple calli (Supplemental Fig. S6C). These results indicated that MdEIL1 promoted the anthocyanin accumulation in an MdMYB1-dependent pathway.

To provide further evidence for MdEIL1-modulated fruit coloration by regulating MdMYB1, MdMYB1-TRV suppression vectors (TRV1+MdMYB1-TRV2) were obtained and TRV vectors (TRV1+TRV2) were used as a control. As shown in Figure 5, D and E, overexpression of *MdMYB1* or *MdEIL1* promoted anthocyanin accumulation in apple skin around the injection sites, whereas *MdMYB1* suppression inhibited anthocyanin accumulation. MdEIL1-promoted fruit coloration was inhibited significantly in the *MdEIL1-pIR/MdMYB1-TRV* coinjected sites, which was further confirmed by RT-qPCR analysis (Fig. 5F). These results implied that MdEIL1 might promote anthocyanin accumulation and fruit coloration in an MdMYB1-dependent pathway.

Red-Flesh Apples Promote Ethylene Production

In apples, red flesh occurs due to the constitutive expression of *MdMYB10*, an allele of MdMYB1 (Ban et al., 2007; Espley et al., 2007). *MdMYB10* transgenic apples emit a more volatile aroma (Espley et al., 2007), which is similar to the effects of ethylene (Song and Bangerth, 1996), indicating that MdMYB1 also may regulate ethylene synthesis and signal transduction in addition to being regulated by ethylene. To investigate this hypothesis, 140-DAFB red-flesh apples and non-red-flesh apples were used to detect anthocyanin contents and ethylene production. The results showed that the red-flesh apples had higher anthocyanin contents and ethylene production rates (Fig. 6, A–C). Ethylene production was inhibited significantly when red-flesh and non-red-flesh apples were treated with 1-MCP (Fig. 6C). Compared with the non-red-flesh apples, expression levels of anthocyanin synthetic and regulatory genes (*MdMYB1*, *MdDFR*, *MdUFGT*, *MdF3H*, *MdCHI*, and *MdCHS*) and ethylene synthetic and regulatory genes (*MdERF3*, *MdACS1*, *MdACS3a*, *MdACS4*, *MdACS5a*, *MdACS5b*, and *MdACS6*) were up-regulated extensively in red-flesh apples (Fig. 6, D and E). These results indicated that ethylene production rates were induced and determined by the MdMYB1 locus in red-flesh apples.

MdMYB1 Activates the Transcription of *MdERF3*

Elevated ethylene production rates in red-flesh apples led us to consider whether MdMYB1 was responsible for the production of ethylene. We first analyzed ethylene production and the expression levels of ethylene synthetic genes using the *MdMYB1* overexpression (MdMYB1-OX) and suppression (MdMYB1-Anti) apple calli. Overexpression of *MdMYB1* increased ethylene production and up-regulated the expression of ethylene synthetic genes (*MdERF3*, *MdACS1*, *MdACS3a*, *MdACS4*, *MdACS5a*, *MdACS5b*, and *MdACS6*) in MdMYB1-OX. In contrast, *MdMYB1* suppression decreased ethylene production and repressed the expression of these genes in the MdMYB1-Anti transgenic calli (Supplemental Fig. S7, A–C).

As an important MYB TF, MdMYB1 regulates target genes by binding the CAACGG/CCGTTG sequence in apples (Hu et al., 2016). Interestingly, a potential binding site was found in the *MdERF3* promoter (Fig. 7A;

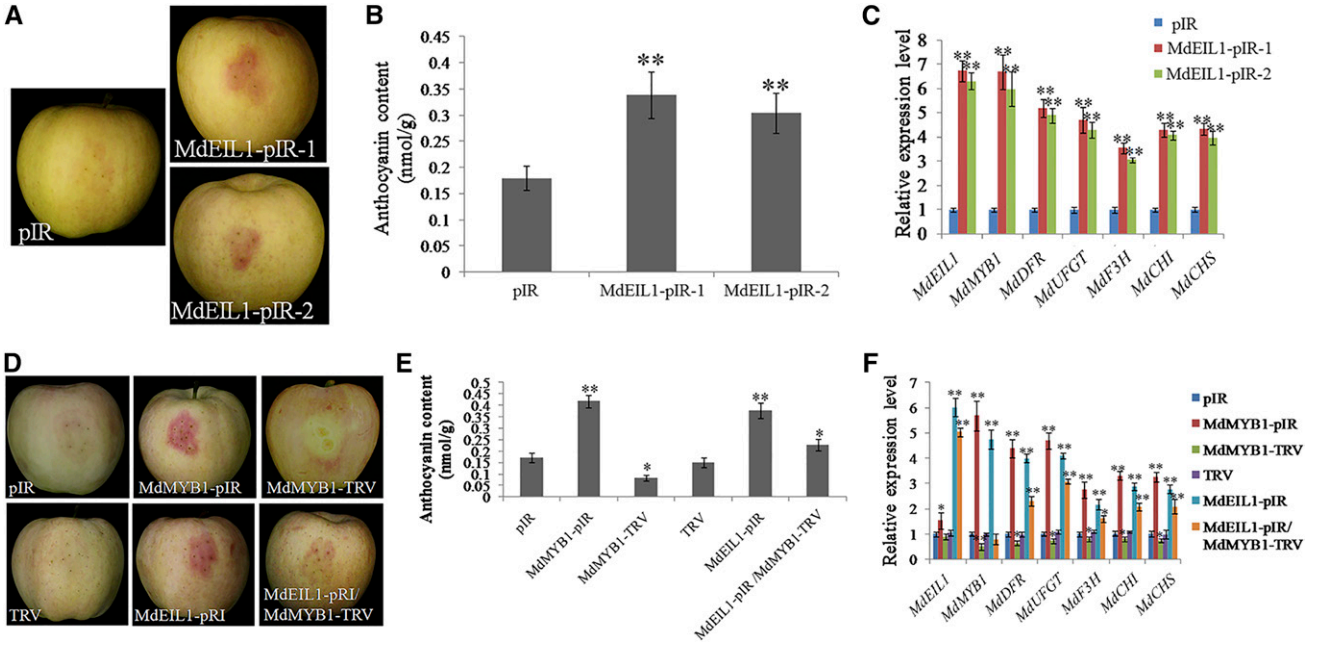

Figure 5. MdEIL1 promotes anthocyanin biosynthesis in an MdMYB1-dependent manner. Overexpression of *MdEIL1* promotes anthocyanin biosynthesis. A, Apple peel injection assays. At 120 DAFB, cv Red Delicious apples were debagged, injected with the mixed vectors, and stored in a phytotron at 15°C with constant high light (70 μmol m^{-2} s^{-1}) for 4 d. pIR, IL60-1+IL60-2; MdEIL1-pIR, IL60-1+MdEIL1-IL60-2. B and C, Detection of anthocyanin contents (B) and expression analysis of anthocyanin biosynthetic genes (*MdMYB1*, *MdDFR*, *MdUFGT*, *MdF3H*, *MdCHI*, and *MdCHS*; C) in fruit peels around the injection sites. The anthocyanin content of pIR was set as a control. The value for pIR was set to 1. The apple peel injection assay was performed in three replicates. RT-qPCR was performed with three technical replicates and three biological replicates. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: **, $P < 0.01$. Overexpression of *MdEIL1* promotes anthocyanin biosynthesis in an MdMYB1-dependent manner. D, Apple peel injection assays. At 120 DAFB, cv Red Delicious apples were debagged, injected with the mixed vectors or *A. tumefaciens* solutions, and stored in a phytotron at 15°C with constant high light (70 μmol m^{-2} s^{-1}) for 4 d. pIR, IL60-1+IL60-2; MdMYB1-pIR, IL60-1+MdMYB1-IL60-2; MdMYB1-TRV, TRV1+MdMYB1-TRV2; MdEIL1-pIR, IL60-1+MdEIL1-IL60-2; MdEIL1-pRI/MdMYB1-TRV, IL60-1+MdMYB1-IL60-2/TRV1+MdMYB1-TRV2. E and F, Detection of anthocyanin contents (E) and expression analysis of anthocyanin biosynthetic genes (*MdMYB1*, *MdDFR*, *MdUFGT*, *MdF3H*, *MdCHI*, and *MdCHS*; F) in fruit peels around the injection sites. The anthocyanin content of pIR was set as a control. The value for pIR was set to 1. The apple peel injection assay was performed in three replicates. RT-qPCR was performed with three technical replicates and three biological replicates. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: *, $P < 0.05$ and **, $P < 0.01$.

Supplemental Table S2). To evaluate whether MdMYB1 regulated ethylene production by binding to the promoter of *MdERF3*, a ChIP-PCR assay was conducted using *35S:MdMYB1-GFP* transgenic apple calli. The results demonstrated that *MdMYB1* overexpression enhanced the enrichment of the promoter of *MdERF3* (Fig. 7B), indicating that MdMYB1 actually bound to the *MdERF3* promoter in vivo.

To further verify the binding of MdMYB1 to the promoter of *MdERF3*, EMSA and Y1H experiments demonstrated that MdMYB1 bound directly to the *MdERF3* promoter in vitro (Fig. 7, C and D).

Subsequently, the transcriptional regulation of *MdERF3* by MdMYB1 was investigated using a transient transactivation assay in tobacco leaves. The results showed that coexpressed MdMYB1 plus the promoter of *MdERF3* increased luminescence, whereas MdMYB1 failed to activate *MdERF3* when replacing the CCGTTG motif with GGAGGT (Fig. 8, A and B). These results indicated that MdMYB1 activates the transcription of *MdERF3* by binding its promoter. In addition, the ethylene release by apple calli was tested. As shown in Figure 8C, overexpression of *MdMYB1* increased ethylene release, whereas the suppression of *MdERF3* in *MdMYB1-OX* calli significantly inhibited MdMYB1-modulated ethylene release, indicating that MdMYB1 promoted ethylene biosynthesis in an MdERF3-dependent pathway.

DISCUSSION

Ethylene is an important hormone in regulating fruit ripening, especially in climacteric fruit (Adams-Phillips et al., 2004; Oraguzie et al., 2004; Gapper et al., 2013; Li et al., 2017). Recently, a series of genes involved in fruit ripening were identified, most of which are ethylene-related genes, which are responsible for fruit

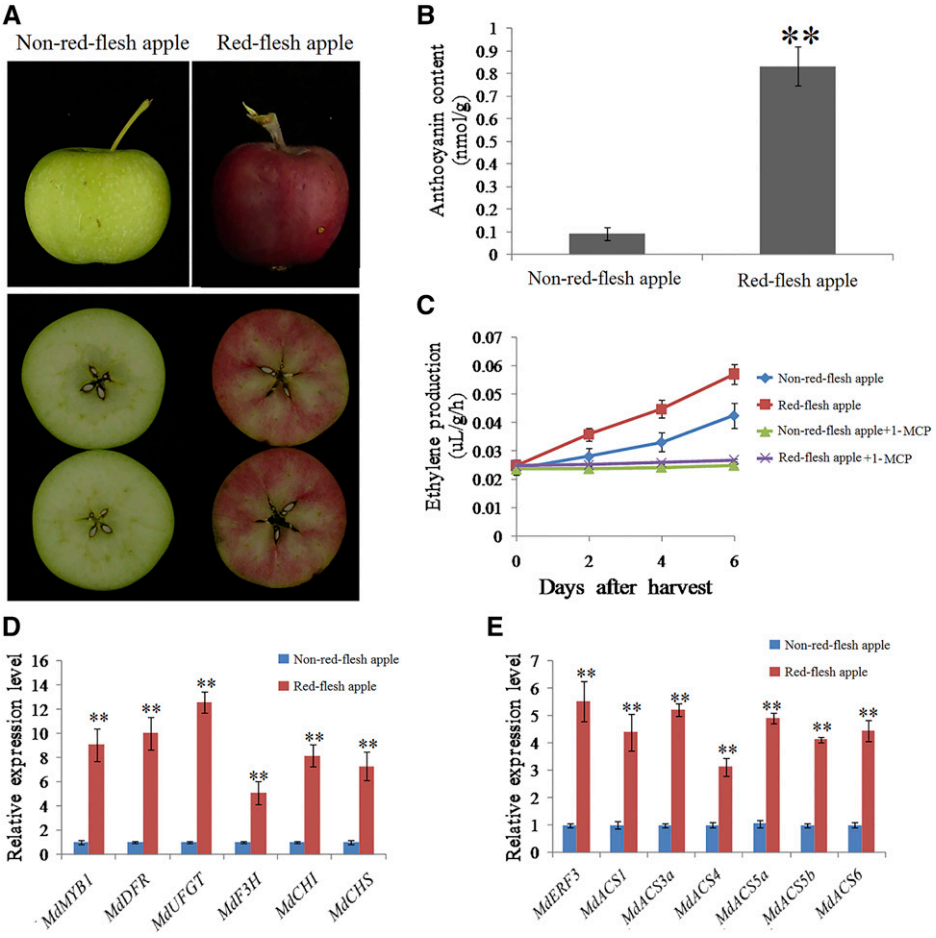

Figure 6. Analysis of anthocyanin content and ethylene production in red-flesh and non-red-flesh apples. A to C, Representative red-flesh and non-red-flesh apples (A) and measurements of anthocyanin content (B) and ethylene production (C). Apple cross-breeding groups (red-flesh and non-red-flesh apples) were harvested at 140 DAFB for anthocyanin measurements. They were treated or untreated with 1-MCP (1 μL L^{-1}) and stored at room temperature for 6 d for ethylene measurements. The anthocyanin content of non-red-flesh apples was used as the control. The assay was performed in three replicates. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: **, $P < 0.01$. D and E, Expression of anthocyanin biosynthetic genes (*MdMYB1*, *MdDFR*, *MdUFGT*, *MdF3H*, *MdCHI*, and *MdCHS*; D) and ethylene biosynthetic genes (*MdERF3*, *MdACS1*, *MdACS3a*, *MdACS4*, *MdACS5a*, *MdACS5b*, and *MdACS6*; E) in untreated red-flesh and non-red-flesh apples. The value for non-red-flesh apple was set to 1. RT-qPCR was performed with three technical replicates and three biological replicates. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: **, $P < 0.01$.

firmness (Goulao et al., 2008), pigmentation (Kondo et al., 2002), and other biological processes. In apples, ethylene treatment markedly increases the enzymatic activity of anthocyanin synthetic structural genes and anthocyanin accumulation during fruit ripening (Faragher and Brohier, 1984). However, the molecular mechanism of how ethylene promotes anthocyanin accumulation and fruit coloration during fruit ripening is unclear. In this study, we found that ethephon treatment markedly enhanced anthocyanin accumulation and fruit coloration. In addition, expression levels of *MdEIL1*, which encodes an important TF in the ethylene signaling pathway, and *MdMYB1*, a critical regulator in anthocyanin synthesis, were apparently induced by ethephon. Moreover, it was suggested that MdEIL1 plays a role in ethylene biosynthesis, anthocyanin accumulation, and fruit coloration mediated by MdMYB1.

MdEIL1 Interacts with the MdMYB1 Promoter to Regulate Ethylene-Modulated Anthocyanin Accumulation and Fruit Coloration

A previous study demonstrated that anthocyanin accumulation and fruit coloration are accompanied by rapid ethylene production during fruit ripening (Faragher and Brohier, 1984). However, less is known about how anthocyanin biosynthesis and fruit ripening

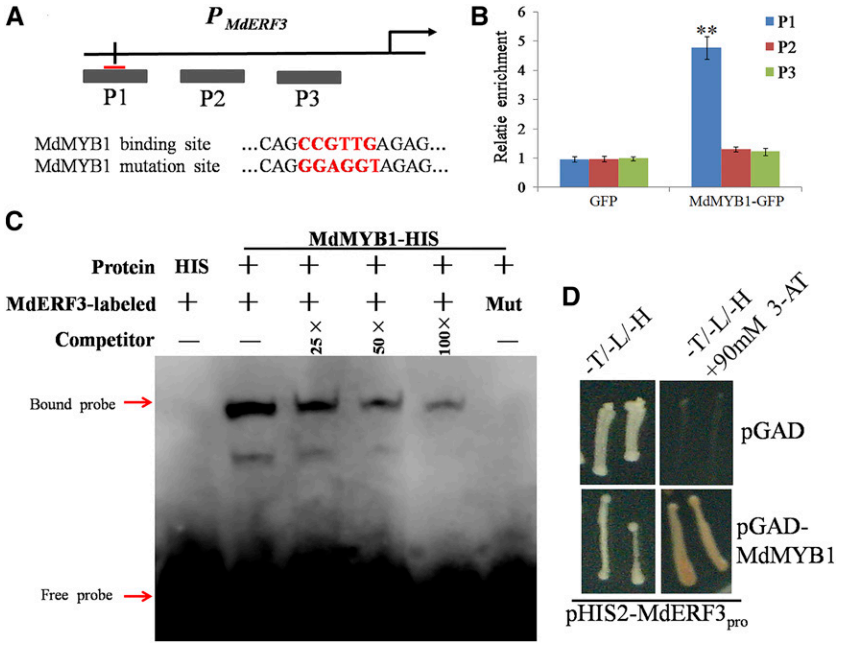

Figure 7. Binding of MdMYB1 to the *MdERF3* promoter. A, Schematic diagram of the *MdERF3* promoter showing the potential MdMYB1 binding sites. The predicted CCGTTG sequences are indicated by the black line. In the mutated probe (Mut), the CCGTTG motif was replaced by GGAGGT. B, ChIP-PCR assay of MdMYB1 binding to the promoter of the *MdERF3* gene. Chromatin from the empty vector control (GFP) and *35S:MdMYB1-GFP* apple calli (MdMYB1-GFP) were immunoprecipitated with and without anti-GFP antibodies. Three regions (P1, P2, and P3) were examined by RT-qPCR. The enrichment of GFP was set to 1. RT-qPCR was performed with three technical replicates and three biological replicates to examine the enrichment of *MdERF3* fragments. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: **, $P < 0.01$. C, EMSA results showing that the MdMYB1-HIS fusion protein bound directly to the *MdMYB1* promoter. Unlabeled probes were used as competitors. In the mutated probe (Mut), the CCGTTG motif was replaced by GGAGGT. D, Y1H assay showing MdMYB1 interaction with the *MdERF3* promoter. The promoter of *MdERF3* was fused to the pHIS2 vector, and the *MdMYB1* gene was fused to the pGAD vector. The columns represent the addition of the pHIS2-MdERF3$_{pro}$ vector. The rows represent the addition of the pGAD and pGAD-MdMYB1 vectors.

are regulated during fruit maturation. In grape (*Vitis vinifera*), ethylene triggers the expression of gene related to anthocyanin biosynthesis and enhances the color of grape skins (El-Kereamy et al., 2003). Our findings showed that ethylene treatment apparently induced anthocyanin biosynthesis and fruit coloration in apples and induced the transcription of *MdMYB1* and anthocyanin biosynthetic genes (Figs. 1A and 2), indicating that MdMYB1 may play a role in ethylene-modulated anthocyanin biosynthesis and fruit coloration.

As primary response regulators, EIN3/EIL proteins play important roles in ethylene-mediated plant growth and development (Chao et al., 1997; Solano et al., 1998; Zhang et al., 2011; Shen et al., 2016). Considering that the expression of *MdEIL1* was induced by ethylene, it is reasonable to speculate that *MdEIL1* may participate in ethylene-modulated anthocyanin biosynthesis and fruit coloration, which was proved through transgenic apple calli and Arabidopsis (Fig. 5, A–C; Supplemental Fig. S5). ATGTA is the core ethylene-responsive element that is essential for binding by EIN3 (Zhang et al., 2011), which also was present in the promoter sequence of *MdMYB1* (Fig. 3; Supplemental Fig. S1). Subsequently, a series of experiments demonstrated that MdEIL1 interacted directly with the MdMYB1 promoter and transcriptionally activated its expression rather than a protein interaction between MdEIL1 and MdMYB1 or other anthocyanin biosynthesis regulators (Figs. 3 and 4; Supplemental Figs. S3 and S8); this ultimately promoted anthocyanin accumulation and fruit coloration through an MdMYB1-dependent pathway (Fig. 5, D–F; Supplemental Fig. S6). These findings describe a molecular mechanism for the regulation of anthocyanin biosynthesis and fruit coloration by ethylene as mediated by the MdEIL1-MdMYB1 signal pathway.

A Positive Feedback Regulatory Loop Mediated by MdMYB1 in Ethylene Biosynthesis

In apples, MdMYB1 and its alleles have been identified as key regulators in anthocyanin biosynthesis and fruit coloration (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). Our previous research demonstrated that MdMYB1 also regulates malate accumulation by

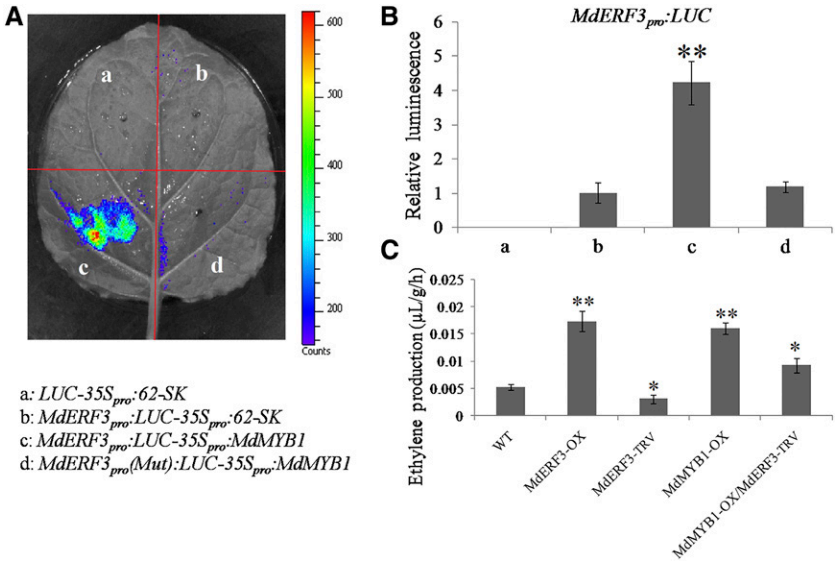

Figure 8. MdMYB1 positively regulates the expression of *MdERF3*. A, Transient expression assays showing that MdMYB1 promoted the expression of *MdERF3*. The promoter fragment of *MdERF3* was cloned into the pGreenII 0800-LUC vector to generate the reporter construct. The effector (35S$_{pro}$:MdMYB1) was generated by recombining *MdMYB1* into the pGreenII 62-SK vector. In MdERF3$_{pro}$(Mut), the CCGTTG motif was replaced by GGAGGT. B, Quantitative analysis of luminescence intensity. The value for column b (MdERF3$_{pro}$:LUC-35S$_{pro}$:62-SK) was set to 1. The transient expression assay was performed in three replicates. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: **, $P < 0.01$. C, Ethylene production of apple calli. Wild-type apple calli (WT), *MdERF3* overexpression calli (MdERF3-OX), *MdMYB1* overexpression calli (MdERF3-OX), *MdERF3-TRV* transient single-transgenic calli (MdERF3-TRV), and *MdERF3-TRV* transient calli in the background of *MdMYB1-OX* (MdMYB1-OX/MdERF3-TRV) were used for measurements of ethylene production. The ethylene production of the wild type was used as the reference. The assay was performed in three replicates. The results represent means of these three replicates. Error bars indicate SD. Asterisks denote Student's *t* test significance: *, $P < 0.05$ and **, $P < 0.01$.

directly facilitating the gene expression of a related tonoplast transporter (Hu et al., 2016). In addition to apples, the homologs of *MdMYB1* have been well studied in Arabidopsis (Borevitz et al., 2000) and other fruit tree species (Allan et al., 2008; Jaakola, 2013), especially in grapes (Kobayashi et al., 2004), peaches (Zhou et al., 2016), and pears (Yao et al., 2017), and were shown to act as positive regulators in anthocyanin accumulation and fruit coloration.

Recently, *PyMYB114*, a homolog of *MdMYB1*, was identified as a candidate responsible for the red skin color of Chinese pears. PyMYB114 interacted with the ERF/APETALA2 TF PyERF3 to coregulate anthocyanin biosynthesis and fruit coloration. The transcript abundance of PyERF3 was correlated significantly with that of PyMYB114 (Yao et al., 2017), indicating that, in addition to protein interactions, PyMYB114 transcriptionally regulated the expression of *PyERF3*. Interestingly, the previous study also found that, compared with the wild type, *MdMYB10* transgenic apple fruits exhibited a phenotype with a smaller fruit size and tendencies of cracking, which may be attributable to the hastening of the ripening; the fruits also emitted a more volatile aroma (Espley et al., 2007). These physiological phenotypes in *MdMYB10* transgenic apples are similar to the effects of ethylene during fruit ripening (Song and Bangerth, 1996), indicating a possible correlation between MdMYB10 and ethylene.

In this study, elevated ethylene production was found in the red-flesh apples (Fig. 6). A series of in vivo and in vitro experiments showed that MdMYB1 actually bound to the promoter of *MdERF3* and activated its expression, then positively regulated ethylene synthesis (Figs. 7 and 8). These data demonstrate the regulatory mechanism of the elevated ethylene production in the red-flesh apples and indicate a positive feedback regulatory loop mediated by MdMYB1 in ethylene synthesis. This positive feedback regulatory loop may bring about a rapid and remarkable upregulation of ethylene production, leading to the subsequent increase in ethylene- and signaling-modulated fruit ripening mediated by MdMYB1.

Role of MdEIL1-MdMYB1 Signaling in Plant Genetic Evolution and Biological Adaptation Modulated by Ethylene

To adapt to the environment, fruit trees have evolved a complex developmental process. During the initial stages of fruit development, the synthesis of ethylene is suppressed. A low level of ethylene inhibits the

softening of fruits and the degradation of chlorophyll. Ethylene synthesis is activated when the seeds mature, promoting fruit softening, soluble accumulation, and fruit coloration (Liu et al., 2015). The fully ripe fruits are edible and brightly colored to attract animals to spread more offspring (Shang et al., 2011), which is a perfect evolutionary strategy for fruit trees.

In this study, we found that, during fruit maturation, the ethylene-MdEIL1 signal promotes MdMYB1-mediated anthocyanin synthesis and fruit coloration and ethylene synthesis through the MdMYB1-MdERF3 pathway; this is followed by strengthening of the ethylene-MdEIL1 signal-mediated fruit ripening, which promotes seed dissemination and species reproduction. This signal cascade partially explains the regulation network for anthocyanin accumulation and fruit ripening mediated by ethylene. However, less is known about the activation of the ethylene signal during fruit ripening.

The Differential Expression of *MdMYB1* May Affect the MdEIL1-MdMYB1-MdERF3 Transcriptional Cascade

Apple is a typical climacteric fruit, showing a burst of respiration and increased ethylene production during fruit ripening. Our data showed that ethylene production promoted the transcription of *MdMYB1* and the accumulation of anthocyanin in the red-skinned apple, which partially requires the function of MdEIL1. However, ethylene production could not promote the accumulation of anthocyanin in the non-red-skinned apple, the underlying mechanism of which remains unknown.

Previous studies showed that the expression of *MdMYB1* in red-skinned apple is much higher than that in non-red-skinned apple (Takos et al., 2006). Different apple species have sequence polymorphisms in the *MdMYB1* promoter region, which are used to design molecular markers to differentiate between red-skinned and non-red-skinned apples (Takos et al., 2006; Yuan et al., 2014). We speculated that the polymorphism in the *MdMYB1* promoter region might affect MdEIL1 binding to the *MdMYB1* promoter, which leads to the differential expression of *MdMYB1* in the red-skinned and non-red-skinned apples. Recent studies reported that DNA methylation is involved in the regulation of the transcription of *MYB1* in apple and pear (Wang et al., 2013; Tian et al., 2017). Different methylation levels in the *MdMYB1* promoter region of striped apples result in different anthocyanin contents in red and green stripes, and green stripes have higher DNA methylation levels than red stripes (Telias et al., 2011). Interestingly, cv Granny Smith apple, a green-skinned apple cultivar, turns red quickly when removing bagging treatments during fruit ripening, which also is associated with the DNA methylation level in the *MdMYB1* promoter region (Zhang et al., 2013). Based on these studies, we speculate that the sequence polymorphism and DNA methylation level in the *MdMYB1* promoter region may affect the ethylene-regulated *MdMYB1* expression and anthocyanin accumulation, which contributes to the formation of red-skinned and non-red-skinned apples. However, this hypothesis needs further investigation.

The Interplay between the Ethylene Signal and MdMYB1 in the Regulation of Ethylene Biosynthesis, Anthocyanin Accumulation, and Fruit Coloration

Taking into account the observations in this study and the results of existing research on the regulation of the ripening process (Giovannoni, 2004; Lin et al., 2009; Klee and Giovannoni, 2011; Gapper et al., 2013), we propose the underlying mechanism that regulates the ethylene release and the fruit coloration during fruit ripening (Fig. 9). In this model, at the onset of fruit ripening, the ethylene signal is activated, which then induces the expression and protein stability of MdEIL1; MdEIL1 then interacts with the promoter of MdMYB1 and transcriptionally activates its expression. The activated MdMYB1 regulates anthocyanin accumulation and fruit coloration by regulating the expression of genes in the anthocyanin biosynthetic pathway. In addition to pigment accumulation, MdMYB1 regulates ethylene release by inducing the transcription of *MdERF3*, a critical regulator in ethylene biosynthesis, thus regulating ethylene synthesis and further strengthening the ethylene-mediated anthocyanin accumulation and fruit coloration.

MdMYB1 is a TF that has multiple internal and external responses in addition to ethylene (Takos et al., 2006; Li et al., 2012, 2017; An et al., 2017a; Zhou et al., 2017). Thus, an elaborate regulatory network between ethylene signaling and other cues mediated by MdMYB1 is central to fruit ripening. It is also important to identify other factors that participate in MdEIL1-MdMYB1-mediated ethylene biosynthesis and fruit coloration to unveil the regulatory mechanism between ethylene and MdMYB1 during fruit ripening. Overall, our findings provide insight into the transcriptional regulatory mechanism of the synergistic interaction of the ethylene signal with the MdMYB1 TF to regulate ethylene synthesis and fruit coloration. These results provide important information for fruit breeding with regard to improved coloration, softening, and storage ability.

MATERIALS AND METHODS

Fruit Materials and Treatments

'Red Delicious' apples (*Malus domestica*) were used for the ethephon and 1-MCP treatments, as well as the injection assays. For the ethephon treatment of the fruits, 120-DAFB apples were dipped into different concentrations (0, 250, 500, and 1,000 mg L^{-1}) of the ethephon solutions for 1 min. After drying naturally, they were kept in individual and closed containers. The containers were stored in a phytotron at 24°C with constant light (70 µmol m^{-2} s^{-1}) for 6 d. For the 1-MCP treatment, the apples were exposed to 1 µL L^{-1} 1-MCP for 12 h at room temperature in an air-tight container. After the 1-MCP treatment, the air-tight container was transferred to a phytotron at 24°C with constant

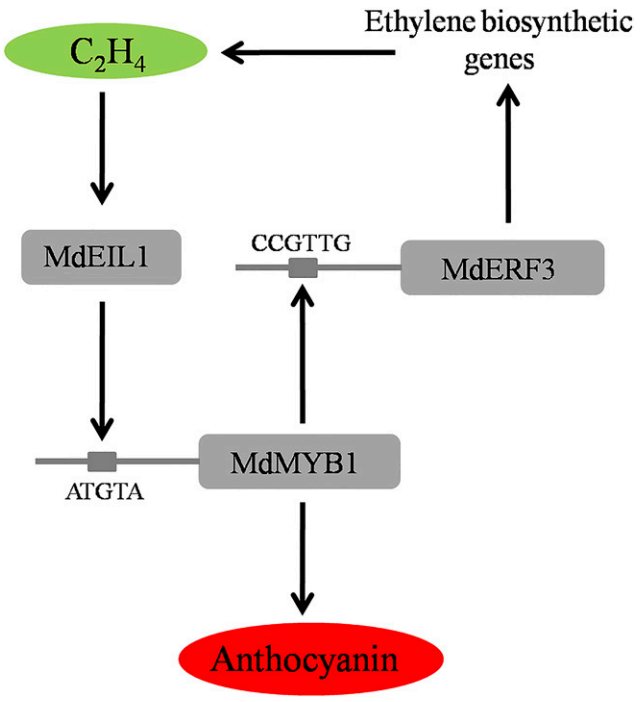

Figure 9. Proposed model of the mechanism regulating anthocyanin accumulation and ethylene release during fruit ripening. MdEIL1 binds directly to the promoter of *MdMYB1* and enhances its action, leading to increased anthocyanin accumulation. In addition, MdMYB1 binds to the promoter of *MdERF3* and activates its expression, resulting in enhanced ethylene production. C_2H_4, Ethylene; ATGTA, MdEIL1-binding sequence; CCGTTG, MdMYB1-binding sequence. Solid arrows show positive regulation.

light (70 μmol m^{-2} s^{-1}) for 6 d. During the treatments, anthocyanin contents and ethylene production were measured at certain times. The two groups of fruits were treated simultaneously, and the tests were conducted three times.

Measurements of Anthocyanin Contents and Ethylene Production

The apples were peeled and collected after treatments. The isolated apple peels were dipped into anthocyanin extraction solution (95% [v/v] anhydrous ethanol:1.5 mol L^{-1} HCl, 17:3) for 24 h in darkness. A spectrophotometer was used for the absorbance measurements at 530, 620, and 650 nm. The results were determined based on the following equation: optical density (OD) = $(A_{530} - A_{620}) - [0.1 \times (A_{650} - A_{620})]$ (An et al., 2017a).

The cv Orin apple calli for coloration assays and injection areas for the fruit injection assays were collected for measurements of anthocyanin. The ethylene production of the apples and apple calli was determined as described by Tan et al. (2013) and Li et al. (2016), respectively. The apple fruits or apple calli were placed in individual containers and stored in a phytotron (24°C). One-milliliter syringes were used for the collection of gas samples. The antisense viral vector MdERF3-TRV2 was obtained by cloning the CDS of *MdERF3* into the TRV2 vector. The TRV1 vector was used as an auxiliary plasmid. The TRV vectors were introduced into *Agrobacterium tumefaciens* LBA4404. The mixed vectors and *A. tumefaciens* solutions were transformed into the apple calli by a vacuum air pump. The MdERF3-TRV transient transformation of the apple calli was used for the measurements of ethylene production. All experiments were repeated at least three times.

RT-qPCR Analysis

Apple peels and apple calli were used for RNA extraction using the RNA Plant Plus reagent (Tiangen; An et al., 2017a). The ethephon-treated apple peels and the apple peels around the injection area were collected using a peeler. The expression levels of the anthocyanin synthetic genes and ethylene synthetic genes were tested using specific primers that are shown in Supplemental Table S3.

Generation of Transgenic Apple Calli and Transgenic Arabidopsis

The overexpression vectors MdEIL1-GFP and MdMYB1-GFP were generated by cloning the CDSs of *MdEIL1* and *MdMYB1* into the transformed vector pCAMBIA1300-GFP. The suppression vector MdMYB1-Anti was generated by cloning the DNA fragment of the *MdMYB1* reverse complement into pCAMBIA1300. The individual pCAMBIA1300-GFP was used as a control.

The transgenic apple calli (MdEIL1-OX, 35S:MdEIL1-GFP; MdMYB1-OX, 35S:MdMYB1-GFP; MdMYB1-Anti, 35S:MdMYB1-Anti; and GFP, the empty vector control) were obtained by *A. tumefaciens*-mediated genetic transformation. The wild-type apple calli and *A. tumefaciens*-inserted recombined vectors were incubated for 30 min at 24°C. Subsequently, the transformed apple calli were screened in a selective medium containing antibiotics (An et al., 2017a).

The transgenic Arabidopsis (*Arabidopsis thaliana*) plants were obtained by the floral dip transformation method (Clough and Bent, 1998).

EMSA

The fusion proteins of MdEIL1-HIS and MdMYB1-HIS were generated through prokaryotic expression in vitro. The CDSs of *MdEIL1* and *MdMYB1* were cloned into the PET32a vector containing a His (HIS) target to generate recombined vectors. Then, these recombined vectors were transformed into *Escherichia coli* BL21 (DE3). Three millimolar isopropyl *β*-D-1-thiogalactopyranoside was used to induce protein production. The fusion proteins were purified using the ProFound PolyHIS Protein Kit (Thermo).

The 3′ biotin-labeled probes and the LightShift Chemiluminescent EMSA Kit (Thermo) were prepared for the subsequent EMSA. Briefly, the fusion proteins and biotin-labeled probes were mixed in a binding buffer for 20 min at 24°C. The HIS protein was used as a negative control, and unlabeled probes were used for probe competition (An et al., 2017a).

ChIP-PCR Assays

ChIP-PCR assays were performed as described by An et al. (2017b). The transgenic apple calli *MdEIL1-OX* and *MdMYB1-OX* containing the GFP targets were applied to the ChIP-PCR assays using the EpiTect ChIP OneDay Kit (Qiagen). The empty vector pCAMBIA1300-GFP-overexpressing apple calli (GFP) was used as a control. A GFP-specific antibody was used in this study. The enriched DNA fragments were examined by qPCR using the primers shown in Supplemental Table S3.

Y1H Assays

The CDSs of *MdEIL1* and *MdMYB1* were inserted into the pGADT7 vector to generate the recombined constructs pGAD-MdEIL1 and pGAD-MdMYB1, and the promoter fragments of *MdMYB1* (MdMYB1-3) and *MdERF3* were cloned into the pHIS2 vector. The cotransformed Y2H Gold yeast strains were plated on -Trp/-Leu/-His medium, supplementing an appropriate concentration of 3-AT.

GUS Activity Detection

The reporter plasmid was obtained (MdMYB1-Pro) by cloning the promoter fragment of *MdMYB1* into pBI101-GUS. MdEIL1-pCAMBIA1300-GFP was used as an effector plasmid. The reporter plasmid and effector plasmid were introduced into the wild-type apple calli separately or together.

GUS staining was conducted using a GUS staining buffer. The apple calli were incubated with the GUS staining buffer (1 mM 5-bromo-4-chloro-3-indolyl-*β*-GlcA, 0.1 mM EDTA, 0.5 mM ferroyanide, and 0.1% [v/v] Triton X-100, pH 7) at 37°C in the dark. For the quantitative analysis of GUS activity, approximately 0.5 g of the apple calli was extracted with 1 mL of extraction buffer (50 mM $NaHPO_4$, 10 mM *β*-mercaptoethanol, 10 mM Na_2EDTA, and 0.1% [v/v] Triton X-100, pH 7). The total protein concentration was determined

using the RC DC Protein Assay Kit (Bio-Rad). The extract was added to the GUS reaction buffer and was incubated at room temperature. After the reaction had proceeded for 0, 5, 10, 15, 30, and 60 min, the reaction mixture was added to the stop solution (1 M sodium carbonate). Fluorescence was measured using a spectrofluorometer at 365 and 450 nm.

Transient Expression Assays

The transient expression assays were performed using tobacco (*Nicotiana tabacum*) leaves (An et al., 2017a). The promoter fragments of *MdMYB1* and *MdERF3* were cloned into the pGreenII 0800-LUC vectors to generate the reporter constructs. The effectors ($35S_{pro}$:MdEIL1 and $35S_{pro}$:MdMYB1) were generated by recombining the *MdEIL1* and *MdMYB1* genes into the pGreenII 62-SK vector. The recombinant plasmids were transformed into *A. tumefaciens* LBA4404. The bacteria were mixed and coinjected into the tobacco leaves. A living imaging apparatus was used for luminescence detection.

Coloration Assays of Apple Calli

Wild-type and transgenic apple calli were subcultured in a medium and stored in a phytotron at 24°C with constant light (70 µmol m^{-2} s^{-1}). The treated apple calli were collected for measurements of anthocyanin contents and the detection of gene expression.

Apple Injection Assays

Fruit injection assays were carried out as described previously (Li et al., 2012). The overexpression viral vectors MdEIL1-IL60-2 and MdMYB1-IL60-2 were generated by inserting the CDSs of *MdEIL1* and *MdMYB1* into the IL60-2 vector. The IL60-1 vector was used as an auxiliary plasmid. The antisense viral vector MdMYB1-TRV2 was obtained by cloning the CDS of *MdMYB1* into the TRV2 vector. The TRV1 vector was used as an auxiliary plasmid. The TRV vectors were introduced into *A. tumefaciens* LBA4404. The mixed vectors and the *A. tumefaciens* solutions were injected into the fruit peels. Then, the fruits were stored in a phytotron at 24°C with constant light (70 µmol m^{-2} s^{-1}) for coloration.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: MdEIL1 (MDP0000423881), MdERF3 (MDP0000787281), MdACS1 (MDP0000370791), MdACS3a (MDP0000145123), MdACS4 (MDP0000262872), MdACS5a (MDP0000923426), MdACS5b (MDP0000435100), MdACS6 (MDP0000133334), MdMYB1 (MDP0000259614), MdDFR (MDP0000494976), MdUFGT (MDP0000405936), MdF3H (MDP0000323864), MdCHI (MDP0000759336), MdCHS (MDP0000686666), AtPAP1 (AT1G56650.1), AtPAP2 (AT1G66390.1), AtPAL (AT2G37040.1), AtDFR (AT5G42800.1), AtUFGT (AT5G54060.1), AtCHI (AT3G55120.1), and AtCHS (AT5G13930.1).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Schematic diagram of the *MdMYB1* promoter showing the potential MdEIL1-binding sites (MdMYB1-1, MdMYB1-2, and MdMYB1-3).

Supplemental Figure S2. Phylogenetic analysis and protein sequence alignment of MdEIL1 with Arabidopsis EIN3/EILs.

Supplemental Figure S3. pHIS2-MdMYB1-3_{pro} and pHIS2-MdERF3_{pro} transformed into Y187 yeast strains to screen for optimal 3-AT concentration for reporter gene inhibition.

Supplemental Figure S4. Schematic representation of the LUC reporter vector containing the indicated fragment of *MdMYB1* or *MdERF3* promoters and the effector vector containing *MdEIL1* or *MdMYB1* genes.

Supplemental Figure S5. Overexpression of *MdEIL1* in apple calli and Arabidopsis increases anthocyanin content.

Supplemental Figure S6. MdEIL1 promotes anthocyanin synthesis in an MdMYB1-dependent manner.

Supplemental Figure S7. MdMYB1 regulates ethylene response.

Supplemental Figure S8. MdEIL1 does not interact with MdMYB1, MdbHLH3, and MdbHLH33 in yeast two-hybrid assays.

Supplemental Table S1. MdEIL1 binding motif in the *MdMYB1* promoter.

Supplemental Table S2. MdMYB1 binding motif in the *MdERF3* promoter.

Supplemental Table S3. Primers used for gene expression analysis and vector construction in this study.

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