Ethylene precisely regulates anthocyanin synthesis in apple via a module comprising MdEIL1, MdMYB1, and MdMYB17

Shuo Wang¹,², Li-Xian Li¹,², Zhen Zhang¹,², Yue Fang¹,², Dan Li¹,², Xue-Sen Chen¹,²*, Shou-Qian Feng¹,²*

1 College of Horticulture Science and Engineering, Shandong Agricultural University, Tai’an, Shandong 271018, China
2 State Key Laboratory of Crop Biology, Tai’an, Shandong 271018, China

*Author for correspondence: Xue-Sen Chen, Shou-Qian Feng
Tel: +86 0538 8249338
Email: chenxs@sdau.edu.cn, shouqianlove@sdau.edu.cn

running title:
MdEIL1, MdMYB1 and MdMYB17 control anthocyanin synthesis

© The Author(s) 2022. Published by Oxford University Press. All rights reserved. This is an Open Access article distributed under the terms of the Creative Commons Attribution License http://creativecommons.org/licenses/by/4.0/, which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.
Abstract
Ethylene regulates anthocyanin synthesis in ripening apple fruit via the antagonistic activities of the R2R3-MYB repressors and activators. However, the molecular mechanism underlying this process remains unknown. In this study, ethylene significantly induced the expression of the R2R3-MYB gene MdMYB17 in apple fruit. Moreover, MdMYB17 was revealed to be an important repressor of anthocyanin synthesis. Specifically, MdMYB17 binds directly to the promoters of the ethylene-induced genes MdMYB1 and MdEIL1, which encode positive regulators of anthocyanin synthesis, and represses their expression. Additionally, MdMYB1 and MdEIL1 bind to the MdMYB17 promoter to activate its expression. Thus, MdMYB17, MdMYB1, and MdEIL1 form a regulatory module that controls the expression of the corresponding genes. MdMYB17 interacts with MdEIL1. The interaction between MdMYB17 and MdEIL1 attenuates the regulatory effects of MdMYB17 on MdMYB1 and MdEIL1 as well as the regulatory effects of MdEIL1 on MdMYB17. Overall, our results reveal the molecular mechanisms by which MdMYB17, MdMYB1, and MdEIL1 finely mediate ethylene-regulated anthocyanin synthesis in apple fruit.

Keywords: apple, ethylene, MdEIL1, MdMYB1, MdMYB17, anthocyanin

Introduction
Anthocyanins, which are flavonoids that are widely distributed in plants, have important biological functions. For example, they are responsible for the coloration of tissues and organs, while also helping to mediate pollination and reproduction and enhancing plant stress resistance\(^1,4\). The biosynthesis of anthocyanins begins in the phenylalanine pathway and is catalyzed by a series of enzymes encoded by structural genes, including phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanin synthase, and UDP-glucose/flavonoid 3-O-glucosyltransferase (UFGT)\(^5\). The expression of these structural genes is regulated by the MYB–bHLH–WDR protein complex, which is composed of an R2R3-MYB transcription factor (TF), a bHLH TF, and a WD repeat protein\(^6,7\). The R2R3-MYB TFs are key regulators of anthocyanin synthesis and the spatiotemporal expression of the related structural genes. The anthocyanin-related R2R3-MYB TFs have been extensively studied in plants\(^8\).
The R2R3-MYB TFs form one of the largest TF families in plants. The 126 R2R3-MYB TFs identified in *Arabidopsis* have been divided into 22 subgroups on the basis of their structures. The R2R3-MYB TFs in subgroups 4 and 6 play important roles in the regulation of anthocyanin synthesis. More specifically, the R2R3-MYB TFs in subgroup 6 positively regulate anthocyanin synthesis, whereas the R2R3-MYB TFs in subgroup 4 negatively regulate anthocyanin synthesis. Many homologs of the genes encoding the R2R3-MYB TFs in subgroups 4 and 6 have been identified in other plants; the functions of these genes are highly conserved among plant species. For example, in apple, *MdMYB1* (subgroup 6) encodes a key activator of anthocyanin synthesis, whereas *MdMYB16* (subgroup 4) encodes a repressor of anthocyanin synthesis.

Anthocyanin synthesis is influenced by multiple developmental and environmental factors. Recent studies revealed that R2R3-MYB TFs regulate anthocyanin synthesis by responding to endogenous and exogenous signals. High temperatures and nitrate contents inhibit anthocyanin synthesis by decreasing the expression of R2R3-MYB activators of anthocyanin synthesis. In contrast, exposures to light, low temperatures, ethylene, and jasmonic acid promote anthocyanin synthesis by inducing the expression of R2R3-MYB activators. In a recent investigation, Ni et al. (2021) showed that ethylene inhibits anthocyanidin synthesis in pear fruit by down-regulating the expression of the R2R3-MYB activators *PpMYB10* and *PpMYB114* and up-regulating the expression of the R2R3-MYB repressor *PpMYB140*. Therefore, the regulatory effects of ethylene on anthocyanin synthesis vary significantly among species and will need to be further explored.

Ethylene is an important hormone that regulates fruit ripening. In climacteric fruits, a sudden increase in respiration intensity accompanied by a peak in ethylene release during the ripening period rapidly accelerates fruit ripening. Dramatic changes in fruit characteristics occur, including color changes (anthocyanin accumulation), starch degradation, and the accumulation of soluble sugars and aromatic compounds. Ethylene biosynthesis and the associated signaling pathways have been thoroughly studied in the model plants tomato and Arabidopsis. Ethylene synthesis is catalyzed by ACC synthase (ACS) and ACC oxidase (ACO). In signal transduction pathway, ethylene was sensed by its receptor, including ETR1, ETR2, ERS1, ERS2, and EIN4, and then the signal was delivered to the last component ERF transcription factors by EIN2 and EIN3, thereby activating the ethylene response to modulate various growth and developmental
During fruit ripening, there is a close link between ethylene release and anthocyanin accumulation. In apple, MdEIL1, which is a critical component of the ethylene signaling pathway, binds directly to the MdMYB1 promoter to induce expression, which in turn promotes anthocyanin accumulation and fruit coloration. Additionally, MdMYB1 induces ethylene production by activating MdERF3 to up-regulate MdACS expression. MdMYB10 (the allele of MdMYB1) can activate the expression of MdERF106. MdEIL1, MdMYB1, and MdERF3 form a positive regulatory loop that mediates ethylene biosynthesis and anthocyanin accumulation. However, in pear, ethylene inhibits anthocyanin synthesis by activating the expression of a negative regulator of anthocyanin synthesis (PpMYB140) through PpERF105. The R2R3-MYB repressor gene PpMYB18 is highly expressed in ripe peach fruit with high anthocyanin concentrations. Therefore, ethylene regulates anthocyanin synthesis via the antagonistic activities of the R2R3-MYB repressors and activators. However, the underlying mechanism remains unclear.

Apple is typical respiratory climacteric fruit, in which respiration and ethylene production markedly increase during the ripening stage. Many genes associated with ethylene biosynthesis and the related signaling pathways were recently identified in apple, including MdACS1, MdACO1, MdERF2, MdERF3, and MdEIL1. Previous studies showed that apple fruit ripening is accompanied by an increase in ethylene release. Moreover, substantial amounts of anthocyanins begin to accumulate, which leads to fruit coloration. During the fruit ripening stage, ethylene induces anthocyanin synthesis through the MdEIL1–MdMYB1 signaling pathway. However, the molecular mechanism of ethylene regulating anthocyanin synthesis by R2R3-MYB repressors in apple fruit remains unclear. In this study, our analyses indicated MdMYB17 plays an important regulatory role in the ethylene-mediated anthocyanin synthesis in apple. More specifically, MdMYB17 together with MdMYB1 and MdEIL1 form a complex feedback regulatory network that finely controls anthocyanin synthesis. Moreover, MdMYB17 interacts with MdEIL1 to attenuate the regulatory effects of MdMYB17 on MdMYB1 and MdEIL1 as well as the regulatory effects of MdEIL1 on MdMYB1 and MdMYB17. Hence, MdMYB17 negatively regulates anthocyanin synthesis and interacts with MdMYB1 and MdEIL1 to precisely control ethylene-regulated anthocyanin synthesis and maintain appropriate anthocyanin levels. The results of this study provide new insights into the molecular basis of ethylene-regulated anthocyanin synthesis.
Results

Ethylene promotes *MdMYB1* and *MdMYB17* expression and anthocyanin synthesis in apple fruit

To verify the effect of ethylene on the expression of anthocyanin synthesis-related R2R3-MYB activators and repressors in apple, we collected ‘Geneva Early’ apple fruit at 60 days after full bloom (DAFB) and then treated them with ethephon (1,000 mg·L⁻¹) or the ethylene inhibitor 1-methylcyclopropene (1-MCP; 1 µL·L⁻¹). The treated fruit were stored for 6 days in an incubator set at 24 °C with a light intensity of 20,000 lux. During storage, we analyzed ethylene release, anthocyanin accumulation in the pericarp, and the expression levels of *MdMYB1* in apple. Consistent with the findings of an earlier study, the ethephon treatment significantly induced ethylene release, anthocyanin synthesis, and *MdMYB1* expression in the apple fruit, whereas the 1-MCP treatment had the opposite effects (Fig. 1a-d). These results suggest that *MdMYB1* contributes to ethylene-regulated anthocyanin synthesis in apple fruit. We used *Arabidopsis* R2R3-MYB subfamily 4 genes to search the GDR (https://www.rosaceae.org/) databases, and obtained six R2R3-MYB subfamily 4 genes in apple genome (GenBank accession numbers are listed in Supplemental Table 2). Regarding the subfamily 4 R2R3-MYB TF genes in apple, ethephon and 1-MCP induced the expression of *MdMYB17*, *MdMYB16*, *MdMYB308*, and *MdMYB109*, but not *MdMYB57* and *MdMYB308L*. Among the above genes, the *MdMYB17* expression level was up-regulated the most, implying that *MdMYB17* may have an important regulatory role during ethylene-induced anthocyanin synthesis (Fig. 1d). Consequently, we selected *MdMYB17* for the subsequent functional investigation.
**Fig. 1:** Effects of ethephon and 1-MCP on the anthocyanin synthesis in ‘Geneva Early’ apple fruit. 

**a** Phenotypes of ‘Geneva Early’ fruit treated with ethephon and 1-MCP. **b** Anthocyanin contents of ‘Geneva Early’ fruit treated with ethephon and 1-MCP. **c** Ethylene production of ‘Geneva Early’ fruit treated with ethephon and 1-MCP. **d** Expression levels of *MdMYB1* and subfamily 4 R2R3-MYB TF genes in ‘Geneva Early’ apple fruit treated with ethephon and 1-MCP. Samples were analyzed at 0, 3, and 6 days after the ethephon and 1-MCP treatments. Data are presented as the mean ± SD of three independent biological replicates. Asterisks indicate significant differences, as determined by Student’s *t*-test (*P* < 0.05, **P** < 0.01).

**MdMYB17** inhibits anthocyanin synthesis and fruit coloration in apple

To further clarify the *MdMYB17* function related to apple anthocyanin synthesis, we generated apple calli overexpressing *MdMYB17* (*MdMYB17*-OE) and calli in which *MdMYB17* expression was suppressed (*MdMYB17*-RNAi). The *MdMYB17*-RNAi apple calli were red, but the *MdMYB17*-OE and wild-type (WT) calli were not (Fig. 2a-c). Compared with the corresponding expression levels in the WT calli, *MdMYB1*, *MdDFR*, and *MdUFGT* were expressed at lower and higher levels in the *MdMYB17*-OE and *MdMYB17*-RNAi calli, respectively (Fig. 2d, e).
We also analyzed transient gene expression in apple fruit. Compared with the control fruit, the overexpression of *MdMYB17* inhibited the anthocyanin accumulation at the apple fruit injection site. Additionally, the *MdMYB1, MdDFR*, and *MdUFGT* expression levels were significantly down-regulated at the *MdMYB17*-pRI injection site (Supplementary Fig. S1). In contrast, the silencing of *MdMYB17* promoted anthocyanin accumulation at the apple fruit injection site. Moreover, the *MdMYB1, MdDFR, MdUFGT*, and *MdANS* expression levels were significantly up-regulated at the *MdMYB17*-TRV injection site (Supplementary Fig. S2). These results suggest that *MdMYB17* may negatively regulate anthocyanin synthesis in apple.

**Fig. 2:** *MdMYB17* inhibits anthocyanin synthesis in ‘Orin’ apple calli. a Phenotypes of ‘Orin’ apple calli incubated at 14 °C and light (20,000 lux) for 10 days. WT: wild-type; *MdMYB17*-OE: *MdMYB17*-overexpression; *MdMYB17*-Anti: *MdMYB17*-antisense suppression. b Expression levels of *MdMYB17* in ‘Orin’ apple calli. c Anthocyanin contents of ‘Orin’ apple calli. FW: fresh weight. d, e Expression levels of anthocyanin-related genes in ‘Orin’ apple calli. Data are presented as the mean ± SD of three independent biological replicates. Asterisks indicate significant differences, as determined by Student’s *t*-test (*P < 0.05, **P < 0.01).

*MdMYB17* represses the transcription of *MdMYB1, MdDFR*, and *MdUFGT* by binding to their promoters

To investigate the regulatory relationship between *MdMYB17* and *MdMYB1, MdDFR*, and *MdUFGT*, we analyzed the binding of *MdMYB17* to the *MdMYB1, MdDFR*, and *MdUFGT* promoters in yeast one-hybrid (Y1H) assays. The Y1H results indicated that *MdMYB17* binds to the promoters of all three genes (Fig. 3a). The interactions were verified in an electrophoretic
mobility shift assay (EMSA). The \textit{MdMYB1}, \textit{MdDFR}, and \textit{MdUFGT} promoters contain one MYB-binding site (MBS) motif. In this study, we used biotinylated MBS-containing promoter fragments as probes. The EMSA confirmed that MdMYB17 can bind to the \textit{MdMYB1} (Fig. 3b, lane 1), \textit{MdDFR} (Fig. 3c, lane 1), and \textit{MdUFGT} (Fig. 3d, lane 1) promoters. This binding was unaffected by the supplementation of a competitor probe containing mutations. Accordingly, MdMYB17 binds to the MBS motif in the \textit{MdMYB1} (Fig. 3b, lane 1), \textit{MdDFR} (Fig. 3c, lane 1), and \textit{MdUFGT} (Fig. 3d, lane 1) promoters.

To assess the \textit{in vivo} binding of MdMYB17 to the \textit{MdMYB1}, \textit{MdDFR}, and \textit{MdUFGT} promoters, a chromatin immunoprecipitation and quantitative real-time PCR (ChIP-qPCR) analysis was performed using 35S::MdMYB17-GFP transgenic apple calli and empty GFP vector transgenic apple calli as controls. The results reflected the \textit{in vivo} binding of MdMYB17 to the promoters of all three genes (Fig. 3e).

We next analyzed the effect of MdMYB17 on the \textit{MdMYB1}, \textit{MdDFR}, and \textit{MdUFGT} promoter activities by conducting luciferase (LUC) assays. The \textit{MdMYB1}, \textit{MdDFR}, and \textit{MdUFGT} promoter sequences were inserted into the pFRK1-LUC vector so they were fused to the LUC reporter gene. The \textit{MdMYB17} coding sequence (CDS) was inserted into the pHBT-AvrRpm1 vector as an effector. Compared with the controls, the co-expression of 35S::MdMYB17 with proMdMYB1::LUC, proMdMYB1::LUC, and proMdMYB1::LUC resulted in significantly weaker LUC activity (Fig. 3f). These results imply that MdMYB17 represses \textit{MdMYB1}, \textit{MdDFR}, and \textit{MdUFGT} transcription.

Many R2R3-MYB repressors contain the conserved C2/EAR repressor motif in their C terminus, and a conserved LxLxL C2/EAR was also found in the C terminus of MdMYB17 (Supplementary Fig. S3). We conducted LUC assays to investigate the effect of the C2/EAR motif on the \textit{MdMYB1}, \textit{MdDFR}, and \textit{MdUFGT} promoter activities. The mutated \textit{MdMYB17} sequence lacking the C2/EAR motif (MdMYB17m) was inserted into the pHBT-AvrRpm1 vector as an effector. Compared with MdMYB17, the inhibitory effect of MdMYB17m on \textit{MdMYB1}, \textit{MdDFR}, and \textit{MdUFGT} transcription was significantly reduced (Fig. 3f). These findings suggest that the C2/EAR motif in MdMYB17 has a repressing effect.
MdMYB1 positively regulates the expression of MdMYB17

As mentioned above, ethephon can induce the expression of both *MdMYB1* and *MdMYB17*, and
the MdMYB1 and MdMYB17 expression levels are significantly correlated ($R^2 = 0.7877$) (Supplementary Fig. S4). Therefore, we speculated that MdMYB1 may activate the transcription of MdMYB17. To investigate the regulatory relationship between MdMYB1 and MdMYB17, we generated apple calli overexpressing MdMYB1 (MdMYB1-OE). Compared with the WT calli, the overexpression of MdMYB1 significantly increased the anthocyanin accumulation and MdMYB17 expression in the transgenic calli (Supplementary Fig. S5). To verify the regulatory relationship between MdMYB1 and MdMYB17, we performed a Y1H assay, which confirmed that MdMYB1 binds to the MdMYB17 promoter (Fig. 4a), which includes three MBS motifs (MBS1, MBS2, and MBS3). The EMSA results indicated that MdMYB1 binds to MBS3 in the MdMYB17 promoter (Fig. 4b, Supplementary Fig. S6). The ChIP-qPCR analysis revealed the in vivo binding of MdMYB1 to the MBS3 motif of the MdMYB17 promoter (Fig. 4c). The subsequent analysis of the regulatory effect of MdMYB1 on the MdMYB17 promoter in a LUC assay indicated that MdMYB1 promotes MdMYB17 expression (Fig. 4d).

![Fig. 4: MdMYB1 promotes MdMYB17 transcription. a Y1H analysis indicating that MdMYB1 binds to the MdMYB17 promoter. The concentration of 3-AT was 150 ng mL$^{-1}$. The empty vector and the MdMYB17 promoter were used as negative controls. b EMSA analysis indicating that MdMYB1 binds to the MBS3 motif in the MdMYB17 promoter. The hot probe was a biotin-labeled promoter fragment containing the MBS motif, whereas the cold probe was an unlabeled competitive probe (200-fold probe concentration). Mutant probe was unlabeled hot probe containing two nucleotide mutations. c ChIP-qPCR analysis indicating the in vivo interaction between MdMYB1 and the MdMYB17 promoter. ‘Orin’ apple calli overexpressing the GFP sequence was used as a negative control. The ChIP assay was completed using three replicates. d LUC activity analysis indicating that MdMYB1 activates the MdMYB17 promoter. Data are presented as the mean ± SD of three independent biological replicates. Asterisks indicate significant differences, as determined by Student’s t-test (*$P < 0.05$, **$P < 0.01$).]
MdEIL1 positively regulates the expression of MdMYB17, whereas MdMYB17 represses the expression of MdEIL1.

Earlier studies identified MdEIL1 as a core gene for ethylene-regulated anthocyanin synthesis during apple fruit ripening. In this study, we generated ‘Orin’ apple calli overexpressing MdEIL1. Consistent with previous results, MdEIL1 overexpression significantly increased anthocyanin accumulation and up-regulated MdMYB17 expression in transgenic apple calli (Supplementary Fig. S7). Furthermore, MdEIL1 expression was substantially up-regulated by the ethephon treatment (Supplementary Fig. S8). A Y1H assay performed to further clarify the regulatory relationship between MdEIL1 and MdMYB17 demonstrated that MdEIL1 binds to the MdMYB17 promoter (Fig. 5a), which contains two ATGTA motifs (ATGTA1 and ATGTA2). An EMSA indicated that MdEIL1 binds to the ATGTA1 motif of the MdMYB17 promoter (Fig. 5b, Supplementary Fig. S9). The ChIP-qPCR analysis confirmed the in vivo binding of MdEIL1 to the ATGTA1 motif of the MdMYB17 promoter (Fig. 5c). The LUC assay results suggested that MdEIL1 promotes MdMYB17 expression (Fig. 5d).

Fig. 5: MdEIL1 promotes MdMYB17 transcription. a Y1H analysis indicating that MdEIL1 binds to the MdMYB17 promoter. The concentration of 3-AT was 120 ng mL⁻¹. The empty vector and the MdMYB17 promoter were used as negative controls. b EMSA analysis indicating that MdEIL1 binds to the ATGTA1 motif in the MdMYB17 promoter. The hot probe was a biotin-labeled promoter fragment containing the ATGTA motif, whereas the cold probe was an unlabeled competitive probe (300-fold probe concentration). Mutant probe was unlabeled hot probe containing two nucleotide mutations. c ChIP-qPCR analysis indicating the in vivo interaction between MdEIL1 and the MdMYB17 promoter. ‘Orin’ apple calli overexpressing the GFP sequence was used as a negative control. The ChIP assay was completed using three replicates. d LUC activity analysis indicating that MdEIL1 activates the MdMYB17 promoter. Data are presented as the mean ± SD of three independent biological replicates.
We also observed that *MdEIL1* was expressed at significantly lower levels in *MdMYB17*-OE apple calli than in WT calli. In contrast, *MdEIL1* was more highly expressed in *MdMYB17*-RNAi apple calli than in WT calli (Supplementary Fig. S10). Thus, MdMYB17 appears to repress *MdEIL1* transcription. To investigate the regulatory relationship between MdMYB17 and MdEIL1, we performed a Y1H assay, which revealed that MdMYB17 binds directly to the *MdEIL1* promoter (Fig. 6a). The *MdEIL1* promoter contains two MYB-recognition elements (MREs), namely MRE1 and MRE2. An EMSA indicated that MdMYB17 binds to MRE2 in the *MdEIL1* promoter (Fig. 6b, Supplementary Fig. S11). The ChIP-qPCR analysis confirmed the *in vivo* binding of MdMYB17 to MRE2 in the *MdEIL1* promoter (Fig. 6c). The LUC assay suggested that MdMYB17 inhibits *MdEIL1* expression (Fig. 6d).

The interaction between MdEIL1 and MdMYB17 affects the transcription of *MdEIL1*, *MdMYB17*, and *MdMYB1*
As a key component of the ethylene signaling pathway, EIN3/EIL interacts with multiple functionally important proteins to regulate plant growth, development, and stress resistance\(^\text{34,35}\). Therefore, MdEIL1 may interact with MdMYB17 to influence the regulatory effect of ethylene on anthocyanin synthesis. To assess this possibility, we examined the interaction between MdEIL1 and MdMYB17 in a yeast two-hybrid (Y2H) assay. The recombinant pGBK7 plasmid containing the \(\text{MdEIL1} \) CDS was self-activating. Therefore, we inserted the \(\text{MdEIL1} \) and \(\text{MdMYB17} \) CDSs into the pGADT7 and pGBK7 vectors, respectively. The Y2H assay proved that \(\text{MdEIL1} \) can interact with \(\text{MdMYB17} \) (Fig. 7a). We then purified the polyhistidine (His)-tagged \(\text{MdMYB17} \) (\(\text{MdMYB17-HIS} \)) fusion protein and the glutathione-S-transferase (GST)-tagged \(\text{MdEIL1} \) (\(\text{MdEIL1-GST} \)) fusion protein for pull-down assays, in which \(\text{MdEIL1-GST} \) was pulled down by \(\text{MdMYB17-HIS} \), implying \(\text{MdEIL1} \) interacts with \(\text{MdMYB17} \) (Fig. 7b).

To investigate the \textit{in vivo} interaction between \(\text{MdEIL1} \) and \(\text{MdMYB17} \), we performed a bimolecular fluorescence complementation (BiFC) assay. The co-expression of \(\text{MdMYB17}^\text{N} \) and \(\text{MdEIL1}^\text{C} \) in onion epidermal cells resulted in a strong yellow fluorescent protein (YFP) signal, indicative of the \textit{in vivo} interaction between \(\text{MdMYB17} \) and \(\text{MdEIL1} \) (Fig. 7c). Next, we performed a co-immunoprecipitation (Co-IP) analysis using apple calli. The immunoprecipitation of \(\text{MdMYB17-HA} \) by \(\text{MdEIL1-FLAG} \) in apple calli confirmed the \textit{in vivo} interaction between \(\text{MdMYB17} \) and \(\text{MdEIL1} \) (Fig. 7d).

Luciferase assays were completed to determine how the interaction between \(\text{MdMYB17} \) and \(\text{MdEIL1} \) affects the \(\text{MdMYB17} \) or \(\text{MdEIL1} \) promoter activity. The LUC activity of the \(\text{MdMYB17} \) promoter was significantly lower when \(\text{MdMYB17} \) and \(\text{MdEIL1} \) were co-expressed than when only \(\text{MdEIL1} \) was expressed (Fig. 7e). In contrast, the LUC activity of the \(\text{MdEIL1} \) promoter was higher when \(\text{MdMYB17} \) and \(\text{MdEIL1} \) were co-expressed than when only \(\text{MdMYB17} \) was expressed (Fig. 7f). Previous studies demonstrated that \(\text{MdEIL1} \) directly activates \(\text{MdMYB1} \) expression. Therefore, we investigated how the interaction between \(\text{MdMYB17} \) and \(\text{MdEIL1} \) influences the \(\text{MdMYB1} \) promoter activity. The LUC assay results indicated that the interaction between \(\text{MdMYB17} \) and \(\text{MdEIL1} \) attenuated the activation of the \(\text{MdMYB1} \) promoter by \(\text{MdEIL1} \) and the repression of the \(\text{MdMYB1} \) promoter by \(\text{MdMYB17} \) (Fig. 7g).
Fig. 7: MdMYB17 interacts with MdEIL1. a Y2H analysis indicating that MdMYB17 interacts with MdEIL1. −L−T: SD medium lacking Leu and Trp; −A−H−L−T: SD medium lacking Leu, Trp, His, and Ade; X-α-gal: SD medium containing X-α-gal. The empty AD vectors were used as the negative control. b Pull-down analysis indicating that MdMYB17 interacts with MdEIL1. An anti-GST antibody was used for immunoblot analyses. c BiFC analysis indicating the in vivo interaction between MdMYB17 and MdEIL1. Empty YFPN and YFPC vectors were used as negative controls. Scale bar = 50 μm. d Co-IP analysis indicating the in vivo interaction between MdMYB17 and MdEIL1. Both MdMYB17-HA and MdEIL1-FLAG were transiently co-expressed in protoplasts prepared from ‘Orin’ apple calli. The samples were precipitated using anti-FLAG agarose beads. e-g LUC analysis indicating that MdMYB17–MdEIL1 weakens the effects of MdEIL1 or MdMYB17 on the MdMYB17 (e), MdEIL1 (f), and MdMYB1 (g) promoters. Data are presented as the mean ± SD of three independent biological replicates. Asterisks indicate significant differences, as determined by Student’s t-test (*P < 0.05, **P < 0.01).
Discussion

Ethylene is an important hormone that promotes fruit ripening and affects fruit quality. In recent years, a series of ethylene-related genes have been identified. The functional characterization of these genes indicated they are involved in various physiological processes, including fruit coloration\textsuperscript{22,36}. An ethephon treatment of apple fruit significantly enhances anthocyanin synthesis and coloration\textsuperscript{22}. However, the molecular mechanisms by which ethylene regulates anthocyanin synthesis in ripening apple fruit have not been thoroughly characterized. In the present study, ethylene induced the expression of \textit{MdMYB17}, which encodes an R2R3-MYB TF that represses anthocyanin synthesis. Additionally, \textit{MdMYB17} together with \textit{MdMYB1} and \textit{MdEIL1} formed a regulatory module that finely mediated the regulation of anthocyanin synthesis by ethylene in apple fruit.

\textit{MdMYB17} is a subfamily 4 R2R3-MYB anthocyanin synthesis repressor gene

Plants contain many R2R3-MYB TFs, which vary considerably in terms of structures and functions. R2R3-MYB TFs include crucial regulators of anthocyanin synthesis\textsuperscript{9}. Additionally, the subgroup 6 members \textit{AtPAP1} and \textit{AtPAP2} as well as their homologs are positive regulators of anthocyanin synthesis; the underlying regulatory mechanisms have been extensively studied\textsuperscript{10,12}. However, the mechanisms mediating the effects of subgroup 4 R2R3-MYB TFs, which negatively regulate anthocyanin synthesis, have only recently been elucidated\textsuperscript{14}. In the present study, we identified six genes encoding subgroup 4 R2R3-MYB TFs in the apple genome. Of these genes, \textit{MdMYB17} was the most responsive to ethylene. Moreover, the transgenic assay confirmed that \textit{MdMYB17} can inhibit anthocyanin synthesis. Thus, \textit{MdMYB17} is important for ethylene-regulated apple fruit coloration.

The C2 repressor motif, which is also known as the EAR repression domain, contains a conserved DLNxxP or LxLxL core sequence and is present in various TFs with repressive functions\textsuperscript{37}. The C2/EAR motif is important for maintaining the inhibitory function of the repressors. Earlier studies proved that this motif can convert transcriptional activators into repressors, and that the complete or partial deletion of the EAR sequence will diminish or eliminate the repressive activity of a repressor\textsuperscript{38-40}. In addition to the C2/EAR motif, TLLFR is another repressor motif. This motif was first detected in \textit{AtMYBL2}, which is a strong inhibitor of flavonoid synthesis\textsuperscript{41}. Cavallini et al. (2015) reported that the R2R3-MYB TFs \textit{MYBC2-L1},
MYBC2-L2, and MYBC2-L3 also contain TLLFR motifs, which may explain their strong repressive effects on anthocyanin synthesis\textsuperscript{42}. The MdMYB17 examined in this study contains the LxLxL C2/EAR motif, but not the TLLFR motif. The transgenic assays combined with promoter activity analyses revealed that MdMYB17 directly represses the expression of anthocyanin synthesis-related genes. Moreover, deleting the C2/EAR motif diminishes the repressive activity of MdMYB17, reflecting the importance of the C2/EAR motif for MdMYB17 functionality. Additionally, our findings indicate that the inhibitory function of the C2/EAR motif is conserved.

There is a complex regulatory relationship between MdMYB17 and MdMYB1

There is evidence suggesting that anthocyanin biosynthesis is jointly regulated by the R2R3-MYB activators and repressors. For example, PpMYB18, which encodes an R2R3-MYB TF that represses anthocyanin synthesis, is co-expressed with PpMYB10.1, which encodes an anthocyanin synthesis activator, during peach fruit coloration; the expression of PpMYB18 is induced by PpMYB10.1\textsuperscript{30}. In strawberry, the FcMYB1 expression level is highest when the anthocyanins accumulate to their peak levels during the fruit coloration period\textsuperscript{43}. In the present study, we determined that the expression levels of MdMYB17 (R2R3-MYB TF that represses anthocyanin synthesis) and MdMYB1 (R2R3-MYB TF that activates anthocyanin synthesis) were up-regulated by ethylene during the fruit coloration stage. This implies that anthocyanin synthesis in apple fruit is regulated by MdMYB17 and MdMYB1. The results of our promoter binding assay and LUC assay revealed that MdMYB17 represses MdMYB1 transcription, whereas MdMYB1 induces MdMYB17 transcription. Thus, a feedback regulatory loop between MdMYB1 and MdMYB17 controls the expression of the corresponding genes. The dynamic balance between MdMYB1 and MdMYB17 expression levels and their regulatory effects on anthocyanin synthesis are maintained by this mechanism.

In this study, we found that ethylene induces the expressions of R2R3-MYB repressor MdMYB17, but it also strongly promotes apple anthocyanin synthesis. Additionally, ethylene induces R2R3-MYB activator MdMYB1 expression. Similarly, ethephon treatments can up-regulate the expression of the R2R3-MYB repressor PpMYB140 in pear. But, ethephon treatments inhibited the accumulation of pear fruit anthocyanins and down-regulate the expression of the R2R3-MYB activators PpMYB10 and PpMYB114\textsuperscript{24}. These results suggest that the changes in the anthocyanin contents of apple and pear fruits treated with ethephon are consistent with the
expression of the R2R3-MYB activator, but not the R2R3-MYB repressor. It is possible that R2R3-MYB activator's activation effect on anthocyanin synthesis exceeds that of the repression effect of R2R3-MYB repressor in fruits.

The mechanism underlying the MdMYB17 response to ethylene is complex

The mechanism underlying the response of the R2R3-MYB repressor to ethylene is unclear. In this study, the ethephon treatment up-regulated the MdMYB17 expression level via the ethylene signal transducer MdEIL1, indicating that the inhibitory effect of MdMYB17 on anthocyanin production is regulated by ethylene. Anthocyanins have important functions related to plant resistance to diseases and UV irradiation, but the excessive accumulation of anthocyanins may lead to an imbalance in the primary and secondary metabolic activities of plants. Ethylene can strongly induce anthocyanin synthesis. We speculate that the up-regulated expression of MdMYB17 can prevent the excessive accumulation of anthocyanins and maintain ethylene-induced apple anthocyanin synthesis at an appropriate level.

The ethylene receptor inhibitor 1-MCP can disrupt ethylene signal transduction to minimize the physiological effect of ethylene (e.g., inhibition of anthocyanin synthesis). An et al. (2018) reported that an ethephon treatment up-regulates the MdEIL1 and MdMYB1 expression levels and promotes anthocyanin synthesis in apple. In contrast, a 1-MCP treatment inhibits the expression of MdEIL1 and MdMYB1 and anthocyanin synthesis. Our findings are consistent with those of An et al. (2018). However, the 1-MCP treatment up-regulated MdMYB17 expression, but had the opposite effect on MdEIL1 expression. Therefore, the mechanism by which ethylene up-regulates the expression of MdMYB17 through MdEIL1 cannot explain why 1-MCP up-regulates the expression of MdMYB17. Additionally, Ni et al. (2021) also observed that 1-MCP up-regulates the expression of the R2R3-MYB repressor PpMYB140 in ‘Hongzaosu’ pear. These results indicate that 1-MCP up-regulating the expression of the R2R3-MYB repressor may be a common phenomenon in fruits. However, the underlying molecular mechanism remains to be characterized.

We speculate that although 1-MCP decreases the MdEIL1-mediated up-regulated expression of MdMYB17, it may modulate the expression of other regulatory genes, which may lead to the up-regulated expression of MdMYB17.

Relationship between MdMYB17 and MdEIL1 during ethylene-regulated anthocyanin synthesis and fruit coloration in apple
Ethylene is crucial for regulating anthocyanin accumulation and coloration in apple fruit. An et al. (2018) investigated the molecular basis of ethylene-induced anthocyanin synthesis in apple. They reported that MdEIL1 activates MdMYB1 expression and ethylene promotes anthocyanin synthesis and apple fruit coloration through the MdEIL1–MdMYB1 signaling pathway22. In the present study, we observed that MdEIL1 binds directly to the MdMYB17 promoter to activate expression, indicating that ethylene also inhibits anthocyanin synthesis through the MdEIL1–MdMYB1 signaling pathway. It is likely that MdEIL1 positively and negatively affects ethylene-regulated anthocyanin synthesis in apple. We also confirmed that MdMYB17 represses the expression of MdEIL1 by directly binding to its promoter. Therefore, the relationship between MdEIL1 and MdMYB17 probably influences the regulatory effect of MdEIL1 on anthocyanin synthesis.

Previous studies proved that MdMYB1 does not interact with MdEIL122. However, in the present study, we revealed MdMYB17 interacts with MdEIL1, indicative of a direct regulatory relationship between MdEIL1 and the anthocyanin synthesis-related R2R3-MYB TFs at the protein level. Luciferase reporter assays indicated that the co-expression of MdMYB17 and MdEIL1 proteins attenuates the negative effects of MdMYB17 on the MdEIL1 and MdMYB1 promoters and the positive effects of MdEIL1 on the MdMYB1 and MdMYB17 promoters. Accordingly, the interaction between MdMYB17 and MdEIL1 is critical for maintaining the MdEIL1, MdMYB17, and MdMYB1 expression levels as well as anthocyanin synthesis.

On the basis of the data presented herein, we propose the following working model for the roles of MdEIL1, MdMYB17, and MdMYB1 related to ethylene-regulated anthocyanin synthesis in apple fruits (Fig. 8). In this model, the initiation of apple fruit maturation activates the ethylene signal. Additionally, the MdEIL1–MdMYB1 regulatory pathway that promotes anthocyanin synthesis and the MdEIL1–MdMYB17 regulatory pathway that inhibits anthocyanin synthesis are induced. The activation of MdMYB17 expression by MdMYB1 and MdEIL1 results in the feedback regulation of MdEIL1 and MdMYB1 expression. At the same time, the MdMYB17–MdEIL1 protein interaction weakens the regulatory effects of MdMYB17 on MdMYB1 and MdEIL1 and of MdEIL1 on MdMYB17 and MdMYB1. Moreover, some studies revealed that MdMYB1 activates MdERF3 expression, which can promote anthocyanin synthesis22,45. In response to light, MdMYB1 is phosphorylated by the light-induced MdMPK4, which stabilizes the TF and promotes anthocyanin synthesis46. Light also increases anthocyanin
synthesis by inducing the expression of the ethylene signal transducer MdERF109. These findings indicate that the regulation of anthocyanin synthesis in apple fruits involves many genes that respond quickly to developmental and environmental signals.

![Fig. 8: Proposed model of ethylene-regulated anthocyanin synthesis in apple fruit. MdEIL1 directly activates MdMYB1 expression to increase structural gene expression and anthocyanin accumulation. Additionally, MdEIL1 directly activates MdMYB17 expression to inhibit structural gene expression and anthocyanin accumulation. In contrast, MdMYB17 directly represses MdMYB1 and MdEIL1 expression to inhibit anthocyanin accumulation. Furthermore, MdMYB17 and MdEIL1 form a protein complex that down-regulates the effects of MdMYB17 on MdMYB1 and MdEIL1 as well as the regulatory effects of MdEIL1 on MdMYB17. In addition, MdMYB1 directly activates MdERF3 expression to increase anthocyanin accumulation and the production of the phosphorylated MdMYB1 protein.](https://academic.oup.com/hr/advance-article/doi/10.1093/hr/uhac034/6532229)

**Materials and methods**

**Plant materials and treatments**

*Malus domestica* cv. ‘Geneva Early’ fruit were harvested at 60 DAFB and divided into three groups. The first group was left untreated (i.e., control). The second group was treated with 1,000 mg·L⁻¹ ethephon solution for 1 min. The third group was treated with 1 μL·L⁻¹ 1-MCP for 12 h. All fruit were then stored at room temperature (24 °C) and exposed to constant light (20,000 lux) for 6 days. The fruit anthocyanin content and ethylene production were measured at 0, 3, and 6
days during the storage period. Three fruits were used as a biological replicate and three biological replicates were set at each sampling time-point for the subsequent measurements.

Apple fruit (cv. ‘Yinv’) were harvested at 50 and 140 DAFB for injection assays. ‘Orin’ apple calli were used for Agrobacterium tumefaciens-mediated transformation.

**Determination of anthocyanin contents and ethylene production**

Anthocyanin contents were measured according to the pH differential method. Briefly, 1 g powdered apple peel was mixed with 10 mL 1% (v/v) HCl–methanol at 4 °C for 24 h. Then, 1 mL supernatant was added to 4 mL KCl buffer (pH 1.0) and 4 mL NaAc buffer (pH 4.5). The absorbance (at 510 and 700 nm) of the mixture was determined using the UV-2450 spectrophotometer (Shimadzu, Kyoto, Japan).

Ethylene production was analyzed using the Clarus 580 GC system (PerkinElmer, Waltham, MA, USA) as previously described.

**qPCR**

Total RNA was extracted, cDNA was synthesized, and a qPCR analysis was performed as previously described. The iCycler iQ5 system (Bio-Rad Laboratories, CA, USA) was used for the qPCR. MdActin was used as the internal control, and the expression levels were calculated using the \( (C \Delta A C t) \) method. Details regarding the qPCR primers are provided in Supplemental Table S1.

**Generation of transgenic apple calli**

The full-length *MdMYB1*, *MdMYB17*, and *MdEIL1* CDSs were inserted into the pRI101-AN vector containing a GFP tag to obtain the overexpression vectors *MdMYB17*-pRI, *MdMYB1*-pRI, and *MdEIL1*-pRI. Additionally, 390 bp sense and antisense sequences of the *MdMYB17* CDS were inserted into the pFGC1008 vector to obtain the RNAi construct *MdMYB17*-RNAi. The recombinant plasmids were inserted into *A. tumefaciens* strain LBA4404 cells. Analyses of the overexpression of *MdMYB17*, *MdMYB1*, and *MdEIL1* and the silencing of *MdMYB17* in apple calli were performed as previously described. Each successfully infected callus line was used as a biological replicate. Three biological replicates were analyzed for the gene overexpression or RNAi assays.

**Fruit injection assay**

Fruit injection assays were performed as previously described. A 390 bp sequence of the
MdMYB17 CDS was inserted into the TRV vector to obtain the antisense virus vector MdMYB17-TRV\textsuperscript{53}. The overexpression vector MdMYB17-pRI was constructed as mentioned above. The recombinant plasmids were inserted into \textit{A. tumefaciens} strain LBA4404 cells. The vectors and the \textit{A. tumefaciens} solutions were injected into apple fruit peels. The injected fruit were stored at 24 °C and exposed to constant light (20,000 lux) for 5 days. Ten injected fruits were used as a biological replicate. Each fruit injection assay was performed using three biological replicates.

\textbf{Y1H assay}

The full-length \textit{MdMYB17}, \textit{MdMYB1}, and \textit{MdEIL1} CDSs were inserted into the pGADT7 vector to obtain the recombinant vectors \textit{MdMYB17-pGAD}, \textit{MdMYB1-pGAD}, and \textit{MdEIL1-pGAD}. The \textit{MdMYB17}, \textit{MdMYB1}, \textit{MdDFR}, \textit{MdUFGT}, and \textit{MdEIL1} promoter fragments were inserted into the pHIS2 vector. Yeast strain Y187 cells harboring the recombinant pHIS2 plasmids were cultured on SD/-Trp/-Leu/-His medium containing 3-amino-1,2,4-triazole (3-AT).

\textbf{EMSA}

The full-length \textit{MdMYB17}, \textit{MdMYB1}, and \textit{MdEIL1} CDSs were inserted into the pET-32a vector containing a His tag to obtain the recombinant plasmids, which were then inserted into \textit{Escherichia coli} BL21 (DE3) cells for the production of fusion proteins. The fusion proteins were purified using the His-tagged Protein Purification kit (CWbio, Beijing, China). The EMSAs were conducted using the LightShift Chemiluminescent EMSA kit (Thermo Scientific, Waltham, MA, USA). All probes were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China).

\textbf{ChIP-qPCR analysis}

Transgenic calli harboring \textit{MdMYB1}, \textit{MdMYB17}, and \textit{MdEIL1} fused to a GFP tag were obtained as described above. The ChIP assays were performed using the EZ-ChIP Chromatin Immunoprecipitation kit (Millipore/Upstate, MA, USA) as previously described\textsuperscript{52}. Apple calli overexpressing the GFP sequence was used as a control. The enriched DNA fragments were examined by qPCR. The primers are provided in Supplemental Table S1.

\textbf{LUC analysis}

The LUC analysis was performed as previously described\textsuperscript{52}. The full-length \textit{MdMYB1}, \textit{MdMYB17}, and \textit{MdEIL1} CDSs were inserted into pHBT-AvrRpm1 to obtain the effectors. The \textit{MdMYB1}, \textit{MdMYB17}, \textit{MdDFR}, \textit{MdUFGT}, and \textit{MdEIL1} promoter fragments were inserted into
pFRK1-LUC-nos to obtain the reporters. Protoplasts were prepared from ‘Orin’ apple calli. The LUC activity values were recorded using the Victor X4 Multimode Plate Reader (PerkinElmer, Norwalk, CT, USA).

Y2H assay

The full-length *MdMYB17* CDS was inserted into the pGBK7 vector, whereas the full-length *MdEIL1* CDS was inserted into the pGADT7 vector. The Y2HGold yeast strain cells harboring the recombinant plasmids were cultured on SD/−Trp/−Leu/−His/−Ade medium containing X-α-gal.

BiFC assay

The BiFC assay was performed as previously described\(^5\). The full-length *MdMYB17* CDS without the stop codon was inserted into the pSPYNE-35S vector, whereas the *MdEIL1* CDS was inserted into the pSPYCE-35S vector. The recombinant plasmids were inserted into *A. tumefaciens* LBA4404 cells. The YFP signals were detected using the DS-Ri2 confocal laser scanning microscope (Nikon Corporation, Tokyo, Japan) at an excitation wavelength of 488 nm.

Pull-down assay

The full-length *MdMYB17* CDS was inserted into the pET-32a(+) vector containing a His tag, whereas the *MdEIL1* CDS was inserted into the pGEX-4T-1 vector containing a GST tag. The recombinant plasmids were inserted into BL21 cells to produce fusion proteins. The MdMYB17-HIS protein and MdEIL1-GST protein were incubated together along with the His-tagged bait protein. Proteins were eluted in elution buffer and then analyzed in an immunoblot using anti-His and anti-GST antibodies (Abmart, Shanghai, China).

Co-IP assay

The Co-IP assay was performed as previously described\(^5\). The full-length *MdMYB17* CDS was inserted into the pHBT-AvrRpm1-HA vector containing a HA tag, whereas the *MdEIL1* CDS was inserted into the pHBT-AvrRpm1-FLAG vector containing a FLAG tag. The MdMYB17-HA and MdEIL1-FLAG constructs were used for the co-transfection of ‘Orin’ protoplasts prepared from apple calli. After a centrifugation, the supernatant was mixed with anti-FLAG agarose beads. A western blot analysis was performed using anti-HA and anti-FLAG antibodies.

Acknowledgements

We thank Liwen Bianji (Edanz) (www.liwenbianji.cn/) for editing the English text of a draft of
this manuscript. We thank Aide Wang and Tong Li (Shenyang Agricultural University), and Jianping An (Shandong Agricultural University) for their help in this work. This work was supported by the National Natural Science Foundation of China (31872940), the National Key Research and Development Program of China (2018YFD1000105), and the Agricultural Improved Seed Project of Shandong Province (2019LZGC008).

Conflict of interests
The authors declare no competing interests.

Contributions
S-QF designed the research. SW, L-XL, ZZ, YF and DL performed experiments, conducted fieldwork and analyzed the data. S-QF and X-SC analyzed the data. S-QF wrote the manuscript.

Data availability
All data supporting the findings of this study are available within the paper and within its supplementary data.

References
6. Koes, R., Verweij, W. & Quattrociatto, F. Flavonoids: a colorful model for the regulation and


(Suppl) 14, S131–S151 (2002).


