microRNA172 targets APETALA2 to regulate flavonoid biosynthesis in apple (Malus domestica).

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Abstract
MicroRNA172 (miR172) plays a role in regulating a diverse range of plant developmental processes, including flowering, fruit development and nodulation. However, its role in regulating flavonoid biosynthesis is unclear. In this study, we show that transgenic apple plants over-expressing miR172 show a reduction in red coloration and anthocyanin accumulation in various tissue types. This reduction was consistent with decreased expression of APETALA2 homolog MdAP2_1a (a miR172 target gene), MdMYB10, and targets of MdMYB10, as demonstrated by both RNA-seq and qRT-PCR analyses. The positive role of MdAP2_1a in regulating anthocyanin biosynthesis was supported by the enhanced petal anthocyanin accumulation in transgenic tobacco plants overexpressing MdAP2_1a, and by the reduction in anthocyanin accumulation in apple and cherry fruits transfected with an MdAP2_1a virus-induced-gene-silencing construct. We demonstrated that MdAP2_1a could bind directly to the promoter and protein sequences of MdMYB10 in yeast and tobacco, and enhance MdMYB10 promoter activity. In Arabidopsis, over-expression of miR172 reduced flavonoid (including anthocyanins and flavonols) concentration and RNA transcript abundance of flavonoid genes in plantlets cultured on medium containing 7% sucrose. The anthocyanin content and RNA abundance of anthocyanin genes could be partially restored by using a WT MdAP2_1a, which had lost the miR172 target sequences at mRNA level, but not restored by using a WT MdAP2_1a. These results indicate that miR172 inhibits flavonoid biosynthesis through suppressing the expression of an AP2 transcription factor that positively regulates MdMYB10.

Introduction
Plants contain multiple MicroRNA172 (miR172) genes that can produce conserved mature miR172 sequences in different tissues and at different developmental stages [1–3]. The mature miR172 sequences target mRNA of a subfamily of APETALA2 (AP2) gene by sequence complementation and then repress gene expression by inhibiting translation or initiating degradation of the target mRNA in Arabidopsis [4–6].

Although the RNA sequence of mature miR172 is highly conserved across the plant kingdom, this miRNA has a diverse range of functions. These diversified functions are likely associated with the differences in expression patterns of miR172 genes in different plant species, and the evolution of its target AP2 genes. Altering the expression of an miR172 gene can affect many aspects of plant development as well as reponses to abiotic and biotic stresses. As AP2 genes are repressors of flowering, miR172 can promote flowering in a number of plant species, including Arabidopsis, maize, and rice [4, 5, 7, 8]. AP2 governs floral organ development [9] and organ size [10], and so miR172 over-expression can also alter floral organ and fruit development [1, 3, 6, 11, 12]. Interestingly, miR172 positively regulates Arabidopsis silique growth [1] but negatively regulates apple fruit growth [3], demonstrating that its function can be quite different between plant species. It has also been shown that miR172 is involved in the regulation of other aspects of plant development such as soybean nodulation [13] and potato tuberization [14]. Altered responses to abiotic and biotic stresses, as a consequence of miR172 overexpression, have included an enhanced tolerance to water deficit and salt stress in Arabidopsis [15] and resistance to disease infection in tomato [16].

The flavonoid biosynthesis pathway produces many plants secondary metabolites, including flavones, flavonols, and anthocyanins. Of these compounds, anthocyanin has been studied extensively in recent years because of its importance in the attractiveness of flowers and fruit and potential health benefits of fruit [17, 18]. Studies have identified the MBW (MYB, bHLH, and WD40) complex of transcription factors that regulates
the transcription of structural genes in the anthocyanin biosynthesis pathway [19, 20]. MYB proteins involved in the MBW complex are highly conserved amongst many plant species and fall into the R2R3 class subgroup, containing two conserved DNA-binding domains [20]. These MYBs are often a limiting factor of the MBW complex and their over-expression can significantly enhance red coloration in a number of plant species, including Arabidopsis [21], apple [22–24], and strawberry [25]. However, it is less clear what genetic regulators control these anthocyanin-promoting MYBs.

In apple (Malus domestica), three MYB genes have been confirmed to regulate anthocyanin biosynthesis. The first gene was discovered by three different groups and named as MdMYB1 [24], MdMYB10 [23] and MdMYBA [26], representing three different alleles of the same gene [27]. All three alleles are important for red fruit skin, and a variant of MdMYB10 containing six small repeats in the promoter region is important for red fruit flesh and red leaves [22]. A long-terminal-repeat retrotransposon insertion in the promoter region of MdMYB1/10/A is responsible for activating the gene expression in fruit skin [28]. The second gene, MdMYB110a, is a paralog of MdMYB10 and has an allele underlying a pink fruit flesh trait [29]. The third gene, MdMYB3, falls in a different subgroup (subgroup 4) from MdMYB10/A/1 and MdMYB110a (subgroup 6) of the MYB transcription factor superfamily, and is important for regulating flower colour [30].

Several studies have demonstrated that miRNAs may act as regulators of phenylpropanoid biosynthesis. It has been shown that the miR156-SPL9 network directly influences anthocyanin production through targeting genes encoding the MYB transcription factor PAP1 (Production of Anthocyanin Pigment 1) and dihydroflavonol 4-reductase [31, 32]. It has been reported that the miR159, miR319, and miR828 families deregulate MYB transcription factors in apple, tomato (Solanum lycopersicum), grape (Vitis vinifera) and red sage (Salvia miltiorrhiza) [2, 33–35]. So, although a number of miRNAs have been shown to regulate flavonoid production, it has not been established in any plant that miR172 or its AP2 target play such a regulatory role in this biosynthesis.

The study presented here shows that miR172 plays a role in the regulation of flavonoids production. We found that over-expression of miR172 in stable apple and Arabidopsis transgenic plants reduced red coloration and altered the levels of flavonoid compounds. These phenotypic changes were consistent with the altered expression patterns of genes in the anthocyanin biosynthesis branch and other branches of the flavonoid biosynthesis pathways.

Results
Over-expression of miR172 reduced red coloration in multiple apple tissue types

To examine the function of miR172, six independent apple transgenic plant lines were produced with a gene construct to over-express miR172 and grown to maturity in a greenhouse. miR172 over-expression (miR172OX) was identified the four transgenic lines [3]. In addition to the discovery of a reduced fruit size phenotype described in our previous study, we discovered that flavonoid accumulation in these transgenic plants was also reduced. Upon visual inspection of the transgenic plants over-expressing miR172, the level of red colour appeared to be reduced in several plant tissue types (Fig. 1). In tissue culture conditions, leaves of in vitro shoots of WT plants displayed red coloration when sucrose concentration of the media was elevated to 7%, whereas this red coloration was not observed when the plants were grown on media containing the standard concentration of 3% sucrose. Sucrose-induced anthocyanin biosynthesis was not observed in the leaves of three independent miR172OX lines tested (Fig. 1a, b, c). In greenhouse conditions, red coloration was not observed on young stems or leaf petioles of four independent transgenic plants or the PCR-positive seedlings of the fertile transgenic plant, but was evident on young stems and leaf petioles of WT control plants (Fig. 1d). A reduction of red coloration was also observed in mature fruit skin of miR172OX plants (Fig. 1e). The fruit skin of miR172OX plants showed a strong russetting that was not observed on fruit skin of WT controls (Fig. 1e; Fig. S1).

Chemical analyses using HPLC showed that the level of catechin, epicatechin, procyanidin and cyanidin-3-galactoside were significantly reduced in the transgenic fruit skin (Fig. S2a, b, c, d). This reduction of cyanidin-3-galactoside, the major anthocyanin in apple, is consistent with the reduction of red coloration. In contrast, the levels of phloridzin and chlorogenic acid were significantly increased (Fig. S2e, f). These two compounds, although synthesised within the general phenylpropanoid biosynthesis pathways, are produced from separate side branches that compete for the flavonoid pathway precursor, p-coumaryl CoA. Together, the changes in coloration and chemical composition of the skin indicated that elevated expression of miR172 inhibited anthocyanin biosynthesis and increased the level of other flavonoid compounds.

Expression of anthocyanin biosynthetic genes were inhibited in miR172 transgenic apple plants

To reveal how miR172 over-expression may change gene expression, transcriptome analyses were performed using mRNA isolated from mature fruit skin (FS). Six RNA-seq libraries were produced using the fruit skin from WT and miR172OX plants. For each library, 46 to 57 million clean reads were generated, producing~8 Gb nucleotide sequences (Table S2). Comparing miR172OX_FS libraries to WT_FS libraries, 4383 genes were significantly up-regulated and 2903 genes were significantly down-regulated (DESeq2 p value <0.05) (Fig. S3). Of these differentially expressed genes (DEGs), 2411 genes were up-regulated and 1687 genes were
miR172 inhibits flavonoid biosynthesis through suppressing the expression of AP2 in Arabidopsis

As it is slow in producing stably transformed apple plants and growing the plants to produce fruit, we selected Arabidopsis plants for further characterisation of miR172 function in regulation of flavonoid biosynthesis. To test whether miR172 inhibits flavonoid biosynthesis in Arabidopsis, we transformed Arabidopsis plants using the apple miR172 over-expression construct because miR172 and its targeting site are highly conserved across plant species [1]. Six of 70 independent Arabidopsis T1 transgenic plants containing the miR172 gene construct were analysed using RT-PCR to determine the miR172 level. Three plants with high miR172 expression levels were selected to produce T3 generation homozygous transgenic plants. When grown in tissue culture medium containing 7% sucrose, plants of these three T3 lines did not show red coloration whereas WT plants clearly showed red coloration due to anthocyanin accumulation (Fig. 2a, b). The transgenic plants also showed significant reduction of the level of flavonols compared to the WT plants (Fig. 2c). In contrast, the transgenic plants

down-regulated by greater than two-fold (log₂-fold-change > 1 or < −1, Table S3–4).

The apple genome contains eight AP2-like genes with miR172 target sequences, and the complementary sequences between miR172 and AP2-like genes were described in a previous study [3]. A phenology analysis using the protein sequences of these genes and six Arabidopsis counterparts revealed that the apple genes were grouped into 4 distinct clades (Fig. S4). Of the eight apple genes, MdAP2_1a (MD15G1286400) was most closely related to Arabidopsis AP2 and was expressed at the highest level, while MdAP2_1b and MdAP2_2a were expressed at a very low level, and the other five genes (MdAP2_2b, 3a, 3b, 4a, and 4b) were not expressed the FS tissues from WT and miR172OX apple plants (Fig. 1). Differential expression analysis showed that MdAP2_1a was down-regulated (DESeq2, p-value < 0.05) in miR172OX fruit skin, but the level of down-regulation was less than two folds (log₂FoldChange of −0.83, Table S5). This small down-regulation of MdAP2_1a mRNA level may be explained by that miR172 represses AP2 expression mostly by inhibiting translation but initiating degradation of the target mRNA at a much low level in Arabidopsis and apple [4, 36], although most other plant miRNAs repress gene expression by initiating degradation of the target mRNAs [37].

MdMYB10 is the key anthocyanin activators in apple fruit skin. The transcript level of MdMYB10 was dramatically reduced in miR172OX fruit skins with a log₂FoldChange value of −4.3 (Fig. 1f, 1g, Table S4). This strong reduction of MdMYB10 expression was confirmed by RT-PCR analysis (Fig. S5a). Besides MdMYB10, anthocyanin biosynthetic pathway genes were also dramatically down-regulated in the miR172OX_FS compared to WT_FS (Fig. 1g, Fig. S5).
showed an early flowering phenotype that is consistent with results of previous studies [5]. In the leaves of the WT plants, the miR172 expression level was extremely low but significantly increased in the leaves of the three transgenic lines (Fig. 2d). In contrast, the AtAP2 expression level showed a trend of reduction in two transgenic lines and a significant reduction in one line (Fig. 2e). The expression levels of regulatory and biosynthetic genes in the anthocyanins and flavonols biosynthetic pathway were also significantly reduced in the transgenic plants compared to WT control plants. This reduction was not so dramatic for the AtPAP1 gene (encoding a MYB transcription factor) and genes (AtCHS, AtCHI, AtF3H and AtFLS1) for upstream steps of the biosynthesis pathway, but dramatic, almost to complete suppression, for the downstream steps of the biosynthesis pathways genes (AtDFR, AtANS, AtUFGT and AtFGT1) (Fig. 2f-n). This result indicates that over-expression of miR172 inhibits flavonoid biosynthesis in Arabidopsis, which is consistent with the results of over-expression of miR172 in apple.

To confirm AP2 being the target of miR172 for regulating flowering time in Arabidopsis, a strategy is developed involving in using a synonymous mutant gene of AP2 to recover the normal flowering time of the transgenic plant over-expressing miR172 [4]. The synonymous mutant AP2 is created by changing several nucleotides important for miR172 targeting so that the mutant mRNA is no longer targeted or repressed by miR172, but still produce the normal protein. The same strategy was used here to test whether miR172 regulates flavonoid biosynthesis through its target AP2-like
genes. Transgenic Arabidopsis plants were produced with a gene construct for over-expressing MdAP2_1a, and a synonymous mutant of MdAP2_1a (MdmAP2_1a) created to eliminate the miR172 target sequence in the mRNA (Fig. S6). MdmAP2_1a is closely related to Arabidopsis AP2 and should have AP2 function. After crossing the miR172OX transgenic plants with the MdmAP2_1a and MdmAP2_1a transgenic plants, respectively, and confirming the hybrid status of the plants (Fig. S7), we found that red coloration under the miR172 over-expression condition could be partially restored by the MdmAP2_1a but not the MdAP2_1a transgene (Fig. 3a). qRT-PCR showed that in the hybrid plants there was partial restoration of the expression levels of the anthocyanin biosynthetic genes we tested (Fig. 3b). Together these results indicate that maybe miR172 inhibits anthocyanin biosynthesis by targeting the AP2-like gene in apple.

**AP2 homologues positively regulated anthocyanin biosynthesis.**

To provide direct evidence for AP2-like gene regulating anthocyanin biosynthesis, we choose to over-express MdAP2_1a in Nicotiana tabacum "NC89". "NC89" was chosen because it produces flowers with light-pink petals, indicating that anthocyanin biosynthesis system is function but weak, the system may be further enhanced. We produced 29 transgenic tobacco plants with the CaMV35S-MdAP2_1a gene construct, and identified at least four plants by RT-PCR analysis as expressing MdAP2_1a (Fig. S8). Two of the MdAP2_1a expressing plants, OE#22 and OE#24, were analysed in detail. The petals of OE#22 and OE#24 showed an earlier red coloration in unopened flowers and a stronger red coloration in opened flowers, when compared to WT control plants. (Fig. 4a). These transgenic plants showed significantly higher levels of anthocyanin accumulation in petals than the WT plants (Fig. 4b). These increased levels of red-coloration and anthocyanin content were correlated with the level of expression of the MdAP2_1a transgene (Fig. 4c). We conducted a qRT-PCR assay of anthocyanin biosynthetic and regulatory genes in the petals of the WT, OE#22, and OE#22 tobaccos. The results showed significantly elevated expression levels of the tobacco MYB regulator NtAN2 (Fig. 4d), two tobacco bHLH regulators NtAn1a, NtAn1b (Fig. 4e-f) and tobacco genes of the anthocyanin biosynthetic pathway, including NtCHS, NtCHI, NtF3H, NtF3'H, NtDFR, NtANS, and NtUFGT (Fig. 4g-m). Together, these results indicate that an AP2-like gene can enhance anthocyanin biosynthesis by increasing the transcription of anthocyanin biosynthetic and regulatory genes.

To determine if knock-down of MdAP2_1a expression can reduce anthocyanin biosynthesis, we silenced MdAP2_1a using virus-induced gene silencing (VIGS). At 15 days after Agrobacterium-infiltration of a VIGS construct of MdAP2_1a into the fruit of the apple cultivar "Jinxiuhong", reduced red-coloration around the infiltration sites was evident. However, there was no clear reduction at the infiltration sites when using an empty VIGS construct as a control (Fig. S9a). RT-PCR analysis showed that the VIGS reduced the MdAP2_1a transcript level, and also resulted in reduced levels of MdMYB10 and MdUFGT expression (Fig. S9b). The MdAP2_1a VIGS construct was also used to knock-down AP2 expression in cherry because the 345 bp of MdAP2_1a coding sequences used in the VIGS construct shared identical sequence with the cherry homologue.


PavAP2 [39], in a region greater than 24 nt (Fig. S10). After infiltration of the VIGS construct, fruit of the sweet cherry “Summit” showed a reduction of red coloration (Fig. S9c) and expression level of sweet cherry “Summit” showed a reduction of red coloration and protein sequences PavMYB10 and MdAP2_1a with the results of regulators of anthocyanin biosynthesis. Tobacco plants, indicate that MdAP2_1a may interact with MdMYB10. Y2H assays showed that the full length MdAP2_1a protein interacted with the full length MdMYB10 protein was able to activate the MdMYB10 promoter as demonstrated by a dual luciferase reporter assay (Fig. 6b) and a GUS reporter assay (Fig. 6c) in N. benthamiana leaves. Together, these results indicated that MdAP2_1a might regulate anthocyanin biosynthesis through directly binding to the promoter and enhancing the expression of the key anthocyanin regulatory gene MdMYB10.

Figure 4. Anthocyanin accumulation in the petals of tobacco plants over-expressing MdAP2_1a. a. Flowers of WT and two transgenic tobacco lines (OE#24 and OE#24) expressing MdAP2_1a were photographed at five developmental stages (S1, unfolding bud, S2, folding bud, S3, early bloom stage, S4, half bloom stage, S5, full bloom stage). The plants shown in (a) were analyzed to determine their total anthocyanin levels in the petals at the S5 stage by the HCl-methanol method described by 38 (b) and the relative transcript levels of the transgene MdAP2_1a (c) and the tobacco genes NtAN2 (d), NtAn1a (e), NtAn1a (f), NtCHS (g), NtCHI (h), NtF3H (i), NtF3'H (j), NtDFR (k), NtANS (l) and NtUFGT (m) by RT-PCR analyses. Error bars represent standard deviation of three biological replicates, and significant difference at p < 0.05 level is indicted by different lowercase letters based on Fisher’s Least Significant Difference (LSD) test.

**Discussion**

miR172 negatively regulated flavonoid biosynthesis in plants

miR172 is reported to play a role in a range of plant development processes. Its role in the regulation plant secondary metabolism is not yet reported. Here we show that miR172 negatively regulates flavonoid biosynthesis, including anthocyanin, using apple and Arabidopsis. Constitutive expression of miR172 reduced the level of flavonoids in the fruit skin of transgenic apple plants (Fig. 1f). In addition to fruit skin, a reduction in anthocyanin accumulation is also observed in the epidermis of young stem and leaf petioles when the plants are grown in greenhouse conditions, as well as in young shoots grown on tissue culture media with elevated sucrose levels (Fig. 1a-d), all of which suggest a major change in the anthocyanin biosynthetic pathway. In Arabidopsis, sucrose-specific signalling activates the...
PAP1 gene, a homolog to MdMYB10, which elevates anthocyanin biosynthesis [41]. Our results further show that over-expression of miR172 inhibits anthocyanin biosynthesis in Arabidopsis plants grown on tissue culture media with elevated sucrose levels (Fig. 2a-b). The data presented in this study suggest that high level of miR172 mutes the ability of MdMYB10 to activate anthocyanin biosynthesis in response to sucrose signalling. Several miRNAs are known to play a role in the regulation of plant color formation, especially in the biosynthesis of anthocyanin. Specific miRNAs inhibit the expression of target transcription factors related to anthocyanin biosynthesis by negatively regulating their mRNA levels, and thereby regulating plant color. For example, in Arabidopsis, miR156 positively regulates anthocyanin biosynthesis by targeting SPL transcription factors [31], and miR828 regulates the expression of PAP1 / MYB75, PAP2 / MYB90 and MYB113, and thus affects the accumulation of anthocyanin [42]. In tomato, miR858 inhibits anthocyanin accumulation in leaves, stems and vegetative buds of transgenic plants by negatively regulating SIMYB7 expression [33]. In apple, miR828 and miR858 are predicted to target a number of MYB genes, including MdMYB10, using bioinformatics tools [43]. Plants use multiple miRNAs to directly or indirectly regulate the expression of anthocyanin MYB genes. These sophisticated regulatory mechanisms may be required to ensure an appropriate degree of anthocyanin pigmentation in different environmental conditions and at different developmental stages.

Transcription analysis showed that the transcript level of MdMYB10 was significantly reduced in the skin of fruit from transgenic apple plants over-expressing miR172 (Fig. S5a). MdMYB10 is a positive regulator of the expression of the structural genes in the anthocyanin biosynthesis pathway [22–24], therefore a reduction in
### Table: Constructs

| Constructs | ProMdMYB10-pAbAi |
|------------|------------------|
| AD-MdAP2_1a |                  |
| AD-MdAP2_1a |                  |
| AD-MdAP2_1a |                  |
| AD-MdAP2_1a |                  |
| AD-Empty   |                  |
| AD-Rec-P53  |                  |
| AD-Empty   |                  |

### Figure 6: MdAP2_1a interacted with and activated MdMYB10 promoter.

- **a.** In yeast one-hybrid (Y1H) analysis, the complete protein of MdAP2_1a and three fragments (MdAP2_1a339–549, MdAP2_1a1–182) were tested for interaction with the MdMYB10 promoter. Yeast cells co-transformed with the constructs named on the left were cultured on non-selective medium SD/-Leu/-Aba (left panel) and selective medium SD/-Leu+/AbA200 (right panel), in a dilution series of 100, 10–1, 2 × 10–2, 10–2, 2 × 10–3, and 10–3 (i-vi). Yeast growth on SD/-Leu+/aureobasidin A (AbA200) medium (row 2) reveals the physical interaction between MdAP2_1a339–549 and MdMYB10 promoter.
- **b.** The graph shows the Luc/Ren ratio of a dual-luciferase assay of MdAP2_1a activation on MdMYB10 promoter. N. benthamiana leaf co-transformations were carried out with three combinations of plasmid constructs: 1: Empty vector, (pSAK778 and pGreenII 0800-LUC 2: ProMdMYB10 in pGreenII 0800-LUC 3: ProMdMYB10 in pGreenII 0800-LUC together with MdAP2_1a in pSAK778. Error bars represent standard deviation of three biological replications and significant difference at the 0.05 level is indicted by different lowercase letters based on Fisher’s Least Significant Difference (LSD) test.
- **c.** Images show GUS staining of six N. benthamiana leaves transiently transformed with *Agrobacterium tumefaciens* GV3101 harboring constructs as listed on the left. The blue color indicates GUS activity.

### Figure 6: MdAP2_1a interacted with and activated MdMYB10 promoter. a. In yeast one-hybrid (Y1H) analysis, the complete protein of MdAP2_1a and three fragments (MdAP2_1a339–549, MdAP2_1a183–338, and MdAP2_1a1–182) were tested for interaction with the MdMYB10 promoter. Yeast cells co-transformed with the constructs named on the left were cultured on non-selective medium SD/-Leu/-Aba (left panel) and selective medium SD/-Leu+/AbA200 (right panel), in a dilution series of 100, 10–1, 2 × 10–2, 10–2, 2 × 10–3, and 10–3 (i-vi). Yeast growth on SD/-Leu+/aureobasidin A (AbA200) medium (row 2) reveals the physical interaction between MdAP2_1a339–549 and MdMYB10 promoter. **b.** The graph shows the Luc/Ren ratio of a dual-luciferase assay of MdAP2_1a activation on MdMYB10 promoter. N. benthamiana leaf co-transformations were carried out with three combinations of plasmid constructs, 1: Empty vector, (pSAK778 and pGreenII 0800-LUC 2: ProMdMYB10 in pGreenII 0800-LUC 3: ProMdMYB10 in pGreenII 0800-LUC together with MdAP2_1a in pSAK778. Error bars represent standard deviation of three biological replications and significant difference at the 0.05 level is indicted by different lowercase letters based on Fisher’s Least Significant Difference (LSD) test. **c.** Images show GUS staining of six N. benthamiana leaves transiently transformed with *Agrobacterium tumefaciens* GV3101 harboring constructs as listed on the left. The blue color indicates GUS activity.

### MdMYB10 expression is consistent with the concomitant reduction in transcript abundance of the biosynthetic genes tested in this study, including *MdPAL*, *MdCHS*, and *MdDFR* (Fig. 1g, Fig. S5b-d). The reduced expression of these pathway genes is evident in the greatly reduced flavonoid-related metabolites, including flavan-3-ols (catechin and epicatechin) and the oligomeric compounds (procyanidin) (Fig. S2). In contrast to anthocyanin and other flavonoids, phloridzin and chlorogenic acid are significantly increased in the skin of apple fruit from the miR172 transgenic plants (Fig. S2). The higher level of phloridzin is consistent with the development of russet on the skin of transgenic fruit (Fig. S1).

### Chemical analyses of flavonoid compounds have shown that high concentrations of dihydrochalcones, such as phloridzin, are found in russet cultivars [44, 45].

### PAL and CHS are required for the synthesis of flavonoids and phloridzin, with the former enzyme also indirectly required for the synthesis of chlorogenic acid. It is unclear why the concentrations of phloridzin and chlorogenic acid are increased while the transcript level of PAL and CHS decrease in the skin of fruit from the miR172 transgenic apple plants. One possibility is that downregulation of some flavonoid biosynthetic genes in the miR172 overexpressing lines, results in the metabolic flux being redirected from anthocyanin, proanthocyanin and flavonol biosynthesis to the phloridzin and chlorogenic acid branch pathways. Our results suggest that despite the down-regulation of PAL and CHS there remains sufficient levels of PAL and CHS enzymes necessary for the increased synthesis of phloridzin and chlorogenic acid.

### miR172 regulated flavonoid biosynthesis by suppressing its target gene AP2-like

It is known that AP2 genes and their closely related homologs are the targets of miR172 for regulation of flower and fruit development, and leaf and root growth [1, 3–5, 8, 46]. However, it was not clear if AP2 is also the target of miR172 for regulating anthocyanin biosynthesis. In apple, miR172 has sequence complementarity...
sufficient to potentially target and inhibit the expression of eight AP2 genes and two other genes [3]. Having established that miR172 negatively regulates flavonoid biosynthesis we asked if this was mediated through its negative regulation of AP2 targets.

Results of this study have shown that miR172 inhibits flavonoid biosynthesis by suppressing AP2-like gene. The direct evidence is from the Arabidopsis experiments where flavonoid biosynthesis in Arabidopsis plants was inhibited by over-expressing miR172 (Fig. 2a). Using a mutation strategy similar to that used to confirm AP2 genes are targets of miR172 for regulation of flowering [4], the inhibition of anthocyanin biosynthesis is released by using a synonymous mutant of MdAP2_1a, which lacks the miR172 target sequences in the mRNA (Fig. 3a). This mutation produced a fully functional AP2 transcription factor that is not post-transcriptionally regulated by miR172. In contrast, the inhibition of anthocyanin biosynthesis cannot be released by using a WT MdAP2_1a gene because expression of the WT gene can be suppressed by miR172 over-expression.

Indirect evidence for AP2 being the miR172 target for regulating anthocyanin biosynthesis is from experiments showing that AP2 homologs can positively regulate anthocyanin accumulation in apple, cherry and tobacco plants. Fruit anthocyanin accumulation and red coloration are reduced by downregulating expression of MdAP2_1a in apple and PauAP2 in cherry using VIGS (Fig. S9). In addition, over-expression of MdAP2_1a in transgenic tobacco plants can accumulate the expression of petal anthocyanin by 40%–108% (Fig. 4). Thus, these findings together suggest that MdAP2_1a possesses a novel function in regulating anthocyanin biosynthesis. Although most AP2/ERFs are involved in plant growth and development, such as flower development [47, 48], fruit development [49, 50], seed development [10, 51], and seed germination [52, 53], a strawberry AP2/ERF family gene, FaRAV1, was shown to positively regulate anthocyanin accumulation by activation of FaMYB10 [54].

AP2 homologs positively regulated anthocyanin biosynthesis by directly interacting with MdMYB10

AP2 transcription factors can be a transcriptional activator or repressor of target genes [55, 56]. In the regulatory network of nitrogen fixation symbiosis in common bean, PhvAP2_1 activates senescence-related genes to accelerate the process of nodule senescence [57]. In this study, the miR172-AP2 regulatory network controls anthocyanin biosynthesis, with MdAP2_1a, in all likelihood by up regulating MdMYB10. MdMYB10 is a key activator of anthocyanin biosynthesis [22, 23] and its expression level was dramatically reduced in transgenic apple plants over-expressing miR172 (Fig. S5a). Experiments to test if MdAP2_1a can directly interact with the MdMYB10 protein and/or promoter sequences demonstrated that MdAP2_1a can bind to the MdMYB10 pro-
binding of MdMYB1 to the promoters of downstream target genes [58]; and MdSIZ1, a small ubiquitin-like modifier E3 ligase, that responds to the low temperature signal and phosphorous deficiency by interacting and stabilising the MdMYB1 protein to promote anthocyanin accumulation and red fruit coloration [62]. In peach, BL (a NAC transcription factors) forms a heterodimer with PpNA1 to induce the expression of PpMYB10.1, which promotes the accumulation of anthocyanin in peach flesh [63]. In kiwifruit, AcSVP3 can inhibit the synthesis of petal anthocyanin by inhibiting the expression of AcMYB110a and AcMYB10 [64]. In this study, we broaden the range of anthocyanin-related transcription factor classes by demonstrating the interaction of AP2 with an anthocyanin activating MYB.

In conclusions, despite miR172 having been functionally studied in many plant species, its role in flavonoid biosynthesis has not been studied previously. Here, we have demonstrated that miR172 negatively regulates flavonoid accumulation, including anthocyanins, in apple and Arabidopsis by suppressing AP2-like. MdAP2_1a is a positive regulator of anthocyanin accumulation by direct interaction with the MdMYB10 promoter and its protein sequence. These findings establish a novel miR172-AP2-MYB regulatory network of flavonoid accumulation (Fig. 7).

Materials and methods

miR172 apple transgenic plants

Agrobacterium-mediated transformation methods [65] using a binary vector containing the CaMV35S-miR172 gene construct (Fig. S11) as previously described [3] and leaf tissue of the apple cultivar “Royal Gala” were used to produce transgenic apple plants. Six independent transgenic lines were produced and grown to maturity in a greenhouse. Four of them were identified over-expressing miR172 [3]. One plant line was fertile and pollinated with “Granny Smith” apple pollen to produce fruit and seed, as were a number of WT “Royal Gala” plants. The transgenic and WT seed of “Royal Gala” x “Granny Smith” were stratified at 4°C and 16-h photoperiod (30 μmol m−2 s−1). The shoots were photographed after four weeks under these conditions to capture and compare the leaf coloration.

Phenylpropanoid compound analysis

For high performance liquid chromatography (HPLC) analysis, about 400 mg of fruit skin, 800 mg of fruit flesh from transgenic and WT apple plants, and the transgenic and WT Arabidopsis seedlings were harvested, weighed and snap frozen in liquid nitrogen. Samples were then processed according to the methods previously described [67]. In brief, the samples were freeze-dried overnight, extracted in 100% methanol and 0.1% HCl for 3 h in the dark, and centrifuged at 4500 g for 5 min to pellet cell debris. After centrifugation, 0.5 ml supernatants were collected into a new tube for vacuum drying. The dried pellets were re-suspended in 0.5 ml of 20% methanol before syringing through 0.45 μM filters to remove insoluble material. Polyphenols were quantified by HPLC-DAD by using a 5 point calibration curve based on chemical standards obtained from Sigma Aldrich.

Transcriptome sequencing and analysis

Three biological replicates were collected from mature fruit skin of WT and miR172 transgenic “Royal Gala”. From these tissue samples, RNA was isolated using the Spectrum Plant Total RNA kit (Sigma-Aldrich) according to the manufacturer’s instructions. 5 μg total RNA was used to construct strand-specific poly-A RNA libraries as described by [68]. Libraries were sequenced using HiSeq2000 sequencing system (Illumina) to produce paired-end reads per library ranging from 20–27 million. Reads were aligned to the GDDH13 reference genome [69] using Bowtie2 [70]. FPKM (Fragments Per Kilobase of transcript per Million mapped reads) and differential gene expression analyses were carried out following previously reported methods [71].

Arabidopsis transformation, crossing, molecular and phenotypic analyses

Three constructs were used in Arabidopsis transformation. The CaMV35S-miR172 gene construct was previously developed for apple transformation [65]. The CaMV35S-MdAP2_1a construct was generated by cloning the CDS of MdAP2_1a (MD15G1286400) into the binary plant transformation vector pSAK778 [72] between the Spe1

Inducing flavonoid production in leaves of in vitro grown apple shoots

Miro-propagated shoots of three transgenic lines and WT “Royal Gala” were tested for flavonoid accumulation on an apple maintenance medium as previously described [65] but with 3% or 7% sucrose. In a tissue culture container, four shoots of a transgenic line and four WT shoots were cultured in two rows side by side. Each transgenic line together with the WT were tested in three containers of each sucrose concentration in an experiment that was repeated three times. The cultures were incubated in a plant growth room with controlled conditions set at 24°C and 16-h photoperiod (30 μmol m−2 s−1). The shoots were photographed after four weeks under these conditions to capture and compare the leaf coloration.
and XhoI restriction enzyme sites. The CDS sequence of MdAP2_1a was amplified from cDNA derived from fruit skin tissues of the apple cultivar “Huashuo” by PCR using primers MdAP2_1a-F and MdAP2_1a-R (Table S1). The CaMV35S-MdAP2_1a construct was generated by cloning a mutated CDS of MdmAP2_1a-F / MdmAP2_1a-R and MdAP2_1a-F / MdAP2_1a-R (Table S1) to create a miR172-resistant form of MdAP2_1a (MdmAP2_1a). The three constructs were transferred into Agrobacterium tumefaciens strain GV3101 by electroporation. Arabidopsis thaliana ecotype Columbia-0 was transformed using the Agrobacterium-floral dipping methods previously reported [73]. Transgenic plants were selected on MS tissue culture medium containing 100 mg/L kanamycin. T3 generation homozygous lines were generated and used for all analyses.

F1 hybrid seeds were produced by manual pollination of T3 CaMV35S-AP2_1a (MdAP2_1a) and CaMV35S-AP2_1a (MdmAP2_1a) transgenic plants with pollen from CaMV35S-miR172 (miR172) transgenic plants. The F1 seeds were germinated on MS medium containing 100 mg / L kanamycin. The successful hybrid status of the plants was confirmed by PCR and transcript analysis using primers OE-AP2_1a-F / OE-AP2_1a-R and OE-miR172-F / OE-miR172-R listed in Table S1 to show the co-presence of MdAP2_1a, or MdmAP2_1a and miR172 gene sequences, respectively.

For induction of anthocyanin biosynthesis, Arabidopsis seeds (T3, F1 and WT) were germinated on MS medium containing 7% sucrose. Although wild type Arabidopsis does not produce anthocyanin on tissue culture medium containing a standard level of sucrose (3%), but produce anthocyanin on medium with higher level of sucrose (7%) [41]. Accumulation of anthocyanin in the leaf tissues was shown by photographing and chemical analyses at about 25 days after germination. The same leaf tissues were also used for RNA extraction and subsequent RT-PCR analyses. The primers used were listed in Table S1.

### Transgenic expression system in N. benthamiana

To verify the interaction between miRNAs and their targets in vivo, we used A. tumefaciens infiltration to co-express miRNAs and their targets in tobacco. Three constructs were used in the transient transformation. The CaMV35S-miR172 gene construct (Ov-miR172) was previously developed for apple transformation [65]. The PCR amplified fragment of miR172-AP2 target site “CUGCACAGUACGAGAUUC” and miR172-mAP2 target site “CUGCACAGUACGAGAUUC” was obtained with the primer pairs rMdAP2_F / rMdAP2_R and rMdmAP2_F / rMdmAP2_R respectively according to the instruction of the Annealing Buffer for DNA Oligos (https://www.beyotime.com/, D0251, China) [75]. The DNA Oligos products were inserted into a green fluorescent protein (GFP) gene over-expression vector pMS4 to obtain the construct of CaMV35S-MdAP2_1a (Ov-MdAP2_1a) and CaMV35S-MdmAP2_1a (Ov-MdmAP2_1a). The above constructs were transferred into A. tumefaciens strain GV3101 by electroporation. Transient expression in tobacco was carried out as described previously [76]. The pART27 vector was used as a control [3]. The primers used in the experiment were listed in Table S1.

### RNAi transient tests in apple and sweet cherry fruits using the VIGS system

Using NCBI CD-Search Tool, the conserved domain of eight AP2-like protein was identified (Table S5). Therefore, a specific fragment (345 bp) of MdAP2_1a coding region was amplified using PCR primers pTRV2-MdAP2_1a-F and pTRV2-MdAP2_1a-R (Table S1) and cDNA templates synthesised from RNA extracted from the fruit skin of “Huashuo” apple. The PCR products were sequenced and cloned into pTRV2 VIGS vector to form pTRV2-MdAP2_1a. The vector was transferred into A. tumefaciens GV3101 and used for the VIGS experiments, as described previously [77, 78]. Briefly, the A. tumefaciens was grown to saturation in liquid Yeast Extract Peptone (YEP) medium, and the culture was centrifuged. Subsequently, the cell pellet was suspended in 10 mM MgCl2, 10 mM MES (pH 5.8), and 150 μM acetoxyringone to a final OD 600 of 0.8. A. tumefaciens strains containing pTRV1 were mixed with strains harboring pTRV2 or its derivatives, and incubated at room temperature without shaking for 2.5 h before infiltration of fruit.
For the apple VIGS experiments, 40 bagged fruit of the cultivar “jinxiuhong” were on the tree at time of infiltration, and randomly divided into two groups, one group were infiltrated with the empty vector (pTRV1:pTRV2 = 1:1, v/v) the other group with pTRV2-MdAP2_1a (pTRV1:pTRV2-MdAP2_1a = 1:1, v/v). Four points evenly spaced around the middle of the fruit surface were selected and each point was infiltrated with about 200 μl of an A. tumefaciens mixture using a 1 ml syringe with a needle. Fifteen days after infiltration, fruit tissues in the injected areas of five fruit were collected for RNA extraction before photographing to record the visible silencing effects.

For the sweet cherry (Prunus avium) VIGS experiments, two groups of 25 fruit of the cultivar “Summit” on the tree at 30 DAFB were infiltrated with the empty vector (pTRV1:pTRV2 = 1:1, v/v) or pTRV2-PavAP2_1a (pTRV1:pTRV2-MdAP2_1a = 1:1, v/v). At 27 days after infiltration, fruits were photographed and sampled for extracting RNA that was used for RT-PCR analyses.

Gene expression analysis by RT-PCR

Total RNA was extracted from apple or tobacco tissues, of WT and transgenic lines using the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich) according to the manufacturer’s instructions. First-strand cDNA synthesis was carried out using the Quantitect® Reverse Transcription Kit (Qiagen) with the appropriate primers. Relative expression of the genes was measured using the Roche LightCycler 480 system (version 1.5) (Roche) and analysed using the LightCycler software according to the methods previously reported [79], normalized using apple MdEF1a [80], Arabidopsis SS [81], and tobacco NtActin and NtEF1a [82]. miR172 expression level was concurrently determined in each sample by stem-loop RT-qPCR [83, 84] and normalised to apple MdActin and MdEF1a [80], Arabidopsis SS [81] and β-tubulin [85]. Relative expression level of genes was calculated using the the Change Ct method. The mean value represents average value with standard deviation bar from technological replicates and biological replicates. Primer sequences used are listed in Table S1.

Yeast one-hybrid assay

The Y1H assay was performed using a Matchmaker™ Gold Yeast One-Hybrid Library Screening System kit (Clontech, San Francisco, USA). The promoter sequence of MdMYB10 was amplified using PCR primers listed in Tables S1 and cloned into the pAbAi vector. The full length CDS of MdAP2_1a and its three partial sequences (AP2_1a1–182, AP2_1a183–338, AP2_1a339–549) were amplified using PCR primers listed in Table S1 and cloned into the pGADT7 vector. The ProMdMYB10-pAbAi vector was linearized and transferred into the Y1HGold yeast strain to test the promoter auto-activation according to the system user manual. The Y1HGold strain transformed with the promoter construct was re-transformed with the MdAP2_1a-pGADT7 plasmid or one of the MdAP2_1a1–182, MdAP2_1a183–338- or MdAP2_1a339–549-pGADT7 plasmids or the empty vector pGADT7 as a negative control. Over-night yeast cultures were normalized to an OD600 of 1.0 and then spotted on the selection medium in a dilution series of 10^0, 10^-1, 2×10^-2, 10^-2, 2×10^-3, and 10^-3.

Yeast two-hybrid assay

Full-length coding sequence and three partial sequences of the MdAP2_1a protein (AP2_1a1–182, AP2_1a183–338, AP2_1a339–549) were amplified using PCR primers listed in Table S1 and each cloned into the pGADT7 vector (Clontech, https://www.clontech.com). Full-length coding sequence of MdMYB10 was amplified and cloned into pGBK7T. The two types of constructs were co-transformed into yeast strain Y2H Gold using the LiCl-PEG method, according to the manufacturer’s manual (http://www.weidibio.com/). Over-night yeast cultures were normalized to an OD600 of 1.0 and spotted on the selection medium in a dilution series of 10^2, 10^-1, 2×10^-2, 10^-2, and 2×10^-3. The transformants were selected on SD/-Leu/-Trp medium. The interactions were tested on SD/-Leu/-Trp/-His/-Ade. The co-transformation using pGADT7-T and pGBK7T-Lam or pGADT7-T and pGBK7T-S3 were used as negative and positive controls, respectively.

Bimolecular fluorescence complementation (BIFC)

The full-length CDS of MdAP2_1a was amplified with primers BIFC-MdAP2_1a-nYFP-F and BIFC-MdAP2_1a-nYFP-R (Table S1) and cloned into pSPYNE to generate MdAP2_1a-nYFP. The full CDS of MdMYB10 was amplified using primers BIFC-MdMYB10-cYFP-F and BIFC-MdMYB10-cYFP-R (Table S1) and cloned into pSPYCE to generate MdMYB10-cYFP. Both constructs were transferred in to A. tumefaciens GV3101 that were then used to transform N. benthamiana leaf tissues using a method previously described [86]. Following the transformation, yellow fluorescent protein (YFP) fluorescence was detected using methods described in a previous study [77].

Co-IP assay

For Co-IP assay, the CDS of MdAP2_1a and MdMYB10 were fused to three repeats of HA and Flag tag, respectively, and cloned into the plant transformation vector pBWA(V)HS between the CaMV35S promoter and ocs terminator in sense orientation. Both constructs were transferred into A. tumefaciens GV3101 that were then used to infiltrate the young leaves of N. benthamiana using a method previously described [86]. At two days after infiltration, the leaves infiltrated with the two constructs separately and in combination were harvested for protein extraction and Co-IP assay using a method previously described [87].

Dual luciferase reporter assay

The promoter sequence of MdMYB10 was PCR amplified from genomic DNA of “Huashuo” and cloned into
pGreenII 0800-LUC vector to transcriptionally fuse it with the luciferase (LUC) reporter gene. pGreenII 0800-LUC also contains a CaMV35S-Renilla_LUC. The full-length coding sequence of MdAP2_1a was amplified from skin cDNA of "Huashuo" and cloned into pSAK778 vector under the CaMV35S promoter. The two constructs were separately transferred into A. tumefaciens GV3101(pSoup) cells (http://www.weidibio.com) that were cultured and mixed at a volume ratio of 5:1 (5 35S-MdAP2_1a, 1 ProMdMYB10-Luc). The mixed cells were incubated at room temperature without shaking for 2.5 h, before they were infiltrated into N. benthamiana leaves [88].

Firefly LUC and Renilla LUC activities of the CaMV35S-Renilla_LUC and ProMdMYB10-LUC were assayed by the Dual-Luciferase Reporter Assay System (E1910, Promega, https://www.promega.com), according to the manufacturer’s instructions. After treatment for 2.5 d, 1 cm leaf discs around the infiltration sites (six technical replicates) were removed and ground in 70 μl of passive lysis buffer. Subsequently, 50 μl of this crude extract was assayed in 50 μl of LUC assay buffer, and the resultant chemiluminescence was measured (LUC). Stop &Glo® Reagent (50 μl) was added, and a second chemiluminescence measurement was recorded (REN). Absolute luminescence units were measured using a multifunctional microplate reader, SpectraMax®IIIx Platform (MOLECULAR DEVICES, USA), with a 10 s shake and delay 10 min before measuring. Three biological replicates were performed for individual experiments. Leaf discs infiltrated with a mixture of cells containing pSAK778 and pGreen II 0800-LUC were used as a negative control. Primer sequences used to PCR amplify the CDS of MdAP2_1a and promoter of MdMYB10 are listed in Table S1.

Analysis of GUS activities

The reporter plasmid, ProMdMYB10, was cloned in to pBI121GUS vector. The effector plasmid, 35S-MdAP2_1a in pSAK778, was the same as used in the dual-luciferase assay described above. A. tumefaciens cells containing the reporter plasmid were mixed equally (v/v) with those harboring the effector plasmid, and used for A. tumefaciens-mediated transient gene expression assays in Nicotiana benthamiana leaves as described previously [89]. At 2.5 days after the transient transformation, 1 cm leaf discs around the injection sites were collected and histochemically stained with 5-bromo-4-chloro-3-indolyl b-D-glucuronide (X-Gluc) for 24 h at 37°C using the Gusbblue kit (GT0391, Huayueyang Biotech Co., Ltd., Beijing, China), and then incubated in 70% ethanol to remove the chlorophyll before photographing.

Statistical analyses

All analyses were carried out using analysis of variance (ANOVA) of statistical analysis system, GenStat version 18 [90] and SPSS 17.0 Statistics (SPSS Inc., Chicago, IL, USA). Post-hoc differences between means were determined using Fisher’s least significant difference (LSD) test [91] at the 5% significance level.

Acknowledgements

This work was supported by National Key Technology Research and Development Program of China (2018YFD1000100), the New Zealand Ministry of Business, Innovation and Employment, and the Agricultural Science and Technology Innovation Program of China (CAAS-ASTIP-2016-RIP-02). We thank Monica Dragulescu and Gnanaseela Wadasinghe for plant care, Tim Holmes and Wara Bullôt for photography, Lindy Guo for advice in statistical analysis, and Niels Nieuwenhuizen and Kui Lin-Wang for helpful comments on the manuscript.

Author contributions

J.Y., A.G. and Z.Y. conceived the research plan and designed the experiments; T.D. performed most of the experiments; T.D. and J.Y. carried out data analyses; S.T., A.D., B.P. and R.E. analysed apple transgenic plants; R.Z., Z.L., Z.Y., A.A., Z.Z., H.W., M.W., H.D., C.L. and J.L. collected experimental materials and interpreted the data; J.Y. and T.D. wrote the manuscript with contributions of all the authors; All the authors read and approved the final manuscript.

Data availability

The data supporting the findings of this study are available from the corresponding author (Jia-Long Yao), upon request.

Conflicts of interest

The authors declare no competing interests.

Supplementary data

Supplementary data is available at Horticulture Research Journal online.

Supplementary information

accompanies the manuscript on the Horticulture Research website http://www.nature.com/hortres.

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