The Balance of Expression of Dihydroflavonol 4-reductase and Flavonol Synthase Regulates Flavonoid Biosynthesis and Red Foliage Coloration in Crabapples

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Red leaf color is an attractive trait of Malus families, including crabapple (Malus spp.); however, little is known about the molecular mechanisms that regulate the coloration. Dihydroflavonols are intermediates in the production of both colored anthocyanins and colorless flavonols, and this current study focused on the gene expression balance involved in the relative accumulation of these compounds in crabapple leaves. Levels of anthocyanins and the transcript abundances of the anthocyanin biosynthetic gene, dihydroflavonol 4-reductase (McDFR) and the flavonol biosynthetic gene, flavonol synthase (McFLS), were assessed during the leaf development in two crabapple cultivars, ‘Royalty’ and ‘Flame’. The concentrations of anthocyanins and flavonols correlated with leaf color and we propose that the expression of McDFR and McFLS influences their accumulation. Further studies showed that overexpression of McDFR, or silencing of McFLS, increased anthocyanin production, resulting in red-leaf and red fruit peel phenotypes. Conversely, elevated flavonol production and green phenotypes in crabapple leaves and apple peel were observed when McFLS was overexpressed or McDFR was silenced. These results suggest that the relative activities of McDFR and McFLS are important determinants of the red color of crabapple leaves, via the regulation of the metabolic fate of substrates that these enzymes have in common.

Flavonoids are a class of plant secondary metabolites that collectively have diverse functions, including providing protection against abiotic stresses, particularly UV-irradiation, and biotic factors, such as phytophagous insects, as well as attracting pollinators1,2. They also have considerable value as components of the human diet3 and are used in the pharmaceutical industry since they have antioxidative, anticancer and anti-inflammatory properties4,5. Flavonoids have been extensively studied in a range of land plants and have been divided into nine structural subclasses: chalcones, flavones, flavonols, dihydroflavonols, flavandiols, anthocyanins, proanthocyanidins, flavonoid iso-flavonoids and aurones5. Moreover, many genes involved in the flavonoid biosynthetic pathway have been identified and characterized. For example, the genes encoding chalcone synthase (CHS), chalcone isomerase (CHI), F3H flavanone 3-hydroxylase (F3H), flavonoid 3′-monooxygenase (F3′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), uridine diphosphate glucose-flavonoid 3-O-glucosyl transferase (UGFT), flavonol synthase (FLS), leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) have been characterized in several plant species6–14.

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The flavonoid biosynthetic pathway lies downstream of the phenylpropanoid pathway and leads to the formation of anthocyanins and flavonols (Fig. 1). Chalcone synthase (CHS; EC 2.3.1.74) uses beta-coumaroyl-CoA and 3 Malonyl-CoA as substrates to form naringenin chalcone. This condensation reaction is a key step in the pathway leading to the formation of flavonoids. Next, the F3H converts naringenin to dihydrokaempferol. Then dihydrokaempferol and dihydroquercetin are converted to kaempferol and quercetin by FLS, respectively. DFR and FLS also catalyze competing reactions to generate products leading to the spectrum of downstream anthocyanins and flavonols.

MYB family transcriptional factors are known to be involved in regulating the expression of flavonoid biosynthesis genes. For example, in Arabidopsis thaliana, AtMYBL2 interacts with TT8 (TRANSPARENT TESTA 8) to reduce anthocyanin biosynthesis. In the context of flavonoid biosynthesis, AtMYB12 is thought to effect flavonol production by regulation of CHS, CHI, F3H and FLS gene expression levels. In addition, the expression level of the DFR gene TfDFR1 has been shown to be positively correlated with red pigment accumulation in the petals of tulip (Tulipa fosteriana). It has also been reported that the expression level of DFR positively correlates with the abundance of anthocyanins in peanut (Arachis hypogaea).

Flavonols provide another important co-pigment in the colorful organs of terrestrial plants, such as the yellow petals of Lathyrus chrysanthus, and they also influence pollen tube growth. Flavonols are derived from 2, 3-dihydroquercetin and their formation is catalyzed by FLS, which belongs to the 2-oxoglutarate dependent dioxygenase family. Following the identification of an FLS gene from Petunia hybrida, homologous FLS genes have been identified from A. thaliana, Solanum tuberosum, Matthiola incana, Malus domestica and Eustoma russellianum. In addition, it has been reported that increases in transcript levels of an FLS gene from satsuma mandarin (Citrus unshiu), CitFLS, in the fruit peel correlate with flavonol accumulation during fruit development. Recently, it has been found that major floral color changes are a consequence of FLS expression in petunia (Petunia hybrida Vilm.), Lisianthus (Eustoma grandiflorum) and camellia (C. nitidissima). In contrast, an indirect effect of a gallipotia FLS gene (CnFLS1) on anthocyanin accumulation during floral coloration was suggested following flower induction.
an experiment where its overexpression in transgenic tobacco (N. benthamiana) plants resulted in an increase in flavonol content, but a reduction in anthocyanin levels in petals37.

Leaf color is a key determinant of the commercial value of many ornamental plant species; however, much remains to be learnt about the mechanisms of color formation in leaves at the molecular level. The study of pigmentation mechanisms in leaves is therefore significant for both breeding and genetic engineering of ornamental plants. An example of an important ornamental woody plant is Malus crabapple, which belongs to the Rosaceae, Malus Mill family. The numerous plant landscape species in this family provide an excellent source of research material for studying the mechanism of color formation and accumulation, due to their colorful leaves, flowers and fruits38. To date, little is known about the mechanism of anthocyanin and flavonol biosynthesis in ornamental crabapples.

In this current study, we investigated the function of the crabapple DFR (McDFR) and FLS (McFLS) genes in regulating leaf color in different cultivars. We overexpressed and silenced each gene to determine their interaction in controlling flavonol and anthocyanin biosynthesis, and evaluated the gene expression ratio of McDFR and McFLS that is required for leaf color production. We also discuss the metabolic flux between McDFR and McFLS during flavonoid biosynthesis in leaves and fruit. We propose that the finding from this study will assist future attempts to enhance anthocyanin or flavonol accumulation in the leaves of target ornamental species by altering the balance between the McDFR and McFLS enzyme activities.

Results
The anthocyanin and flavonol content of the leaves of two crabapple cultivars. Two extreme leaf color cultivars, ‘Royalty’ and ‘Flame’, have ever-red and ever-green leaves, respectively. We evaluated the abundance of anthocyanins and flavonols in the leaves of these cultivars at 5 development stages of the crabapple leaf growing season by high-performance liquid chromatography (HPLC) (Fig. 2). The chromatography results showed that cyanidin 3-O-glucoside was the predominant anthocyanin, and we found that the major flavonols were quercetin derived compounds, such as quercetin 3-O-diglucoside, quercetin 3-O-glucoside and quercetin 3-O-glycosidase isomer (Fig. 2B). As shown in Fig. 2C, anthocyanin levels in the ever-red leaves of ‘Royalty’ were significantly higher than those in the ever-green leaves of ‘Flame’. A gradual decrease in anthocyanin content was observed in ‘Royalty’ leaves during their development, while anthocyanins were almost undetectable in ‘Flame’ leaves. In contrast, the abundance of flavonols increased during the development of ‘Flame’ leaves, except at stage 5.

The expression levels of anthocyanin and flavonol biosynthetic genes in the leaves of the two crabapple cultivars. To gain insight into the relationship between the expression patterns of anthocyanin biosynthetic genes and anthocyanin/flavonol accumulation, the transcript levels of the key anthocyanin biosynthetic gene McDFR, and the flavonol biosynthesis gene McFLS, were determined by qRT-PCR. The expression of McDFR decreased during the development of both ever-red and ever-green leaves (Fig. 3). In contrast, the transcript levels of McFLS showed an increase in the early developmental stages, and then decreased in stages 4 and 5 in ever-red leaves (Fig. 2A), while only a slight decrease was seen in the last stage of the ever-green leaves (Fig. 3B). This suggested that McDFR expression positively correlated with anthocyanin accumulation during the development of ever-red leaves, and that McFLS expression and flavonol accumulation were positively correlated in leaves of both cultivars.

McDFR plays an important role in the anthocyanin biosynthetic pathway. To further investigate the function of McDFR, we suppressed its expression in the leaves of the red-leaved crabapple cultivar ‘Royalty’ by virus-induced gene silencing (VIGS) using the TRV vector39. Leaves infected with the virus containing the TRV-McDFR construct began to exhibit a green color at 14 days post-infection (dpi), while a more severe phenotype was detected in the new buds of TRV-McDFR infected stem tips at 35 dpi (Fig. 4A). We next analyzed the variation in flavonoid content of the infected leaves by HPLC and observed that in the McDFR silenced areas of the leaves; the levels of anthocyanin were much lower than in non-silenced leaves, while the flavonol content showed a significant increase (Fig. 4B). We also confirmed, by qRT-PCR analysis, that the abundance of endogenous McDFR transcripts was greatly reduced in TRV-McDFR infected leaves. The expression levels of other flavonoid biosynthetic genes, namely CHS (McCHS) and F3’H (McF3’H) were similar in the control and infected leaves, while silencing of the McDFR gene increased the transcript levels of phenylalanine ammonia lyase (PAL; McPAL), CHI (McCHI), FSH (McF3H), FLS (McFLS), ANS (McANS) and UFGT (McUFGT) genes (Fig. 4C).

We also transiently over-expressed the McDFR gene in the stem tips of the ever-green cultivar ‘Strawberry Jelly’, which promoted anthocyanin accumulation at 20 dpi, and a deep green coloration in most of the new buds (Fig. 4A). The anthocyanin content showed a slight increase in the McDFR-overexpressing plants (Fig. 4B) and we confirmed that these plants indeed had higher McDFR transcript levels in the new buds (Fig. 4C). We also detected an increase in the transcript levels of anthocyanin biosynthetic genes and a decrease in McFLS expression, compared with the non-transformed plants (Fig. 4C).

Collectively, these results indicated that McDFR expression is associated with red color formation in crabapple leaves, and that changes in McDFR expression can affect the expression of downstream genes (e.g. ANS and UFGT) involved in the anthocyanin biosynthetic pathway.
Figure 2. Analysis of flavonoid accumulation in 5 developmental stages of leaves from the *Malus* crabapple cultivars 'Royalty' and 'Flame'. S1, 3 days after budding; S2, 9 days after budding; S3, 15 days after budding; S4, 21 days after budding; S5, 30 days after budding. (A) Five leaf developmental stages used for the analysis. (B) HPLC analysis of methanol extracts from crabapple leaf. A1, cyanidin 3-O-glucoside; F1, quercetin 3-O-diglucoside; F2, procyanidin dimer; F3, quercetin 3-O-glucoside; F4, quercetin 3-O-glycosidase isomer; F5, avicularin; F6, acetyl quercetin 3-O-glucoside; F7, acetyl quercetin 3-O-glycosidase isomer; F8, avicularin isomer; F9, quercetin 3-O-rhamnoside; F10, phloridzin. (C) The total anthocyanins content and total flavonols content in 5 developmental stages of leaves of the two crabapple cultivars. Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values (P < 0.05) calculated using one-way analysis of variance (ANOVA) followed by a Duncan's multiple range test.
McFLS is involved in flavonol biosynthesis in crabapple leaves. To confirm the prediction, based on sequence homology, that McFLS is a key flavonol biosynthetic gene, we suppressed its expression in the leaves of ‘Strawberry Jelly’ using the VIGS system and the TRV vector. Approximately 14 days after Agrobacterium infiltration, red coloration was seen in the margin and other areas of the infected leaves (Fig. 5A). HPLC analysis confirmed that the levels of anthocyanins were significantly higher in the silenced leaves than in control leaves infiltrated with TRV alone (Fig. 5B). Finally, as seen in Fig. 4C, the expression of McPAL, McDFR and McANS was up-regulated in infected leaves.

We also transiently overexpressed McFLS in the stem tips of the ever-red leaves of ‘Royalty’. Crabapple stem tips transformed with 35S::McFLS developed new buds with a green color (Fig. 5A), indicating that anthocyanin synthesis was suppressed. This was confirmed by HPLC analysis, which also revealed an increase in flavonol content (Fig. 5B). As expected, we observed an increase in McFLS transcript levels in green buds infiltrated with 35S::McFLS compared to control leaves. This elevated expression of McFLS caused an approximately 1.6-fold decrease in McDFR transcript abundance, and we observed lower levels of expression of other anthocyanin biosynthetic genes (Fig. 5C). To summarize, these results suggested that McFLS expression can promote flavonol accumulation by inhibiting the expression of McDFR, and that McFLS has a competitive relationship with McDFR in the flavonol biosynthetic pathway.
Variation in the expression of McDFR and McFLS in apple fruit. To further characterize the roles of McDFR and McFLS, we infected apple (Malus × domestica 'Fuji') fruits with transgenic Agrobacterium harboring TRV-McDFR, TRV-McFLS, pBI121-McDFR or pBI121-McFLS constructs (Figs 6 and 7). A
Figure 5. Transient expression of McFLS in crabapple. McFLS expression was suppressed by VIGS using the vector pTRV2-McFLS in 'Royalty', or the gene was overexpressed using the vector pBI121-McFLS in 'Strawberry Jelly'. Crabapple leaves injected with the empty TRV and pBI121 vectors and infiltration buffer were used as controls. (A) Phenotype of McFLS silenced or McFLS overexpressing 'Strawberry Jelly' and 'Royalty' leaves. (B) Anthocyanin and flavonol contents at infiltration sites of crabapple leaves in μg/g fresh weight (FW). (C) Relative transcript expression levels in crabapple leaves around the infiltration sites were determined using qRT-PCR. Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values (P < 0.05) calculated using one-way analysis of variance (ANOVA) followed by a Duncan’s multiple range test.
Figure 6. Transient expression of McDFR in apple fruit. McDFR expression was suppressed in apple fruit using the vector pTRV2-McDFR, or the gene was overexpressed using the vector pBI121-McDFR. Apple fruit injected with the empty TRV and pBI121 vectors and infiltration buffer were used as controls. (A) Phenotype of McDFR silenced or overexpressing McDFR apple peels. (B) Anthocyanin and flavonol contents at the infiltration sites of apple peels in μg/g fresh weight (FW). (C) Relative expression levels around the infiltration sites were determined using qRT-PCR. Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values (P < 0.05) calculated using one-way analysis of variance (ANOVA) followed by a Duncan’s multiple range test.
Figure 7. Transient expression of McFLS in apple fruit. McFLS expression was suppressed in apple fruit using the vector pTRV2·McFLS, or overexpressed using the vector pBI121-McFLS. Apple fruit injected with the empty TRV and pBI121 vectors and infiltration buffer were used as controls. (A) Phenotype of McFLS silenced or McFLS overexpressing apple peels. (B) Anthocyanin and flavonol contents at the infiltration sites of apple peels in μg/g fresh weight (FW). (C) Relative expression levels were determined using qRT-PCR around the infiltration sites. Error bars indicate the standard error of the mean ± SE of three replicate measurements. Different letters above the bars indicate significantly different values (P < 0.05) calculated using one-way analysis of variance (ANOVA) followed by a Duncan’s multiple range test.
large increase in anthocyanin accumulation was observed at the sites of TRV-McFLS and pBI121-McDFR over-expression, while the areas of TRV-McDFR and pBI121-McFLS over-expression showed a yellow or white coloration (Figs 6A and 7A). HPLC quantification of the anthocyanin and flavonol content of the infected areas confirmed that the variation in flavonoid content correlated with the observed variation in fruit color (Figs 6B and 7B). Transcript expression analysis of the transiently expressing tissues further revealed that the up-regulation of McDFR expression, or the down-regulation of McFLS expression, was accompanied by a proportional increase in the expression levels of McDFR as well as some of the genes involved in anthocyanin biosynthesis (McCHS, McCHI, McF3H, McF3′H, McDFR, McCHS and McUFGT) and a decrease in the expression levels of McFLS. Importantly, up-regulation of McFLS expression or down-regulation of McDFR expression resulted in fading leaf color and decrease McDFR gene expression (Figs 6C and 7C). Finally, we compared the relationship between the expression of McDFR and the content of anthocyanins, and the results were consistent with the red color formation in leaves and fruit; as well as between the levels of McFLS and the content of flavonols (Figs 5 and 6).

### Discussion

Due to the central role that the DFR enzyme plays in the anthocyanin biosynthetic pathway, DFR genes have been studied in several monocotyledonous and dicotyledonous species, such as Forsythia intermedia, Torenia fournieri, Triticum aestivum, Vitis vinifera and Ascocenda spp., and in some cases at the transcriptional level. The key structural gene, FLS, completes the last step of flavonol synthesis. To our knowledge, although FLS and DFR have been identified and characterized in many land plant species, these genes have yet to be studied in ornamental crabapples. There is growing evidence that anthocyanins and flavonols contribute to the ornamental and economic value of crabapples, which have highly colorful leaves, fruits and flowers. In this study we explored the expression and function of McDFR and McFLS in the biosynthesis and accumulation of anthocyanins and flavonols.

**McDFR and McFLS expression are coordinated and correlate with the accumulation of anthocyanins and flavonols.** In this study, the content of anthocyanins in the leaves of two crabapple cultivars was evaluated, and we observed that it increased in parallel with red pigmentation in several organs/tissues. Furthermore, the anthocyanin levels showed a positive correlation with the expression of McDFR, but a negatively correlation with the expression of McFLS (Table 1). This result is congruent with a recent study showing a negative correlation of CnFLS1 expression and anthocyanin synthesis during floral coloration in the petals of transgenic tobacco expressing this gene. We infer from these observations that there is a competitive relationship between McDFR and McFLS in flavonoid biosynthesis.

**The function of McDFR and McFLS in flavonoid biosynthesis.** Genetic transformation has been used to test the functions of several genes in the flavonoid biosynthetic pathway in model experimental plants, such as tobacco and A. thaliana, as well as in some crop species. For example, overexpression of petunia CHI in tomato fruit was reported to lead to an ~65% increase in flavonol levels, while silencing of a CHS gene in apple fruit resulted in changes in growth and developmental phenotypes. In Gentiana triflora and apples (Malus spp.), the silencing of an ANS gene caused a reduction in anthocyanin content and, consequently, a much weaker color. However, few studies to date have focused on genetic transformation using DFR or FLS genes and none has targeted woody ornamental species, such as crabapple.

Virus-induced gene silencing (VIGS) is a technology that allows the analysis of genes function by suppressing the expression of target genes. In a previous study, we assessed the effect of gene silencing in several plant species, including Nicotiana benthamiana, rose (Rose hybrida) and strawberry (Fragaria ×ananassa). Here, we used tissue cultured seedling buds from different crabapple cultivars (’Royalty’, ’Strawberry Jelly’) to assess the function of McDFR and McFLS through VIGS and overexpression approaches. We determined that silencing of McFLS or overexpression of McDFR promoted the accumulation of anthocyanins, while the opposite results were obtained when McFLS was overexpressed or McDFR expression was silenced. In addition, the abundance of flavonols increased when McFLS was overexpressed or McDFR was silenced. However, it is interesting that levels of flavonols were slightly elevated in transgenic crabapple leaves that were transiently overexpressing McDFR or in which McFLS

| Genes     | McDFR | McFLS | Anthocyanins | Flavonols |
|-----------|-------|-------|--------------|-----------|
| McDFR     | 1.00  | −0.23 | 0.55**       | −0.52**   |
| McFLS     | −0.23 | 1.00  | −0.49*       | 0.27      |
| Anthocyanins | 0.55** | −0.49* | 1.00         | −0.73**   |
| Flavonols | −0.52** | 0.27  | −0.73**      | 1.00      |

Table 1. Correlation coefficients between the levels of expression of two biosynthetic genes and flavonoid content. *Correlation coefficient was significant at the P ≤ 0.05 level. **Correlation coefficient was significant at the P ≤ 0.01 level.
expression was silenced. This phenomenon may be explained if McFLS was still expressed but at lower levels degree in McDFR overexpressed or McFLS silenced crabapple plants. In this scenario the lower expression level of McFLS in these transgenic crabapple plants may have resulted in reduced accumulation flavonol, but not a complete block in flavonol biosynthesis. Moreover, correlation coefficients indicated that the levels of expression of McDFR negatively correlated with those of McFLS, as well as with the abundance of anthocyanins and flavonols (Table 1). We propose that there is a competitive relationship between the expression McDFR and McFLS that results in the production of different classes of flavonoid compounds (i.e. anthocyanins or flavonols) (Figs 4–7).

Since dihydroflavonols are substrates for DFR and FLS, they lie at an important branch point in flavonoid biosynthesis, where precursor substrates are channeled toward either anthocyanin or flavonol production. In this regard, the regulation of DFR expression and the competing DFR and FLS activities may be particularly important. We conclude that the expression of McDFR and McFLS may represent a key mechanism for regulating color in crabapple leaves.

A possible explanation for the expression patterns of McDFR and McFLS in two contrasting varieties. R2R3-MYB transcription factors (TFs), which belong to one of the largest plant TF families, are known to be involved in regulating the biosynthesis of anthocyanins and flavonols in A. thaliana50. A previously study reported that over-expression of the A. thaliana TF AtMYB12 in tobacco (Nicotiana tabacum) resulted in higher levels of flavonols and increased expression of the FLS gene50. Moreover, when AtMYB12 was over-expressed in a tissue-specific manner in tomato, the flavonol biosynthesis pathway was activated51. However, expression of the DFR gene was not induced in AtMYB12 and AtMYB111 over-expressing transgenic plants, as well as in AtMYB111 transgenic lines34,46,51. In apple and crabapple, MYB10, which regulates anthocyanin accumulation and coloration of various organ (e.g. fruit, petals and leaves), can activate the expression of DFR and bind to several the promoters of several anthocyanin biosynthetic genes52,53. In grape berries, expression of the regulatory gene VvMYBFI is light inducible, and is involved in the control of VvFLS1 transcription and flavonol synthesis in fruit34. Thus, we speculated that MYB TFs may similarly regulate flavonol biosynthesis in crabapple, and that their expression levels may vary at different development stages or in response to different environment conditions. Moreover, changes in the transcript levels of MYB TFs control the biosynthesis of flavonoids, by regulating the expression of various members of the flavonoid biosynthetic pathway. We propose that MYB TFs promote anthocyanin biosynthesis by increasing the transcript levels of DFR during fruit or leaf development, leading to red coloration. However, in response to environmental stresses, MYB TFs activate the transcription of FLS resulting in increased production of flavonols.

Conclusion
In this study, we investigated the functions of McDFR and McFLS in regulating leaf color in different crabapple cultivars. We demonstrated that the competitive relationship between the expression of McDFR and McFLS is important for anthocyanin and flavonol synthesis. In addition, the expression of McDFR and McFLS correlated with anthocyanin and flavonol accumulation, as well as with leaf color. The work described in this report may suggest strategies to genetically modify ornamental plants in order to enhance or modulate flavonoid accumulation.

Methods
Plant material and growth conditions. The plant material used included three M. domestica crabapple cultivars: (1) Malus cv. 'Royalty', an ever-red leaf cultivar; (2) Malus cv. 'Flame', an ever-green leaf cultivar. Five year old trees of these cultivars grafted on M. hupehensis were planted in the Crabapple Germplasm Resources Nursery in Beijing University of Agriculture; and (3) Malus cv. 'Strawberry Jelly', an ever-green leaf cultivar, the explants of which were harvested from one-year old branches before spring bud germination, cultured on Murashige and Skoog medium supplemented with 0.1 mg/L 6-Benzylaminopurine (6-BA) and 2 mg/L (2,4-dichlorophenoxy) acetic acid (2,4-D) at 23°C with a 16 h light (200 μmol s⁻¹ m⁻²) /8h dark period. Plants were grown in a greenhouse at 27°C under constant illumination.

Leaves of 'Royalty' and 'Flame' were collected at five different developmental stages (Fig. 1A,B) for gene expression analyses and anthocyanin and flavonol quantification. Wild-type 'Royalty' and 'Strawberry' seedlings were grown in a greenhouse, as above. Apple (M. domestica 'Fujit') fruits were used for analysis of McDFR and McFLS expression. All samples were frozen in liquid nitrogen upon collection, and stored at −80°C until further use.

Construction of VIGS vectors and Agrobacterium-mediated infiltration of crabapple. The coding sequences of McDFR (GenBank: FJ817487) and McFLS (GenBank: KF495602) have previously been deposited in the NCBI (National Center for Biotechnology Information) database. Fragments for the pTRV2-McDFR (540bp) and pTRV2-McFLS (490bp) constructs were amplified by PCR, using gene-specific primers, from a cDNA library derived from Malus crabapple leaves (cv. 'Royalty') using Taq DNA polymerase (TAKARA BIOTECH) according to the manufacturer's instructions. The PCR primers used are shown in Table S1. Virus-induced gene silencing vectors carrying the target gene fragments, as well as pTRV1 and pTRV247–49, were transformed into Agrobacterium tumefaciens strain GV3101 competent cells using a freeze-thaw method55 and selected on kanamycin- rifampicin-containing (50 mg/L)
LB (Luria Bertani media) plates. Positive clones were verified by restriction enzyme digestion and by sequencing the vector-insert junctions. The harvested bacterial cells were then resuspended to an OD$_{600}$ of 0.5 in infiltration medium (10 mM 2-morpholinoethanesulfonic acid [MES], 200 mM acetosyringone, and 10 mM MgCl$_2$) and incubated at room temperature for 3h. Before infiltration, bacteria carrying pTRV1 and pTRV2 were mixed in a 1: 1 volume ratio.

For vacuum infiltration, whole plants were submerged in the Agrobacterium suspension and subjected to a vacuum (−25 kPa). When the rate of air bubbles being released from the plants started to decrease, the vacuum was released quickly to allow bacteria to enter the plant tissues. The vacuum treatment time varied from 30 s up to 3 min, depending on the vacuum source used. After vacuum infiltration, plants were rinsed with sterile water, and cultured on Murashige and Skoog medium. Fifteen plants from each cultivar were treated, and 'Royalty' was used for silencing of McDFR expression, while 'Strawberry Jelly' was used for silencing of McFLS expression. All experiments were repeated three times.

Overexpression of McDFR and McFLS in crabapple leaves and fruits. The full length McDFR and McFLS open reading frames (ORFs) were cloned from the cDNA library described above and inserted into the pBI121 vector$^{53}$ using the XbaI and SacI sites. Primers used for these constructs are shown in Table S1. Transient expression in Malus crabapple leaves was performed using the 'Strawberry' Jelly cultivar and Agrobacterium-mediated transformation, as described above. Agrobacterium cells containing the different constructs were harvested and resuspended in infiltration buffer (10 mM MES, 0.2 mM acetosyringone, and 10 mM MgCl$_2$) to a final concentration of OD$_{600}$ = 0.5. Vacuum infiltration was performed as described above, and infiltration with an empty vector was used as a negative control. Seven days after infiltration, the infected leaves and fruit were collected to observe phenotypic features and to evaluate differences in expression.

RNA extraction. To analyze the effects of VIGS and overexpression on target genes expression, tissue samples from areas showing the silencing and enhancing phenotypes were collected. For controls, corresponding samples were collected from tissues infected by Agrobacterium carrying vectors with no host gene fragment insert, or from non-infected plants. Samples from three independent biological replicates were analyzed. Total RNA was extracted from crabapple leaves using the RNA plant plus Reagent (TIANGEN BIOTECH) according to the manufacturer’s instructions. DNase (TIANGEN BIOTECH) treatment was performed to remove any genomic DNA according to the manufacturer’s instructions. First-strand cDNA was synthesized from total RNA using the Reverse Transcriptase M-MLV (RNase−H−) kit (TaKaRa).

Quantitative RT-PCR analysis. qRT-PCR was performed using the SYBR® Premix Ex TaqTM II (Perfect Real Time) (TaKaRa, Ohtsu, Japan) and the CFX96TM Real Time System (Bio-Rad, USA). The PCR amplification conditions were as previously described$^{56}$, and transcript levels were determined by relative quantification using the Malus 18S ribosomal RNA gene (DQ341382) as the internal control and the $2^\Delta\Delta$CT analysis method was applied. Specific primers (Table S1) for semi-quantitative RT-PCR and qRT-PCR analysis were designed using the primer 5 software$^{57}$.

HPLC analysis. Crabapple leaf samples (approximately 0.8–1.0 g fresh weight) were subjected to extraction with 10 mL extraction solution (methanol: water: formic acid: trifluoroacetic acid= 70: 27: 2: 1)$^{58}$ at 4 °C in the dark for 72 h, shaking every 6 h. The supernatant was isolated by filtration through filter paper and a further filtration through a 0.22 μm Millipore™ filter (Billerica, MA, USA). For the HPLC analysis, trifluoroacetic acid: formic acid: water (0.1: 2: 97.9) was used as mobile phase A and trifluoroacetic acid: formic acid: acetonitrile: water (0.1: 2: 48: 49.9) was used as mobile phase B. The gradients used were as follows: 0 min, 30% B; 10 min, 40% B; 50 min, 55% B; 70 min, 60% B; 30 min, 80% B. Detection was performed at 520 nm for anthocyanin and 350 nm for flavonol$^{58}$, respectively. All samples were analyzed in three biological replicates (extracted from three different batches of leaves).

In this study, we employed HPLC-ESI (±)-MS2 analysis to identify the kinds of compounds by standards and comparing their spectroscopic data to literature$^{59,60}$. Cyanidin-3-O-glucoside, quercetin-3-O-glucoside, avicularin, phloridzin, quercetin (Sigma-Aldrich, Germany), Procyanidin B2 (Sigma-Aldrich, UK) was used as standards.

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Conceived and designed the experiments: J.T. and Y.Y. Performed the experiments: Z.H. and Y.H. Analyzed the data: J.Z. and T.S. Contributed reagents/materials/analysis tools: Y.Y. and T.S. Wrote the paper: J.T., Y.Y. and Z.H.

Additional Information
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