A *Malus* Crabapple Chalcone Synthase Gene, *McCHS*, Regulates Red Petal Color and Flavonoid Biosynthesis

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### Abstract

Chalcone synthase is a key and often rate-limiting enzyme in the biosynthesis of anthocyanin pigments that accumulate in plant organs such as flowers and fruits, but the relationship between CHS expression and the petal coloration level in different cultivars is still unclear. In this study, three typical crabapple cultivars were chosen based on different petal colors and coloration patterns. The two extreme color cultivars, ‘Royalty’ and ‘Flame’, have dark red and white petals respectively, while the intermediate cultivar ‘Radiant’ has pink petals. We detected the flavonoids accumulation and the expression levels of *McCHS* during petals expansion process in different cultivars. The results showed *McCHS* have their special expression patterns in each tested cultivars, and is responsible for the red coloration and color variation in crabapple petals, especially for color fade process in ‘Radiant’. Furthermore, tobacco plants constitutively expressing *McCHS* displayed a higher anthocyanins accumulation and a deeper red petal color compared with control untransformed lines. Moreover, the expression levels of several anthocyanin biosynthetic genes were higher in the transgenic *McCHS* overexpressing tobacco lines than in the control plants. A close relationship was observed between the expression of *McCHS* and the transcription factors *McMYB4* and *McMYB5* during petals development in different crabapple cultivars, suggesting that the expression of *McCHS* was regulated by these transcription factors. We conclude that the endogenous *McCHS* gene is a critical factor in the regulation of anthocyanin biosynthesis during petal coloration in *Malus* crabapple.

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### Introduction

The plant phenylpropanoid biosynthetic pathway leads to the formation of numerous compounds that are involved in diverse physiological and biochemical processes [1]. Some well-studied examples of these compounds include anthocyanins, flavonoids and proanthocyanidins of the flavonoid family, which play a central role in the pigmentation of plant organs, seed germination, UV-B protection and defense against pathogens and biotic stresses [2–9]. Previous studies have focused on anthocyanin biosynthesis in *Arabidopsis thaliana* [9], *Petunia hybrida* [10], *Zea mays* [11] and *Malus domestica* [12–13], and anthocyanin biosynthetic genes have been characterized that are regulated by three classes of transcription factors (TFs): MYB, basic helix-loop-helix (bHLH) and WD40 proteins [14–16]. The reaction catalyzed by chalcone synthase (CHS) is thought to be the key regulatory step in the synthesis of flavonoids by catalyzing the condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA to form naringenin chalcone, a major pigment of many flowers, leaves and fruits [17–19]. Indeed, chalcones provide the structural precursors for a broad range of flavonoids, flavonols, flavanones, anthocyanin glycosides and other derived compounds (Figure 1). Consequently, there has been much interest in CHS and its involvement in many aspects of plant physiology and biochemistry.

The first CHS gene was cloned from parsley (*Petroselinum crispum*) in 1983 [20] and, since then, numerous CHS genes have been isolated, mostly from monocots and dicots, including the legume soybean (*Glycine max*), alfalfa (*Medicago sativa*), pea (*Pisum sativum*), *A. thaliana*, barley (*Hordeum vulgare*), corn (*Zea mays*), grape (*Vitis vinifera*) and others [21–27]. CHS protein sequences are highly conserved among different plants [28], with amino acid homologies of approximately 80–90% [29], and molecular evolution analysis of CHS genes has shown them to be ubiquitous in plants, including early land plants and algae of the Charophyceae [30].

CHS has been well studied in the context of the synthesis and accumulation of anthocyanin pigments and several reports have described the effects of altering CHS expression in transgenic plants. For example, expression of an antisense CHS gene in petunia resulted in flowers that were pale colored, or even white, due to an inhibition of anthocyanin production, and plant fertility
was also affected [17,31]. Similarly, fruits from CHS-silenced strawberry (*Fragaria × ananassa*) lines were reported to have a lighter pink/orange coloration and a significant decrease in the levels of all flavonols, proanthocyanidins and anthocyanins [32]. As another example, fruits from apple (*Malus domestica*) CHS-RNAi knockout lines were shown to have no detectable anthocyanins accumulation and substantially reduced levels of dihydrochalcones and flavonoids [33].

*Malus* crabapple has one of the most economically important sets of ornamental apple germplasm resources. This represents a valuable source of research material, since crabapple exhibits excellent stress resistance and is useful for investigating the mechanism of plant pigmentation, due to the diversity of color in its leaves, flowers and fruits as a consequence of anthocyanins accumulation [34]. In this current study, we compared three crabapple cultivars: two extreme color cultivars, ‘Royalty’ and ‘Flame’ with dark red and white petals respectively, and an intermediate cultivar, ‘Radiant’, with pink petals [35]. To understand better the function of *McCHS* involved in the biosynthesis of anthocyanins and coloration level in the petals of these cultivars, we compared the expression of *McCHS* and other flavonoid biosynthesis pathway structural genes during petal expansion in these three typical cultivars by quantitative real-time PCR (qRT-PCR). Meanwhile, HPLC analysis provided an insight into the accumulation of anthocyanins and other flavonoids compounds in these different crabapple cultivars. The results suggest that the expression of *McCHS* in petals is well controlled, in a tissue- and developmentally specific fashion. We also overexpressed the *McCHS* gene in tobacco to evaluate its activity and the consequences of its expression. To sum up, *McCHS* expression is associated with petal coloration and the expression level of *McCHS* determined the coloration level of petals in

**Figure 1. Scheme of the flavonoid biosynthetic pathway in plants.** Genes encoding enzymes for each step are indicated as follows: PAL, phenylalanine ammonia-lyase; 4CL, cinnamate 4-hydroxylase; CHS, chalcone synthase; CHI, chalcone isomerase; F3′H, flavonoid 3′-hydroxylase; F3H′, flavanone 3′-hydroxylase; F3H, flavanone 3-hydroxylase; DFR, dihydro-flavonol 4-reductase; FNS, flavonol synthase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANS, anthocyanidin synthase; UFGT, UDP-glucose: flavonoid-3′-O-glycosyltransferase.

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different crabapple cultivars. Meanwhile, the expression level of this gene may be regulated by multiple MYB transcription factors.

Results

Petal phenotypes of three Malus crabapple cultivars, ‘Royalty’, ‘Radiant’ and ‘Flame’

The petal phenotypes of three Malus crabapple cultivars, ‘Royalty’, ‘Radiant’ and ‘Flame’, are shown in Figure 2A. ‘Royalty’ petals at stage I were dark red and during flowering the color became more vivid and bright, ultimately reaching maximum color strength at stage IV of full bloom. At the later stage V, after full bloom, the pigmentation faded and dulled (Figure 2B). The petals of ‘Flame’ at stage I showed an obvious light red color, while from stage II to stage V, the color gradually became fainter and eventually the petals turned white (Figure 2D). Interestingly, the petals of ‘Radiant’ were red at stage I, and the color was gradually becoming pink during petal expansion (Figure 2C). Overall, ‘Royalty’ has the most red and vivid flowers, while the ‘Flame’ petals are almost white during petal development, and ‘Radiant’ petals have a significantly color fade process during petal expansion. Next, it is determined how the anthocyanins color the three typical cultivars.

Quantification and identification of flavonoid composition during petal development in three Malus crabapple cultivars

To gain insight into the Malus crabapple petal flavonoid composition and its variation among cultivars, the levels of two anthocyanins (cyanidin and pelargonidin) and five other flavonoids were measured in the petals of the three cultivars (Figure 2E). Consistent with the petal color observations in the red cultivar ‘Royalty’, the abundance of anthocyanins was significantly higher than the other two cultivars, and a gradually decrease in the anthocyanins occurred in petal development, while anthocyanins were only detected in white cultivar ‘Flame’ petals at the first stage. Quercetin and apigenin showed the same spatial and temporal accumulation patterns, and the abundance of these compounds also decreased during petal development in all three varieties. Rutin had a similar profile to quercetin and apigenin and decreased substantially to undetectable levels except for in ‘Flame’ at stage II. In contrast, the catechin content of ‘Flame’ petals was much higher than petals of ‘Royalty’ and ‘Radiant’, and catechin was only found at the first stage of development in ‘Royalty’ and ‘Radiant’ petals. As the precursor for proanthocyanidin, the accumulation of catechin suggests that proanthocyanidin is the main flavonoids compound in colorless tissues and compared with other color-petal cultivars, the proanthocyanidin biosynthetic pathway is primary flavonoids biosynthetic branch pathway in ‘Flame’. In addition, the levels of kaempferol in ‘Royalty’ petals were almost same with those in the other two varieties in the first stage and decreased with the development of petals, the kaempferol contents in ‘Radiant’ and ‘Flame’ were barely detectable from the second stage to the fifth stage (Figure 2E).

Characterization of the Malus crabapple McCHS gene

Based on homology with Malus x domestica sequences and related sequences in the Genome Database for Rosaceae (http://www.rosaceae.org), a gene sequence for the full length CHS (McCHS) was amplified from total RNA of Malus crabapple ‘Royalty’ leaves by RT-PCR and RACE. Specifically, the McCHS cDNA (accession no. FJ399763) is 1,529 bp in length and is predicted to encode a protein of 389 amino acids with a high level of homology to with CHS genes from a range of species (Figure 3A), including Malus domestica (McCHS2; 99% identity), Malus domestica (McCHS1; 99% identity), Malus domestica (McCHS320; 99% identity), Pyrus bretschneideri (PbCHS; 98% identity), Malus domestica (McCHS46; 94% identity) and so on. It appears that chalcone synthase protein sequences have undergone little sequence diversification in the species examined and that they are highly similar in length in different plant species. Other than the putative CHS gene sequences, there are also related sequences that are more divergent from characterized CHS genes and although these genes show high sequence similarity to a Fragaria ananassa CHS (FrCHS; AB201756), e.g. Rubus idaeus (RiPKS-5; EF694718; 97% identity), their functions have yet to be determined. Collectively they are described as polyketide synthase (PKS) genes, a more general description for the CHS and CHS-Like gene family (Figure 3B).

We performed qRT-PCR to determine the expression of McCHS during petal development of the three crabapple cultivars (Figure 3C). Compared with that in colorless cultivars, the relative expression level of McCHS in ‘Royalty’ was much higher, whereas McCHS transcripts were barely detectable in ‘Flame’ petals, except at stage IV. In ‘Royalty’ and ‘Radiant’ the transcript levels decreased gradually among the petal development. The results showed the expression level of McCHS was consistent with the variation of petal color and anthocyanins accumulation in different crabapple cultivars, and the expression intensity of McCHS induced the petal pigmentation level in crabapple. In addition, the color variation in ‘Radiant’ petals was induced by the down-regulation of McCHS transcriptional level.

The expressions of McCHS in leaves and fruits at maturity were also investigated in the three cultivars. The phenotypes of leaf, fruit flesh and fruit pericarp are shown in Figure S1. When comparing leaves, the highest expression of McCHS was in ‘Royalty’, with a value more than twice that of ‘Royalty’ and four times that of ‘Flame’ (Figure 4A). McCHS transcripts were most abundant in the flesh and pericarp of ‘Flame’ fruits, but they were almost undetectable in the pericarp of ‘Royalty’ and ‘Radiant’ (Figure 4A). In fruit flesh, the expression profile of McCHS showed an opposite trend from that of leaves (Figure 4A). These results suggest that the expression of McCHS have different patterns in different tissues/organs among these three cultivars, so we deduced that the expression of McCHS may be also involved in other flavonoids compounds biosynthesis. As an important factor to determine the leaf color, the chlorophyll contents in leaves from the three cultivars were measured at five development stages, the results suggested that the chlorophyll content was higher in ever-red-leaf ‘Royalty’ leaves than spring-red-leaf ‘Radiant’ and ever-green-leaf ‘Flame’, and gradually decreased with the development of leaves in crabapple. So the red leaf color of crabapple is depended on the accumulation of anthocyanin and expression of McCHS, not the chlorophyll content (Figure 4B).

RNA expression profiles of flavonoid biosynthetic genes in Malus crabapple petals

To further confirm how CHS regulated the other anthocyanin biosynthetic genes to affect anthocyanin biosynthesis, we analyzed the expression of anthocyanin biosynthetic genes that are located downstream of McCHS (Figure 5). The expression levels of the downstream genes McF3H, McF3′H, McDFR, McANS and McUFGT were almost all higher in ‘Royalty’ and ‘Radiant’ petals than in ‘Flame’ petals at all developmental stages, with an exception being McUFGT at stage II. To some extent, the expression patterns of McF3H, McF3′H and McDFR showed a similar pattern among the three Malus crabapple cultivars and was closely related to flavonol levels (Figure 2E and 5). While the
expression of McF3H, McF3’H and McDFR in ‘Radiant’ and ‘Flame’ petals was similar to McCHS, but not in ‘Royalty’. Most importantly, McCHS showed the same spatial and temporal expression pattern as McANS and McUFGT in all three cultivars. Taken together, the results of transcriptional level suggested McCHS gene regulate the flavonoid biosynthetic genes to color the plants.

Quantitative PCR expression analysis of putative transcription factor genes in Malus crabapple petals

Transcriptional control of flavonoid biosynthesis is one of the best characterized regulatory systems in plants, and involves integrating both developmental and various biotic and abiotic stress signals and the promoters of flavonoid biosynthetic genes via the control of TFs [37–38]. Previously, we showed that anthocyanin biosynthetic genes are regulated by three classes of TFs [14–16]. To explore the transcriptional regulatory mechanisms of McCHS, we investigated the expression profiles of nine MYB TFs during the five petal developmental stages (Figure 6). For all three crabapple cultivars, the expressions of McMYB1, McMYB2 and McMYB14 were barely detected in petals, except at stage II. The expression levels of McMYB3 and McMYB7 showed a very similar profile in petals from the same cultivar and almost

Figure 2. Flower developmental series of three Malus crabapple cultivars. (A) Typical flower phenotypes of Malus crabapple ‘Royalty’, ‘Radiant’ and ‘Flame’ cultivars through development. Five stages of each cultivar are shown. (B) Color changes in ‘Royalty’ petals. (C) Color changes in ‘Radiant’ petals. (D) Color changes in ‘Flame’ petals. (E) Content of flavonoids and anthocyanin in ‘Royalty’, ‘Radiant’ and ‘Flame’ petals. A spectrophotometric colorimeter was used to measure dynamic changes in petals, and HPLC was used to analyze the flavonoids and total anthocyanin contents. Five stages were tested in this study: (I) 6 days before full bloom; (II) 3 days before full bloom; (III) 1 day before full bloom; (IV) full bloom; and (V) 3 days after full bloom. Error bars indicate the standard error of the mean ± SE of three replicate measurements.
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Figure 3. Sequence characteristics of McCHS and relationships with other CHS proteins. (A) Protein sequence alignment of McCHS and other known anthocyanin biosynthetic proteins from other plant species. Identical residues are shown in black, conserved residues in dark grey and similar residues in light grey. (B) Phylogenetic relationships between McCHS and CHS sequences from other species involved in anthocyanin biosynthesis. Phylogenetic and molecular evolutionary analysis was conducted using MEGA version 5.10 (using the minimum evolution phylogeny test and 1000 bootstrap replicates). (C) Relative expression profile of McCHS at different developmental stages of the petals of three *Malus* crabapple.
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cultivars: ‘Royalty’, ‘Radiant’ and ‘Flame’. Five stages were tested in this study: (I) 6 days before full bloom; (II) 3 days before full bloom; (III) 1 day before full bloom; (IV) full bloom; and (V) 3 days after full bloom. Error bars represent the standard error of the mean ± SE of three replicate reactions. All real-time PCR reactions were normalized using the Ct value corresponding to a Malus crabapple 18S ribosomal RNA gene (DQ341382). The GenBank accession numbers of the proteins are as follows: McCHS (Malus domestica: FJ599763), MdCHS (Malus domestica: AB074485), McCHS-1 (Malus domestica: DQ026297), McCHS-320 (Malus domestica: EU872158), PpCHS (Pyrus bretschneideri; KF148032), MdCHS-46 (Malus domestica: EU872156), MtCHS (Malus toringoides; HQ853494), McCHS-2 (Malus toringoides; JQ582624), MdCHS-23 (Malus toringoides; EU872155), SaCHS (Sorbus aucuparia; DQ286037), FrCHS-2 (Fragaria ananassa; AB201756), RiPKS-5 (Rubus idaeus; EF694718).

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increased with the development of crabapple petals. Furthermore, McMYB4 expression displayed a similar pattern to that of McMYB5 in petals from the same cultivars and decreased at the early development stages and increased at later development stages of crabapple petals. The profiles of McMYB6 and McMYB10 transcript accumulation suggested more specialized expression patterns between ‘Royalty’, ‘Radiant’ and ‘Flame’ petals. In ‘Royalty’ and ‘Flame’, the expressions of McMYB6 and McMYB10 were increased at the early development stages and decreased at later development stages of crabapple petals, but the transcriptions of McMYB6 and McMYB10 were increased with the development of petals in ‘Radiant’.

McCHS showed decreased expression in ‘Royalty’ petals during development, while the expression of McMYB4 increased over the same period, revealing a negative correlation between the McMYB4 and genes in the flavonoid biosynthetic pathway, and the anthocyanins accumulation was negatively regulated by McMYB4. In ‘Radiant’ petals, the expression of McMYB5 was almost consistent with the expression pattern of McCHS and the contents of anthocyanins, flavanol (kaempferol, quercetin and rutin) and apigenin. In ‘Flame’ petals, the expression of McCHS also showed a positive correlation with McMYB5 expression (except for the second petal development stage). Meanwhile, the expression trend of TFs and McCHS were consistent with the accumulation of kaempferol, quercetin, rutin, apigenin and catechin, which were the main flavonoid compounds in white petal cultivar ‘Flame’. These data are consistent with McMYB4, McMYB5 that may be involved in the regulation of McCHS expression during petal development in these cultivars, and these results suggested that due to different accumulation of flavonoids compounds, the McCHS gene have different biosynthesis functions and regulated by different transcription factors.

Overexpression McCHS in tobacco plants

To investigate the temporal and spatial expression of McCHS in plants, transgenic tobacco plants expressing McCHS under the control of a constitutive CaMV35S promoter were generated, and two independent T2 lines were characterized. Compared to wild type, tobacco plants transformed with 35S::McCHS developed a more intense pigmentation in the petals, especially in McCHS-ox-I line (Figure 7A). The lightness value L*, hue value a*, hue value b* of the McCHS-overexpressing tobacco petals were also

Figure 4. Relative expression profile of McCHS in different organs or tissues and chlorophyll content in leaves of the three Malus crabapple cultivars: ‘Royalty’, ‘Radiant’ and ‘Flame’. (A) Relative expression profile of McCHS in different organs or tissues. (B) The Chlorophyll contents in leaves of the three crabapple cultivars. All real time-PCR reactions were normalized using the Ct value corresponding to a Malus crabapple 18S ribosomal RNA gene (DQ341382). Similar material was collected at the same time from the three cultivars. Leaves were all collected at the time of petal stage IV (Figure 2) and the fruits were collected at a fully ripe stage. The Chlorophyll contents of leaves on the tree in vivo were measured by Chlorophyll Content Meter (CL-01, Hansatech, UK). Five Phyllotaxy was measured according to the order of the leaves, respectively. Error bars correspond to the standard error of the mean ± SE of three replicate reactions. 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.

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transcription factors. The expression levels of NtAn1a (doi:10.1371/journal.pone.0110570.g005) at different developmental stages were measured. The ‘H’ value (hue angle) decreased to one-third of that of control plant petals and the ‘a*’ values were 4.0 and 5.0-fold higher than in control plant petals, indicative of a stronger red color (Figure 7B). In addition, we confirmed by microscopy that more anthocyanins accumulated in the petal cells of the transgenic tobacco lines (Figure 7C).

To confirm the biochemical activity of McCHS, anthocyanins content and the expression level of the McCHS gene were detected in the transgenic tobacco petals, (Figure 7D). The anthocyanins content of petals was approximately 3-fold greater in the McCHS-ox-1 line and 2.5-fold greater in the McCHS-ox-3 line compared to control petals. These results are consistent with the visual flower color phenotypes.

The qRT-PCR results confirmed a massive elevation of McCHS transcript levels in the transgenic lines, and the absence of expression in the control plants, as expected. The accumulation of anthocyanins was consistent with the relative expression profile of McCHS in McCHS-overexpressing tobacco petals. Moreover, the over-expression of McCHS in tobacco significantly promoted the expression of the downstream endogenous genes NtF3H, NtF3'H, NtDFR, NtANS, and NtUFGT (Figure 7E). To further explore the molecular regulation mechanism in transgenic tobacco, we tested the expression of several endogenous tobacco anthocyanin regulation factors. Interestingly, the results showed that the massive accumulated McCHS can alter the expressions of these transcription factors. The expression levels of NtAn1a, NtTTG1, MYB305, NtMYC2a and NtMYC2b were increased, and NtAn1b and NtTTG2 were decreased by overexpressed McCHS (Figure 8).

Discussion

CHS, which catalyzes the first committed step in anthocyanin biosynthesis, plays a central role and provides a common chalcone precursor for the production of all intermediates and final products of the flavonoid biosynthetic pathway. Many studies have analyzed the activity of CHS in different plants, including *P. hybrida* [17], *Antirrhinum majus* [39], maize [40], *Arabidopsis* [41], strawberry and tomato [32,42] and apple [33]. The first characterized *chs* mutant, the light white mutant of *A. majus*, whose single CHS gene was knocked out, was reported to lack anthocyanins and UV-absorbing flavonoids [39]. This mutant established a relationship between CHS activity and the white-flowered phenotype. In maize, recessive mutations in both CHS genes of maize, *C2* and *Whp*, result in a white pollen phenotype and male sterility [26,40]. Moreover, down-regulation of *MdCHS* was reported to lead to major changes in plant development, resulting in plants with shortened internode lengths and leaf areas, and a greatly reduced growth rate, as well as a loss of anthocyanins, tannins and phenylpropanoid-related coloration of the stem and fruit skin [33]. But the important role of CHS in petal coloration level in different cultivars is still unknown.

In this current study, we evaluated the expression of McCHS, associated downstream genes and several MYB TFs in three different *Malus* crabapples cultivars, and determined that McCHS shows different expression patterns in various tissues and organs. A positive correlation is identified between the expression of McCHS and the accumulation of anthocyanins in petals, but not in other organs, indicated that the petal coloration level is determined by the expression level of McCHS. The expression of McCHS has more red color in crabapple petals. Meanwhile, the expression of McCHS is responsible for the color fade process during petal expansion in color variation cultivar. The different expression patterns of this gene in accumulation during leaves, petals and fruits development in different cultivars, and different tissues/organisms, which may be explained by diversity flavonoids compounds accumulation during leaves, petals and fruits development in different cultivars, and is regulated by various organ-specific transcription factors. In nectarine, CHS expression was
reported that the expression in the skin was much higher than that in the fruit flesh [36]. The same result was found in apple, the expression of CHS was much higher in the fruit skin than in flesh and green leaves [43]. To sum up, CHS expression was higher in red tissues/organs than in colorless tissues/organs. Several reports showed that ANS [44–46] and UFGT [44,47–49] were two key anthocyanin biosynthetic pathway genes. A positive correlation between the expression of McCHS and its downstream genes McANS, McUFGT was observed. So this is additional evidence that McCHS gene is involved in anthocyanin biosynthesis.

Furthermore, the expression patterns of the downstream genes McF3H, McF3’H and McDFR shows similar pattern to McCHS in ‘Radiant’ and ‘Flame’ petals. This may suggest that the transcript abundance of McCHS may influence the expression of other anthocyanin biosynthetic genes, and thus regulate the production of anthocyanin compounds, or they may be regulated by the same TFs.

NtAn1a, NtAn1b, NtTTG1, NtTTG2, MYB305, NtMYC2a and NtMYC2b have been proved to play an important role in tobacco anthocyanin biosynthesis pathway [50–55]. In our results, overexpressed McCHS can alter the expression of endogenous tobacco anthocyanin regulation factors. So we presume that massive expression of McCHS may be as a feedback signal to activate or inhibit the anthocyanin-related transcription factors, resulting in enhanced anthocyanins accumulation in tobacco.

The expression of CHS is not only responsible for the anthocyanins biosynthesis, but also for the flavonol and other flavonoids compounds biosynthesis in plants. So the transcription level of McCHS is different in various crabapple cultivars, and may be regulated by several MYB transcription factors. We identify a close relationship between McCHS and McMYB4, McMYB5. The three cultivars show individual patterns of correlated expression and some of the results support the conclusions resulting from previously published data. MYB4 functions as a transcription repressor which involved in the regulation of diversity secondary metabolism, such as anthocyanins, lignin, flavonol, proanthocyanidin, and as a target gene that the transcript level of CHS was regulated by MYB4 in A. thaliana, turnip, Kiwifruit, Pinus taeda and so on [56–60]. The transcript levels of MYB5 and all proanthocyanidin-specific genes were previously shown to be down-regulated, while anthocyanin-specific gene expression increased, leading to a switch from proanthocyanidin biosynthesis to anthocyanin biosynthesis in the mature phase of grape berries [61–64]. Transient expression assays have shown that VvMYB3a and VvMYB5b could activate several grapevine flavonoid pathway genes and affect the
biosynthesis of anthocyanins, flavonols, tannins and lignins in reproductive organs when overexpressed in tobacco (Nicotiana tabacum), including CHS gene [65–66]. Meanwhile, the promoter of McCHS has several MYB-binding cis-elements and maybe regulated by MYB TFs [67]. All of these informations support our observation in crabapple that McCHS can be regulated by these transcription factors in different crabapple cultivars due to the various flavonoids compounds accumulation (Figure 2E, Figure 3C and Figure 6).

To confirm that the accumulation of flavonoids was influenced by expression of the McCHS gene, we over-expressed McCHS under the control of a constitutive CaMV35S promoter in tobacco and observed an increase in anthocyanins accumulation, as well as increased expression of downstream endogenous tobacco anthocyanin biosynthesis genes. This may indicate that massive expression of McCHS may regulate the downstream genes to promote anthocyanin accumulation. It has also been shown that overexpression of CHS can lead to an accumulation of flavonoids. Such as, overexpression of a CHS gene in Linum usitatissimum, tomato and potato (Solanum tuberosum) resulted in an increase in total phenolic compounds in Linum usitatissimum [68], an accumulation of naringenin in the tomato fruit flesh and an accumulation of anthocyanins in potato tubers, respectively [69]. Besides overexpression, silencing of CHS gene expression in

Figure 7. Phenotypic analysis of McCHS overexpressing transgenic tobacco flowers and expression profiles of target genes. (A) Typical flower phenotypes of control lines (CK) and McCHS-ox-1 and McCHS-ox-3 tobacco plants overexpressing McCHS. (B) Petal color of control plants (CK) and McCHS-ox-1 and McCHS-ox-3. (C) Microscopic observation of the transgenic tobacco petals. (D) Content of total anthocyanin in petals of control lines (CK) and McCHS-ox-1 and McCHS-ox-3. (E) Relative expression profiles of endogenous anthocyanin biosynthesis genes in transgenic tobacco flowers. A spectrophotometric colorimeter was used to measure color changes in petals and HPLC was used to analyze the total anthocyanin content in the petals of transgenic tobacco. Real-time PCR was used to assess the expression of target genes (NtCHS, NtF3H, NtF3’H, NtDFR, NtANS, NtUFGT) in McCHS-ox tobacco plants. CK refers to wild type tobacco. All real time-PCR reactions were normalized using the Ct value corresponding to the NtActin gene (GQ339768). Error bars correspond to the standard error of the mean ± SE of three replicate reactions. Analysis of relative expression levels of control and McCHS-ox lines and 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.

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several plants has been shown to result in reduced anthocyanin levels in flowers. CHS gene silencing in tobacco [70], chrysanthemum [71], *P. hybrid* [17,72], *carnation* [73], Gutterson rose [73], lisianthus [74] and gentian [75] resulted in plants with very pale pink, or entirely white flowers due to reduced anthocyanin levels, which again is congruent with the results reported here for *Malus* crabapple.

The expression of CHS is regulated by several factors, such as environmental conditions, the variation in the conditions in plants (such as pH), transcription factors and so on [12,76–79]. Recently, the report showed that the different methylation levels of OgCHS promoter altered the expression of this gene in different *Oncidium* orchid cultivars [19]. Therefore, epigenetic modification is a new sight that it can affect the anthocyanin biosynthetic gene expression. Future study is required on the possible regulation mechanism controlling McCHS expression in crabapple.

**Conclusions**

In this study, we have shown evidences that the expression level of McCHS is consistent with the variation of petal color and anthocyanins accumulation in different crabapple cultivars, and the expression intensity of McCHS determined the petal pigmentation level in crabapple specially. On the other hand, the expression of McCHS is responsible for the color fade process during petal expansion in color variation cultivar. The regulation mechanism of McCHS expression is complicated, and several transcription factors might be involved in regulating McCHS expression in crabapple petals. Key future questions that need to be addressed include but not limited to the transcription regulation and epigenetic modification of McCHS expression. Undoubtedly the work described in this report will trigger a series of exciting future research projects to elucidate the mechanisms governing plant coloration.

**Materials and Methods**

**Plant materials**

The petals of *Malus* crabapple cv. ‘Royalty’, ‘Radiant’ and ‘Flame’ were collected at different development stages (I, six days before full bloom; II, three days before full bloom; III, one day before full bloom; IV, full bloom V, three days after full bloom). These trees were grown in the Crabapple germplasm of Beijing University of Agriculture (Changping District, Beijing, China) received standard horticultural practices, and disease and insect control. Control and T2 transgenic tobacco (*Nicotiana benthamiana*) plants were grown in a greenhouse. Flowers were collected

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**Figure 8. Relative expression profiles of endogenous tobacco anthocyanin regulation factors during flower development.** Real-time PCR was used to analyze the transcripts in transgenic tobacco flowers, and all real time-PCR reactions were normalized using the Ct value corresponding to the *NtActin* gene (GQ339768). Error bars correspond to the standard error of the mean ± SE of three replicate reactions. Analysis of relative expression levels of control and McCHS-ox lines and 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. doi:10.1371/journal.pone.0110570.g008
for studies at the full-bloom stage (IV). The leaves of *Malus* crabapple cv. ‘Royalty’, ‘Radiant’ and ‘Flame’ were collected at the same time with petals at the full bloom stage. Fruits were collected at their fully mature stage in October 2012. At sampling time all the plant materials were immediately frozen in liquid nitrogen and stored at -80 °C until RNA or phenolic compounds were extracted.

Color analysis

For flower color evaluation, color components of the CIE L*a*b* coordinate, namely lightness and hue, were measured immediately after flowers were picked with a hand spectrophotometer (Konica Minolta CR-400, Minolta, Japan, Tokyo, Japan). The L* value represents brightness and darkness, the a* value represents greenness and redness as the value increases from negative to positive, and the b* value represents blueness and yellowness. The C* value represents chroma, calculated according to C* = \sqrt{a^2 + b^2}. The hue angle (H) was calculated according to the following equation: H = \arctan \left( \frac{b^*}{a^*} \right) [80]. Three areas of the petal adaxial surface were subjected to color measurement. Values were obtained and averaged from three replicate petals at 5 stages from three different flowers. For leaf color evaluation, the Chlorophyll contents of leaves on the tree in vivo were measured by Chlorophyll Content Meter (CL-01, Hansatech, UK). The same part of leaf adaxial surface was subjected to color measurement. Five Phyllotaxy was measured according to the order of the leaves, respectively. Values were obtained and averaged from three replicate leaves of five Phyllotaxy from three different cultivars.

Identification and quantification of anthocyanins and flavonols

Pigments were quantified by high-performance liquid chromatography (HPLC). One hundred milligrams of petals were homogenized with a mortar and pestle in liquid nitrogen, then 1 ml of extraction solution (methanol: water: formic acid [70:27:2:1, v/v]) was added and the mixture was stored overnight at 4 °C in the dark. The mixture was then centrifuged at 4 °C at 12,000 g for 15 min, and the supernatants were filtered through a 0.22 μm Millipore filter (Billerica, MA, USA). Anthocyanins were identified and quantified using an HPLC1100-DAD system (Agilent Technologies, Waldbronn, Germany). Detection was performed at 520 nm for anthocyanins, 280 nm for flavonoids. A NUCLEODUR C18 column (250 mm x 4.6 mm) (Pretech Instruments, Sollentuna, Sweden) operating at 25 °C was used for separation and compounds were eluted in a mobile phase consisting of solvent A, (trifluoroacetic acid [250 mm x 4.6 mm]) (Pretech Instruments, Sollentuna, Sweden) and solvent B was initially 30% and increased linearly in steps to 35% at 5 min, 40% at 10 min, 50% at 30 min, 55% at 60 min, 60% at 70 min, 30% at 80 min. HPLC analysis was performed as described in Ohno [82].

RNA extraction and Quantitative real-time PCR analysis

Total RNA was extracted from flowers, leaves and fruit flesh and pericarp using an RNA Extract Kit (Aidlab, Beijing, China), according to the manufacturer’s instructions. DNase I (TaKara, Japan) was added to remove genomic DNA and the samples were subjected to cDNA synthesis using the Access RT-PCR System (Promega, USA) according to the manufacturer’s instructions. The expression levels of *McCHS*, *McF3H*, *McF3’H*, *McDFR*, *McANS*, *McUFGT*, *McMYB1*, *McMYB2*, *McMYB3*, *McMYB4*, *McMYB5*, *McMYB6*, *McMYB7*, *McMYB10*, *McMYB14*, *NtActin* (GQ339768) was used as a reference gene for the target genes *McCHS*, *McF3H*, *McF3’H*, *McDFR*, *McANS*, and *McUFGT*, *McMYB1*, *McMYB2*, *McMYB3*, *McMYB4*, *McMYB5*, *McMYB6*, *McMYB7*, *McMYB10*, *McMYB14*, *NtActin* (GQ339768) was used as a reference gene for the target genes *NtCHS*, *NtF3H*, *NtF3’H*, *NtDFR*, *NtANS* and *NtUFGT* were analyzed using the same techniques. Primers are designed by NCBI Primer BLAST and listed in Table S1. *Malus* ribosomal 18S rRNA gene (DQ911382) was used as a reference gene for the target genes *McCHS*, *McF3H*, *McF3’H*, *McDFR*, *McANS*, and *McUFGT*, *McMYB1*, *McMYB2*, *McMYB3*, *McMYB4*, *McMYB5*, *McMYB6*, *McMYB7*, *McMYB10*, *McMYB14*, *NtActin* (GQ339768) was used as a reference gene for the target genes *NtCHS*, *NtF3H*, *NtF3’H*, *NtDFR*, *NtANS* and *NtUFGT*. The resulting cDNA samples for reverse transcription were serially diluted (1, 1/10, 1/100, 1/1000, 1/10000). Real-time RT-PCR analysis was carried out in a total volume of 20 μl, containing 9 μl of 2×SYBR Green qPCR Mix (Takara, Japan), 0.1 μM of each specific primer, and 100 ng of template cDNA. The reaction mixtures were heated to 95 °C for 30 s, followed by 39 cycles at 95 °C for 10 s, 59 °C for 15 s, and 72 °C for 30 s. A melting curve was generated for each sample at the end of each run to ensure the purity of the amplified products. Standard dilution curves were performed for each gene fragment, and all data were normalized to the level of the reference gene transcript. Primers for real-time experiments were designed using primer premier v5.0 software with forward and reverse primers corresponding to two different exons.

Phylogeny and sequence alignment

Protein consensus sequences were determined from the coding sequences of *CHS* and aligned with translated genome reference sequences and published CHS protein sequences from other species with DNAMAN 5.2.2. A phylogenetic tree was produced using MEGA version 5.10 [83] based on the coding sequence alignment of chalcone synthise-like genes, using a minimum evolution phylogeny test and 1,000 bootstrap replicates.

Expression vector construction and tobacco transformation

The entire *McCHS* coding sequence was amplified by PCR with the *MeCHS*-F and *MeCHS*-R primers, using cDNA from ‘Royalty’ petals as a template. The primers for *MeCHS* amplification contained *Spel* and *KpmI* restriction enzyme sites, which were used to clone the coding region of the *MeCHS* gene into the pH121 vector. This was then used to transform *Agrobacterium* strain LBA4404 which in turn was used to transform tobacco (*N. benthamiana*) Wisconsin 38 using the leaf disk method [84]. Transgenic plants were selected based on kanamycin resistance. T2 progeny from the transgenic plants was used for further analysis and compared to wild-type non-transformed lines grown under the same conditions. All the primers used are listed in Table S1.

Statistical analysis

Statistical analysis and graphing was carried out using the OriginPro 8 statistical software (OriginLab Corporation, USA). Microsoft Office PowerPoint 2003 was used for artwork. Error bars represent mean ± SE of three replicate reactions.
Supporting Information
Figure S1 The phenotypes of different organs of the three Malus crabapple.

(DOC)

Table S1 Sequences of primers and relevant accession numbers.

(DOC)

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Author Contributions
Conceived and designed the experiments: JT YY. Performed the experiments: DT JZ TS. Analyzed the data: JZ JT YY. Contributed reagents/materials/analysis tools: YY TS. Wrote the paper: DT JT YY.

References
1. Toni M, Kutchan (2005) A role for intra- and intercellular translocation in natural product biosynthesis. Curr Opin Plant Biol 8: 292–300.
2. Debrajun I, Nes N, Perez P, Devic M, Grandjean O, et al. (2003) Proanthocyanidin-accumulating cells in Arabidopsis thaliana: Regulation of differentiation and role in seed development. Plant Cell 15: 2534–2543.
3. Price SF, Beven PJ, Valladao M, Watson BT (1995) Cluster sun exposure and queretin in Pinot noir grapes and wine. Am J Enol Vitic 46: 187–194.
4. Grönquist M, Bezzarides A, Attigale A, Meinwald J, Eiser M, et al. (2001) Attractive and defensive functions of the ultraviolet pigments of a flower (Helenium heliops). PNAS 98: 13745–13750.
5. Downey MO, Harvey JS, Robinson SP (2004) The effect of bunch shading on berry development and flavonoid accumulation in Shiraz grapes. Aust J Grape Wine Res 10: 25–43.
6. Albert NW, Leis DH, Zhang HB, Irving LJ, Jameson PE, et al. (2009) Light-induced vegetative anthercyanin pigmentation in petunia. J Exp Bot 60: 2191–2202.
7. Czemmel S, Fracke R, Weisshaar B, Cordon N, Harris NN, et al. (2009) The grapevine R2R3-MYB transcription factor VvMYB1 regulates flavonoid synthesis in developing grape berries. Plant Physiol 151: 1513–1530.
8. Panisarisi PA, Aberyshinge IS, Kumar V, Trottier D, Dey D, et al. (2004) Flavonoid biosynthesis in the tea plant Camellia sinensis: properties of enzymes of the prominent epicarpicin and catechin pathways. Arch Biochem Biophys 421: 43–52.
9. Burbulis LE, Shirley BW (1999) Interactions among enzymes of the Arabidopsis flavonoid biosynthetic pathway. PNAS 96: 12929–12934.
10. Quatrrocchi F, Wang J, Van der Woude K, Souter E, de Vetten N, et al. (1999) Molecular analysis of the anthocyanin2 gene of petunia and its role in the evolution of flower color. Plant Cell 11: 1433–1444.
11. Winkel-Shirley B (2001) Flavonoid biosynthesis: A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol 126: 485–493.
12. Chang D, Wang KL, Espley RV, Volk RK, How NM, et al. (2013) An ancient duplication of apple MYB transcription factors is responsible for novel red fruit-flesh phenotypes. Plant Physiol 161: 223–239.
13. Fung PJ, Li MJ, Ma FW, Cheng L (2013) Phenylpropanoid metabolites and expression of key genes involved in anthocyanin biosynthesis in the shaded peel of apple fruit in response to sun exposure. Plant Physiol Bioch 69: 54–61.
14. Richi L, Barrieu F, Bogs J, Kappel C, Delrot S, et al. (2011) Recent advances in the transcriptional regulation of the flavonoid biosynthetic pathway. J Exp Bot 62: 2465–2483.
15. Bai YH, Pattanaik S, Patra B, Werkman RJ, Xie HC, et al. (2011) Flavonoid-biosynthesis in the tea plant Camellia sinensis: properties of enzymes of the prominent epicarpicin and catechin pathways. Arch Biochem Biophys 421: 43–52.
16. Sparvoli F, Martin C, Weisshaar B, Cordon N, Harris NN, et al. (2009) The grapevine R2R3-MYB transcription factor VvMYB1 regulates flavonoid synthesis in developing grape berries. Plant Physiol 151: 1513–1530.
17. Sanjaya MA, de Vos CHR, Martens S, Jonker HH, Rosin FM, et al. (2007) Arabidopsis Proanthocyanidin-accumulating cells. Mol Genet Genomic 71: 429–440.
18. Shirley BW, Bubaskei WL, Storz G, Bruggemann E, Koornneef M, et al. (1995) Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. Plant J 6: 861–877.
19. Lunkerbein S, Coder H, De Vos CHR, Schaa JG, Boone MJ, et al. (2006) Molecular characterization of a stable antiseque chalcone synthase phenotype in strawberry (Fragaria ananassa). J Agr Food Chem 54: 2143–2153.
20. Dure AP, Jones S, Michie TK, Stevenson DE, et al. (2013) Phenotypic changes associated with RNA interference silencing of chalcone synthase in apple (Malus xdomestica). Plant J 74: 398–410.
21. Wang YS, Gao LP, Shan Y, Liu YJ, Tian YW, et al. (2012) Influence of shade on flavonoid biosynthesis in tea (Camellia sinensis (L.)) or syn. Zute. Sci Hort 141: 7–16.
22. Shi XH, Zhang J, Yao YC, Tian J, Song TF, et al. (2012) Isolation and expression of MeiF3H gene in the leaves of crabapple. Acta Physiol Plant 34: 1353–1361.
23. Ravaglia D, Espley RV, Henry-Kirk RA, Andreotti C, Zosi V, et al. (2013) Transcriptional regulation of flavonoid biosynthesis in nectarine (Punica persica) by a set of R2R3 MYB transcription factors. BMC Plant Biol 68: 1471–1485.
24. Grotewold E (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol, 57: 761–780.
25. Czemmel S, Heppel SC, Bogs J (2012) R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. Protoplasma 249: 109–118.
26. Kuckuck H (1936) Uber vier neue Serien multipler Allele bei Antirrhinsum majus. Mol Genet Genomic 71: 429–440.
27. MO YY, Nagel C, Taylor LP (1992) Biochemical complementation of chalcone synthase mutants defines a role for flavonol in functional pollen. PNAS 89: 7213–7217.
28. Espley RV, Hellens RP, Potter RJ, Stevenson DE, Ammal SK, et al. (2007) Expression of key genes involved in anthocyanin biosynthesis in grape (Vitis vinifera L.). Planta 216: 761–780.
29. Shirley BW, Bubaskei WL, Storz G, Bruggemann E, Koornneef M, et al. (1995) Transgene-mediated suppression of chalcone synthase expression in Petunia hybrida results from an increase in RNA turnover. Plant J 6: 861–877.
30. Lunkerbein S, Coder H, De Vos CHR, Schaa JG, Boone MJ, et al. (2006) Molecular characterization of a stable antiseque chalcone synthase phenotype in strawberry (Fragaria ananassa). J Agr Food Chem 54: 2143–2153.
31. Dare AP, Jones S, Michie TK, Stevenson DE, et al. (2013) Phenotypic changes associated with RNA interference silencing of chalcone synthase in apple (Malus xdomestica). Plant J 74: 398–410.
32. Wang YS, Gao LP, Shan Y, Liu YJ, Tian YW, et al. (2012) Influence of shade on flavonoid biosynthesis in tea (Camellia sinensis (L.)) or syn. Zute. Sci Hort 141: 7–16.
33. Shi XH, Zhang J, Yao YC, Tian J, Song TF, et al. (2012) Isolation and expression of MeiF3H gene in the leaves of crabapple. Acta Physiol Plant 34: 1353–1361.
34. Ravaglia D, Espley RV, Henry-Kirk RA, Andreotti C, Zosi V, et al. (2013) Transcriptional regulation of flavonoid biosynthesis in nectarine (Punica persica) by a set of R2R3 MYB transcription factors. BMC Plant Biol 68: 1471–1485.
35. Grotewold E (2006) The genetics and biochemistry of floral pigments. Annu Rev Plant Biol, 57: 761–780.
36. Czemmel S, Heppel SC, Bogs J (2012) R2R3 MYB transcription factors: key regulators of the flavonoid biosynthetic pathway in grapevine. Protoplasma 249: 109–118.
37. Kuckuck H (1936) Uber vier neue Serien multipler Allele bei Antirrhinsum majus. Mol Genet Genomic 71: 429–440.
54. Wang WJ, Liu GS, Niu HX, Timko MP, Zhang HB (2014) The F-box protein
53. Zhu Q, Li B, Mu S, Han B, Cui R, et al. (2013) TTG2-regulated development is
51. Wang Y, Liu R, Chen L, Wang Y, Liang Y, et al. (2009) Nicotiana tabacum
50. Bai YH, Pattanaik S, Patra B, Werkman JR, Xie CH, et al. (2011) Flavonoid-
49. Walker AR, Lee E, Bogs J, Debra AJ, David M, et al. (2007) White grapes arose
47. Kobayashi S, Ikegami A, Tsujimoto T, Kobayashi S, Sato A, et al. (2009) DkMyb4 is
46. Debes MA, Arias ME, Grellet-Bouzonville CF, Wulff AF, Martinez-Zamora
57. Schenke D, Bottcher C, Scheel D (2011) Crosstalk between abiotic ultraviolet-B
64. Cutanda-Perez MC, Ageorges A, Gomez C, Vialet S, Terrier N, et al. (2009)