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Isolation and expression of *McF3H* gene in the leaves of crabapple

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Abstract The molecular mechanisms involved in leaf color variation were investigated in crabapple. Using the total RNA from the leaves of crabapple 'Royalty' as the template, the full cDNA of *F3H* (Flavanone-3-hydroxylase) gene (1,370 bp) was cloned by reverse transcription polymerase chain reaction and rapid-amplification of cDNA ends. The gene was named as *McF3H*, containing a 1,092-bp open reading frame encoding a protein of 364 amino acids. Corresponding genomic DNA sequence was 1,983 bp, containing two introns, and all the cleave sites obeyed the GT–AG rule. The expression of *McF3H* gene following leaf development was determined by real-time quantitative PCR in the leaves of three crabapple varieties, 'Flame' (both young and mature leaves are green), 'Radiant' (young leaves are orange to red and mature leaves are green) and 'Royalty' (both young and mature leaves are red to purple). The results showed that *McF3H* gene was expressed in both red and green leaves. But the expression levels of *McF3H* gene in ever-red-leafed 'Royalty' were significantly higher than in evergreen-leafed 'Flame' at all stages of leaf development. The transcript level in 'Radiant' showed the similar temporal pattern to the variation of leaf color following its leaf development. Also, the anthocyanin accumulation levels in crabapple leaves were consistent with the color variation of leaves. These results suggest that *McF3H* gene is one of the important structure genes related to anthocyanin accumulation in crabapple leaves and the red coloration of crabapple leaf is associated with high expression level of this gene.

Keywords Crabapples · Flavanone-3-hydroxylase · Anthocyanin · Leaf color

Introduction

More and more attention has been paid to the breeding of color-leafed (especially red/purple leafed) crabapples for ornamental use, because purple or red foliage, in addition to flower and fruit, is a novel trait and the ornamental appeal enhances the landscapes aesthetics (Wadl et al. 2010). According to leaf color, the crabapple varieties can be classified into the following three types: ever-red-, evergreen- and spring-red-leafed crabapples (Song et al. 2010). Clarifying the complex mechanisms involved in leaf color variation in crabapple, especially at molecular level, may assist the future breeding programs on color-leafed crabapples to a large degree.

Red coloration of leaf is mainly due to the accumulation of flavonoids (anthocyanins) (Lightbourn et al. 2008), which as the major pigments impart the wide range of red and purple colors observed in plant leaves (Lebowitz 1985; Nguyen and Cin 2009). Numerous publications have confirmed that anthocyanins are derived from a branch of the flavonoid biosynthetic pathway (Mol et al. 1998; Schijlen et al. 2004), which is one of the best characterized of a11 plant secondary pathways (Takos et al. 2006). Considerable research on apple indicated that most of the genes in the anthocyanin biosynthetic pathway, and particularly those for chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase

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(F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose:flavonoid 3-O-glucosyl-transferase (UFGT), are positively associated with the accumulation of anthocyanin and the red coloration in apple skin (Ban et al. 2007; Honda et al. 2002).

Of these genes, CHS gene is at the entry point into the anthocyanin pathway, encoding chalcone synthase to catalyze condensation of 4-coumaroyl-CoA with three molecules of malonyl-CoA (Takos et al. 2006). ANS gene encodes anthocyanidin synthase, which catalyzes the reaction from the colorless leucoanthocyanidin to the colored anthocyanidin (Nakatsuka et al. 2008). Based on this, McCHS and McANS, as the first two studied genes in our laboratory, have been cloned recently from the leaves of 'Royalty' crabapple, an ever-red-leafed variety, and the expression patterns of the two genes have been examined in leaves of some crabapple varieties with different leaf color (Song et al. 2010; Tian et al. 2010). However, there was no significant correlation between the leaf color and expression of McCHS and McANS.

Taken into account that flavanone 3-hydroxylase (F3H) is one of the 'core' enzymes acting at the bifurcation of the anthocyanin and flavonols branches, the F3H gene may represent a pivotal point in the regulation of flavonoid biosynthesis (Shen et al. 2006; Pelletier and Shirley 1996). Also, previous research on Petunia and Antirrhinum revealed that a mutation resulting in a loss of F3H activity prevents the progression along the anthocyanin pathway and white flowers are the consequence (Martin et al. 1991; Britsch et al. 1992; Schijlen et al. 2004). So far the F3H gene has been cloned in a variety of plant species, including Petunia hybrida (Britsch et al. 1992), Hordeum vulgare (Meldgaard 1992), Medicago sativa (Charrier et al. 1995), Zea mays (Deboo et al. 1995), Arabidopsis thaliana (Pelletier and Shirley 1996), and Perilla frutescenes (Gong et al. 1997) and so on. However, the information is limited about the isolation and expression of F3H gene associated with leaf pigmentation difference in crabapple leaves.

As one of the initial steps to elucidate the molecular mechanisms for the leaf color variation in crabapple leaves, we decided to isolate the F3H gene from the leaves of ever-red-leafed variety and compare the gene expression level among three varieties with different leaf colors to test the hypothesis that the red coloration of leaves relates to the high-leveled expression of F3H gene.

Materials and methods

Plant materials

The plant materials used for the experiments included three crabapple varieties: (1) Malus 'Flame' (both young and mature leaves are green), (2) Malus 'Radiant' (young leaves are orange to red and mature leaves are green) and (3) Malus 'Royalty'(both young and mature leaves are red to purple). These 5-year-old crabapple trees grafted on Malus 'Balenghaitang' were planted in Crabapple Germplasm Resources Nursery in Beijing University of Agriculture.

Isolation of McF3H gene

For cloning of cDNA of F3H in crabapple, the total RNA was isolated from young leaves of 'Royalty' using the guanidine thiocyanate solution (Chomczynski and Sacchi 1987). The cDNA was prepared from 1 μg of total RNA using the SMART^{TM} RACE cDNA Amplification Kit (Clontech, USA). The PCR reaction solution contained 1.5 mM $MgCl_2$, 200 μM dNTPs, 500 nM each primer, 10 ng cDNA and 1 U LA Taq DNA polymerase in $1\times$ buffer (Takara, Ohtsu, Japan). The cycling conditions were as follows: 94°C for 1.5 min, $30 \times$ (94°C for 30 s, 50–64°C for 1 min, and 72°C for 2 min) and 72°C for 7 min. The 3′ end of McF3H was amplified with primers UPM (Clontech, USA) and F3H3′1: GGCTGGCTTCATC GTGTC. The 5′ end of McF3H was amplified with primers UPM (Clontech, USA) and F3H5′outer: GGCTTGTCTGG CCACCTCG and F3H5′inner: CCCGGTGACGAATCGG ATATG. The PCR products were directly cloned into the pMD-19 vector (Takara) for sequencing.

Using the DNA and cDNA from the leaves of 'Royalty' as template, McF3H was amplified with primers F3HF1: ATGGCTCCTG CTACTACGCT and F3HR1: CAG-TGGACGA CATTTTTGCTTAG. The PCR conditions were as follows: 1.5 mM $MgCl_2$, 200 μM dNTPs, 500 nM each primer, 10 ng cDNA or DNA and 1 U LA Taq DNA polymerase in $1\times$ buffer (Takara, Ohtsu, Japan). Cycling conditions were: 94°C for 5 min, $30 \times$ (94°C for 30 s, 57°C for 1 min, and 72°C for 3 min) and 72°C for 7 min. The PCR products were directly cloned into the pMD-19 vector (Takara) for sequencing.

Sequence analysis

Comparison and analysis of the sequences were performed with the advanced basic local alignment search tool (BLAST) at the National Center for Biotechnological Information (http://www.ncbi.nlm.nih.gov/). The full-length sequences of DNA and protein were aligned using DANMAN 5.2.2 (Lynnon Biosoft, USA). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Kumar et al. 2004) by a minimum evolution phylogeny test and 1,000 bootstrap replicates.

Expression profile of *McF3H* gene

The total RNA was extracted from each leaf tissue of 'Flame', 'Radiant' and 'Royalty' during different development stage using the guanidine thiocyanate solution (Chomczynski and Sacchi 1987). To avoid DNA contamination, total RNA (1 μg) was treated with RNase-free DNase and reverse transcribed in a 20-μL reaction using Promega reverse transcription system (Promega, USA) according to the instructions. The forward and reverse primers used for the real-time PCR of *McF3H* were, 5′-ACGAAGACGAGCGTCCAAAG-3′ and, 5′-CTCCTCCGATGGCAAAGCAA-3′, respectively. *Malus domestica 18S ribosomal RNA* (accession number: DQ341382) was treated with the same reactions to serve as the control. The forward and reverse primers for the real-time PCR of 18s rRNA were 5′-GAGCCTGAGAAACGGCTACC-3′ and 5′-GTCACTACCTCCCCGTGTCA-3′, respectively. 2 μL of this cDNA was amplified using the Bio-Rad iQ™ SYBR-Green Supermix (Bio-Rad, USA) and 1 μL of each 10 mM primer was in a 20-μL reaction. The PCR amplification was performed using the Bio-Rad CFX96 real-time PCR detection system under the following conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles of 94°C for 20 s, 59°C for 30 s and extension at 72°C for 30 s. Fluorescence was collected in the third step of every cycle. The final denaturation was at 95°C for 1 min, and annealing was at 55°C (ascending 0.15°C per 10 s) and kept for 1 min to draw a melting curve. The amplification specificity was checked using the melting curves. The relative quantification of the samples was determined using the Bio-Rad CFX Manager software, integrating primer efficiencies calculated from a standard curve. Since not all the samples were analyzed in the same run, inter-run calibration was required to correct for possible run-to-run variation. So we set identical samples as inter-run calibrators (IRC) that were tested in every run. The correction was calculated using the mentioned software to remove the run-to-run difference. For the gene expression, the sample showing the highest intensity level was used as reference with a relative value of 1. The final data resulted from averages of three biological replicates and at least two technical repetitions.

Measurement of anthocyanin concentration and leaf color

The crabapple leaf samples were freeze-dried and coarsely ground. 0.8 g leaf powders from each samples were extracted with 10 ml extract solution (methanol:water:formic acid:TFA = 70:27:2:1), at 4°C in the dark for 72 h, shaken every 3 h, and the liquid was separated from the solid matrix by filtration through sheets of qualitative filter paper. The filtrate was further passed through 0.22-μm reinforced nylon membrane filters. Then the filtrate was evaporated at 30°C, and the residue was dissolved in 5 ml of water and then purified by solid-phase extraction cartridge (500 mg, 3 ml) C18 Supelclean ENVI-18 cartridge. The cartridge was successively rinsed with water and methanol. In order to separate anthocyanins from flavonols by HPLC, TFA:formic acid:water (0.1:2:97.9) was selected as mobile phase A and TFA:formic acid:acetonitrile:water (0.1:2:48:49.9) as mobile phase B. The gradients were used as follows: 0 min, 30% B; 10 min, 40% B; 50 min, 55% B; 70 min, 60% B; 80 min, 30% B. The anthocyanins in each sample were measured semi-quantitatively by linear regression of Cynidion 3-*O*-glucoside and quantified at 530 nm as anthocyanin equivalents. All of the samples were analyzed in triplicate.

Color intensity and hue were quantified by measurement with a Minolta chromometer, using the $L*a*b*$ system. The data are shown as a ratio of $a*/b*$, where the change from positive toward negative indicates a shift from red to green (Espley et al. 2007).

Results and discussion

Isolation and sequence analysis of *McF3H* gene

The *McF3H* gene (GenBank accession FJ817486) was successfully isolated from crabapple leaves. The complete cDNA sequence was 1,370 bp long, with a poly(A) tail, and contained a 1,092-bp open reading frame (ORF) encoding a 364-amino acid protein. There was a 5′ untranslated region of 58 bp upstream from the start codon, and the coding region was followed by 3′ untranslated region that was 220 bp downstream from the stop codon.

Specific primers derived from the start and stop codon regions of the cDNA had been designed for PCR to isolate genomic DNA sequence of *McF3H*. Finally, a 1,983-bp PCR product was successfully isolated. It had an identical coding region to the full-length cDNA sequence. Like that in most plant species, the isolated genomic DNA sequence of *McF3H* also had two introns. Intron1 and Intron2 were found at 364/540 bp and 970/1,678 bp position, respectively. It was also shown that the splicing sites of the two introns obeyed the GU/AG rule. In intron 1, there were only two direct repeated sequences, TAAAATTT (+61 and +102 bp) and AGCTATTA (+96 and +117 bp). But in intron 2, many repeated sequences existed. For example, there were 10 bp direct repeat, TAAATAAAAT (+104 and +242 bp), 9 bp mirror repeat, ATACTTTTT (+48 and +367 bp) and 12 bp inverted repeat, ATTTTATTTTTC (+361 and +645 bp).

Characterization of the deduced McF3H protein

The deduced McF3H protein contained 364 amino acids, which was similar to amino acid number found in the F3H protein from most plants (340–380 amino acids) (Pelletier and Shirley 1996). The calculated molecular weight and isoelectric point (pI) of the deduced McF3H protein were predicted to be 40.8 and 5.43 kD, respectively. Like other known 2-oxoglutarate-dependent dioxygenases, the encoded McF3H protein had conserved 2OG-FeII_Oxy domain, His-His-Asp-His and Arg-X-Ser, which is essential for Fe(II) coordination and involved in 2-oxoglutarate binding, respectively (Lukačin and Britsch 1997).

The predicted secondary structure of McF3H protein by SOPMA showed a high similarity to that of strawberry and grape. They all contained α-helices, random coil, β-turn and extended strands. Also, α-helices were evenly distributed in the above structures. The putative secondary structure of McF3H protein contained 34.9% α-helices, 15.9% extended strands, 5.2% β-turn and 44.0% random coils.

The modeling of 3-D structure of McF3H protein using SWISS-MODEL (Schwede et al. 2003) indicated that it resembled McANS protein with a jelly-shape configuration, which matched to structure character of 2-ODDs (Prescott and John 1996). The jelly-shape motif formed the core of the enzyme, where was the binding site of Fe^{2+} and substrate, naringenin. A large amount of α-helixes in C-terminal formed a 'cap structure' on the surface of 3-D model. A larger α-helixe in C-terminal and some α-helixes in the middle of sequence became the important frame of jelly structure. The extension chain located inside and formed the gap surface, which was proposed to play an important role in stabilizing the jellyroll core of 2-ODDs (Britsch et al. 1992; Roach et al. 1995; Britsch et al. 1993).

Molecular homology and evolution analysis

Multi-alignment by DNAMAN5.2.2 showed that McF3H contained the similar main ORF compared to other homology genes (Fig. 1). The deduced McF3H showed more than 60% identities to the counterparts of some other plants. This indicated the conserved evolution of F3H proteins.

But an obvious polymorphism was found in the C-terminal region of F3Hs. The difference in amino acid sequences of F3H proteins among different plants derived from the 31 amino acids in the N-terminal region, which contained the water-repellant amino acid residues, the signal sequence indicating inserting site at membrane.

To investigate the evolutionary relationships among McF3H and some other F3H proteins in plants, the phylogenetic tree was constructed using MEGA 4.0 (Fig. 2). The result showed that the amino acid sequence encoded by McF3H had considerable high homology with those in other plants, especially like pear (AY965342), cherry (AB097151) and strawberry (AY691918). The identity of 93.46, 95.26 and 84.38% was observed in the sequences between crabapple and them, respectively. In addition to this, it suggested that the evolution of F3Hs coincided with that of plants because the plants from the same genus were at the same branch of the phylogenetic tree (Fig. 2). The result suggests that McF3H shared a common evolutionary ancestor with other F3Hs based on conserved structure and sequence characteristics such as amino acid homologies and conserved motifs.

All the above information suggested that our cDNA clone of McF3H indeed coded for the corresponding enzyme, flavanone 3-hydroxylase.

Analysis of McF3H gene expression, anthocyanin accumulation and leaf color

The temporal expression patterns of McF3H gene associated with variation of anthocyanin content and leaf color were investigated in the leaves of three crabapple varieties, 'Flame', 'Radiant' and 'Royalty', which have different leaf color variations during leaf development (Fig. 3).

Expression of McF3H gene was detectable in all the three varieties (Fig. 3a). But striking differences were observed. Transcript levels in 'Royalty' were significantly higher during all stages of leaf development than the levels found in 'Flame' and the levels in 'Radiant' were in between the former two varieties except at the 30th day of leaf expansion (30 DALE). Furthermore, the three varieties showed different temporal expression patterns in McF3H gene. Although the transcript levels in both 'Royalty' and 'Radiant' increased rapidly after the leaf expansion and peaked at 6 DALE, 'Royalty', evidently, kept a longer duration in the peak and showed a slower decrease after peak compared to 'Radiant'. Unlike the former two varieties, 'Flame' sustained its transcripts at much lower levels during all stages of leaf development except with a small peak beginning at late stage (18 DALE).

The levels of anthocyanin accumulation in 'Royalty' were the highest among the three varieties at each stage of leaf development, followed by 'Radiant' and then 'Flame' (Fig. 3b). Also, significant differences in temporal expression patterns of anthocyanin accumulation were found among the three varieties. The anthocyanin accumulation in both 'Royalty' and 'Radiant' increased after leaf expansion and reached the maximum levels at 12 DALE, but a more rapid decrease thereafter was observed in 'Radiant' compared to 'Royalty'. As regard to 'Flame',

Fig. 1

Malus 'Royalty'	MAP....ATTLTSIAHEKTLQQKFVRDED.ERPKVAYNDFSNEIPI.ISLAGID.EVEG.RRGEICKKIVAACEDWGIFQIVDHGVDAELISEMTGLARE	92
Pyrus communis	MAP....ATTLTSIAHEKTLQQKFVRDED.ERPKVAYNDFSNEIPI.ISLAGID.EVEG.RRAEICKKIVAACEDWGVFQIVDHGVDAELISEMTGLARE	92
Prunus persica	........................ERPKVAYNNFSNEIPI.ISLAGID.EVEG.RRADICKKIVEACEDWGIFQIVDHGVDTKLISEMTGLARE	67
Fragaria x ananassa	MAPT...PTTLTAIAGENTLQQSFVRDED.ERPKVAYNQFSIDIPI.ISLSGIE.EVEG.RRAEICKKIVEACEDWGVFQIVDHGVDPKLISEMTRLARE	93
Juglans nigra	..............ED.ERPKVAYNQFSTEIPI.ISLAGID.EVHG.RRTEICQKIVEACEDWGVFQVVDHGVDASLISEMTRLARD	69
Dimocarpus longan	MAP.....ATLTALAQEKTLNASFVRDED.ERPKVAYNEFSKEIPV.ISLAGID.EVDG.RRAEICKKIVEACEDWGVFQIVDHGVDTKLISDMTRLARE	91
Ammi majus	MAP.....TTTLTALAQEKTLNSKFVRDED.ERPKIAYNQFSDEIPV.ISLAGIDDSDD.KRSQICRKIVEACEGWGIFQVVDHGVDTDLISEMTRLARE	92
Glycine max	MAPT...AKTLTYLAQEKTLESSFVRDEE.ERPKVAYNEFSDEIPV.ISLAGID.EVDG.RRREICEKIVEACENWGIFQVVDHGVDQQLVABMTRLARE	93
Brassica napus	MAP....GTLNELAGETKLNSKFVRDED.ERPKVAYNEFSTEIPV.ISLAGID.DVGE.RRGEICRQIVEACBNWGVFQVVDHGVDTSLVADMTRLARD	91
Ipomoea batatas	MAAT...LSTLTALAGEKSLQSSFVRDED.ERPKVGYNEFSDEIPV.ISLKGID.DVNG.RRVQIRNDIVKACEDWGIFQVVDHGVDAGLIGBMTRLSKD	93
Solanum pinnatisectum	...M...ASTLTALANEKTLQTSFIRDEE.ERPKVAYDKFSDEIPV.ISLQGID.DINGSRRSEICEEIVNACEDWGVFQVVDHGVDAELLSQMTKLAKE	91
Gentiana triflora	MAPPP.PPSTLTALSKEKTLQQTFIRDED.ERPKIAYNQFSNEIPV.ISLKGLE...DGDERKGICEKIVEACEDWGVFQIVDHGVDLDVVNBMTRLARE	94
Phyllanthus emblic	..........................SCBDGGIFQVVDHGVDTKLISEMTSLARE	29
Lilium speciosum	MAP.V.ATTFLPTISDEKTLRASFVRDED.ERPKVAYNNFSDDIPI.ISLAGMD..EDGPIRSEICGKIVAACEDWGIFQVVDHGVDAGLVTBMNRLARE	94
Iris x hollandic	MAPGASTPPFLPTSSNETTLPVTFVRRGGEARRVAYNAFSDEIPVGIAGRGSTMPETAGGREEIRRKIVAACEPWGIFQVVDHGVDAGLVVDMARLARE	100
Consensus	ce g fq dhg d m l	

Malus 'Royalty'	FFALPSEEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRHRDYSRWPDKPEAWREVTKKYSDELVGLACKLLGVLSBAMGLDTEALTKACV	192
Pyrus communis	FFALPSEEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRHRDYSRWPDKPEAWREVTKKYSDELVGLACKLLGVLSBAMGLDTEALTKACV	192
Prunus persica	FFALPSEEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRHRDYSRWPDKPEGWREVTQKYSDELVGLACKLLGVLSBAMGLDTEALTKACV	167
Fragaria x ananassa	FFALPPEEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPVRHRDYSRWPDKPEGWDVTTQYSDELVGLACKLLEVLSBAMGLEKEALTKACV	193
Juglans nigra	FFAMPPEEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRTRDYSRWPDKPEGWRKVTEEYSDKLVGLACKLLEVLSBAMGLEKEALTKACV	169
Dimocarpus longan	FFALPPEEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPMRTRDYSRWPDKPEGWIDVTKEYSDKLVGLACKLMEVLSBAMGLEKEALTKACV	191
Ammi majus	FFALPAEEKLRFDMIGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPVPARDYSRWPDKPEGWRSVTEVYSDKLVGLACKLLEVLSBAMGLEKEALTKACV	192
Glycine max	FFALPPDEKLRFDMSGAKKKGGFIVSSHLQGESVQDWREIVTYFSYPKRERDYSRWPDTPEGWRSVTEEYSDKVVGLACKLMEVLSBAMGLEKEGLSKACV	193
Brassica napus	FFALPPEEKLRFDMSGGKKKGGFIVSSHLQGESVQDWREIVTYFSYPVRNRDYSRWPTKPEGWVKVTEEYSERLVGLACKLLEVLSBAMGLEKEALTNACV	191
Ipomoea batatas	FFALPPEEKLRFDMSGGKKKGGFIVSSHLQGEAVKDWREIVTYFSYPVRARDYSRWPDKPEGWRAVTEKYSEKLMDLACKLLEVLSBAMGLEKEALTKACV	193
Solanum pinnatisectum	FFELPPDEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRDYSRWPDKPQGWIAVTEKYSEKLMGLACKLLEVLSBAMGLEKEALTKACV	191
Gentiana triflora	FFDLPPEEKLRFDMSGGKKKGGFIVSSHLQGEAVRDWREIVTYFSYPIKSRDYSRWPDKPEGWKSVTEKYSEQLMNLACKLLEVLSBSMRLEKEALTKACV	194
Phyllanthus emblic	FFALPPEEKLRFDMSGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRNRDYSRWPANPEGWIEVTKKYSDELVGLACKLLEVLSBAMGLEKEALTKACV	129
Lilium speciosum	FFALPEDKLRFDMIGGKKKGGFIVSSHLQGEAVQDWREIVTYFSYPIRVRDYSRWPDKPEGWRAVVEAYSEQLVGLACKLLGVLSBAMGLDKEALTKACV	194
Iris x hollandic	FFALPPEEKLRFDMIGGKKKGGFIVSSHLQGEVVQDWREIVTYFSYPIISRNFSRWPDKEKEWRSVTEKYSEKLMBLACKLLGVLSBAMGLEKEALDQACV	200
Consensus	ff p kl fdm g kkggfivsshlqge v dwreivtyf yp r srwp p w v ys m lackl vlse m l el acv	

Malus 'Royalty'	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDDGKTWITVQPVEGAFVVNLGDHGHLLSNGRFKNADHQAVVNSNSSRLSIATF	292
Pyrus communis	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDDGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRLSIATF	292
Prunus persica	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDDGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRLSIATF	267
Fragaria x ananassa	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGKTWIFTVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNHSRLSIATF	293
Juglans nigra	DMDQKVVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKTWIFTVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNYSRLSIATF	269
Dimocarpus longan	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDNGKTWIFTVQPVDGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNCSRLSIATF	291
Ammi majus	DMDQKVIVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKTWITVQPVEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRMSIATF	292
Glycine max	DMDQKVVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDNGKTWIFTVQPVEAAFVVNLGDHAHYLSNGRFKNADHQAVVNSNHSRLSIATF	293
Brassica napus	DMDQKIVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDDGKTWIFTVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNSSRLSIATF	291
Ipomoea batatas	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDGGKTWIFTVQPVDGAFVVNLGDHGHFLSNGRFKNADHQAVVNSERSRVSIATF	293
Solanum pinnatisectum	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDNGKTWIFTVQPVEGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNSSRLSIATF	291
Gentiana triflora	DMDQKIVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDGGKSWITVQPVDGAFVVNLGDHGHYLSNGRFKNADHQAVVNSNYSRLSIATF	294
Phyllanthus emblic	DMDQKVVVNYYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATRDDCKTWITVQPVDTG..	191
Lilium speciosum	DMDQKVVVNFYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDGGNTWIFTVKPIEGAFVVNLGDHGHFLSNGRFKNADHQAVVNSNSSRLSIATF	294
Iris x hollandic	DMDQKVVVNLYPKCPQPDLTLGLKRHTDPGTITLLLQDQVGGLQATKDGGNTWFIQGSLVVNLGDHAHFLSNGRFKNADHQAVVNSNSSRLSIATF	300
Consensus	dmdqk vn ypkcp pdltlglkrhtdpgtitlll qdqvgglqat d w tv p	

Malus 'Royalty'	QNPAQEAIVYPLSVREGEKPILEAPITYTEMYKKKMSKDLELARLKKLAKEQQ..SQDLEK........AKVDTKPVDDIF	363
Pyrus communis	QNPAQEATVYPLCVREGEKPILEAPITYTEMYKKKMSKDLELARLKKLAKEQQ..LQDLET........AKVDTKPVDDIF	363
Prunus persica	QNP...	270
Fragaria x ananassa	QNPAQEAIVYPLKVREGEKPILEEPMTYTEMYKKKMSKDLELARLKKHAKEQ...LQDSEK........TKLEAKPVDDIF	363
Juglans nigra	QNP...	272
Dimocarpus longan	QNPAPEATVYPLKIREGEKPILEEPITFAEMYRRKMSKDLELARLKKLAKEQKLQLQDNEK........SKLEAKPIEQIL	364
Ammi majus	QNPAPNATVYPLKIREGEKAVMEEPITFAEMYRRKMSKDIEMAKLKKLAKEKLLQEQEAEK......AKLQMTPKSADEIF	367
Glycine max	QNPAPNATVYPLKIREGEKPVMEEPITFAEMYRRKMSKDIEIARMKKLAKEKHLQDLENEKHLQELDQKAKLEAKPLKEIL	374
Brassica napus	QNPAPEATVYPLKIREGEKPILEEPITFAEMYRRKMSRDLELARLKKEEHDH...............KEAAKPLDQII	357
Ipomoea batatas	QNPAPEATVYPLTVREGDKPIMEEPITFAEMYRRKMSKDLELARLKKFAKEQQQIVKAA.......AADKNLETKPIDQIL	367
Solanum pinnatisectum	QNPAPDAKVYPLKIREGEKAIMDEPITFAEMYRRKMSKDLELARLKKQKKQ..............TEEAQLESKPIEEIL	357
Gentiana triflora	QNPAPEATVYPLAIRDGEKPVLDEPITFAEMYRRKMSKDLELARLKKQAKEELKNVEK..........AKIEGKALEEIL	364
Phyllanthus emblic	....	191
Lilium speciosum	QNPAPEATVYPLAIRDGEKPVLDAPMTFSEMYRRKMSKDIELAKLKKLANTEAQEVAKKPT.......EVAVQPGKLDQIL	368
Iris x hollandic	QNPAPDAVYPLAIREGEMPVLDEPIKFTEMYRRKMSRDLELASSRSSPRLHENAKVEE.........AVAQPKGLNGIF	371
Consensus		

Fig. 1 Alignment of deduced amino acid sequences of McF3H from crabapple and some known F3Hs from other species. The *numbers* indicate the positions of amino acid. Identical residues are shown in *black*, conserved residues in *red*, and similar residues in *blue*. The accession number of the F3Hs in the GenBank database are as follows: *Malus* 'Royalty', FJ817487; *Pyrus communis,* AY965342; *Prunus persica*, AB097151; *Fragaria* × *ananassa*, AY691918; *Juglans nigra*, AJ278457; *Dimocarpus longan*, EF468104; *Ammi majus*, AY817678; *Glycine max*, FJ770474; *Brassica napus*, DQ513329; *Ipomoea batatas,* AB023790; *Solanum pinnatisectum*, AY954032; *Gentiana triflora*, AB193311; *Phyllanthus emblic*, EU090062; *Lilium speciosum*, AB201532; *Iris* × *hollandic*, AB265225 (color figure online)

the accumulation of anthocyanin showed the highest level at 0 DALE and then decreased gradually thereafter to even non-detectable level at 24 and 30 DALE.

The variation of leaf color during leaf development were quantified using a ratio of *a*/b**, which changes from positive toward negative indicating a shift from red to
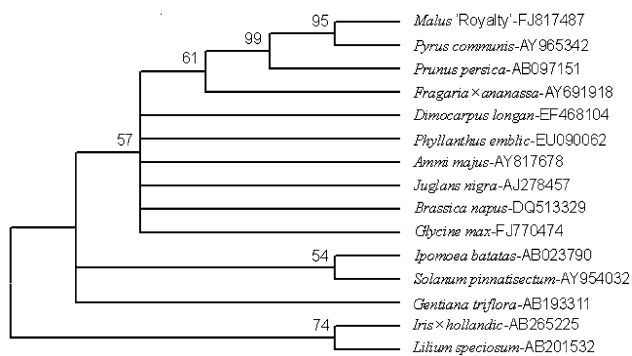
Fig. 2 Phylogenetic tree of McF3H from crabapple and some known F3Hs from others species. *Numbers on branches* indicate percent bootstraps values

green. Of the three varieties, ever-red-leafed 'Royalty' maintained the highest and positive value of a^*/b^* during all stages of leaf development, compared to the lowest and negative value of a^*/b^* in evergreen-leafed 'Flame' (Fig. 3c). But 'Radiant' showed a great change in the ratio

of a^*/b^* following leaf development and leaf color change, with a positive value before 12 DALE (red leaf period) and a negative value thereafter (green leaf period). The variation in ratio of a^*/b^* confirmed the visible leaf color difference among the three varieties and leaf color transition during leaf development (Fig. 3c, d).

Taken together, *McF3H* gene was expressed at much higher levels, producing higher contents of anthocyanin, in red leaves than in green leaves of crabapple at each stage of leaf development. Correlation analysis demonstrated that the expression level of *McF3H* gene related closely to the accumulation level of anthocyanin pigments and variation of red coloration in the crabapple leaves (Fig. 4). Previous research also indicated that *F3H* is one of the first three genes (the other two genes are *CHS* and *CHI*) encoding the early, unbranched segment of the flavonoid biosynthetic pathway (Zuker et al. 2002). In petunia, *F3H* is expressed independently to regulate anthocyanin synthesis (Khlestkina et al. 2009; Quattrocchio et al. 1993). Also, blocking *F3Hs* in *Arabidopsis thaliana* results in decreased levels of

Fig. 3 Temporal variation of *McF3H* expression (**a**), anthocyanin content (**b**), color parameter (**c**) and visual color (**d**) in crabapple leaves at different development stage. Color parameter was shown as a^*/b^* ratio. A shift of a^*/b^* ratio toward negative indicates a color change from *red* toward *green*. DALE represents the day after leaf expansion. The *vertical bars* indicate SD (color figure online)
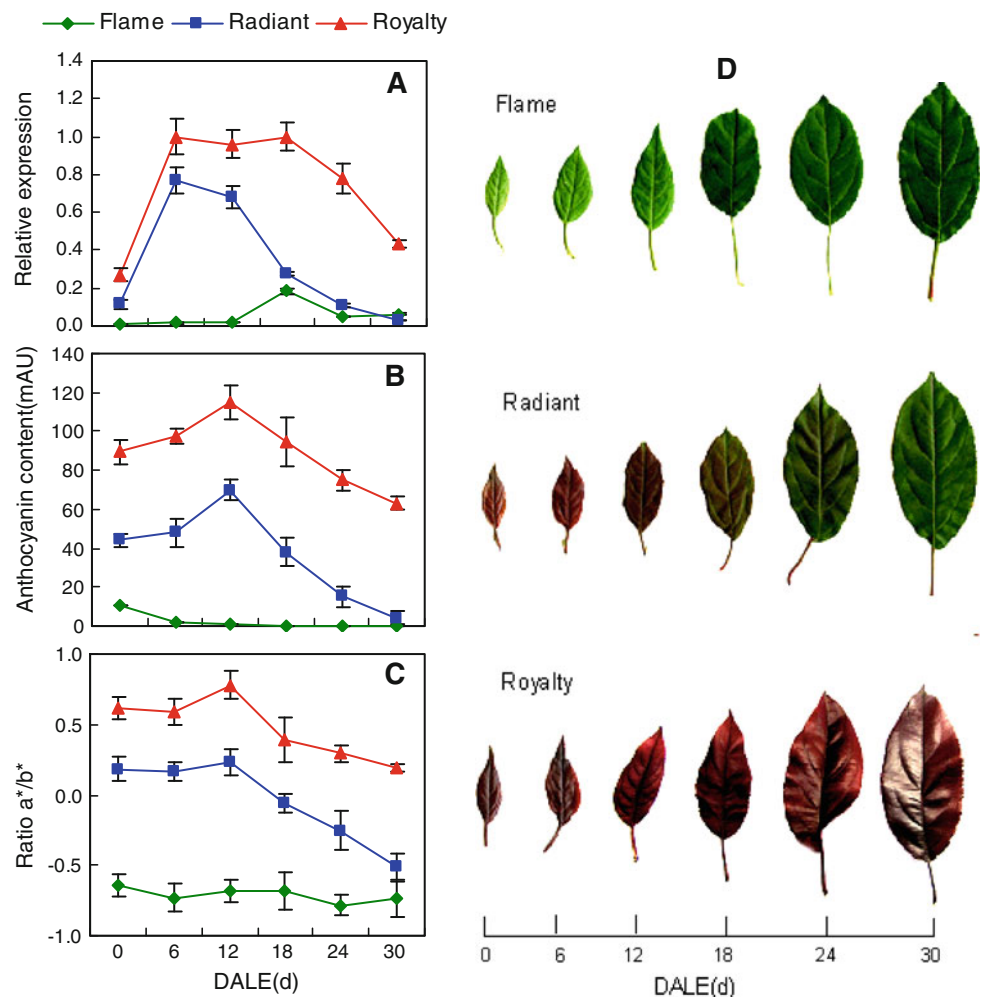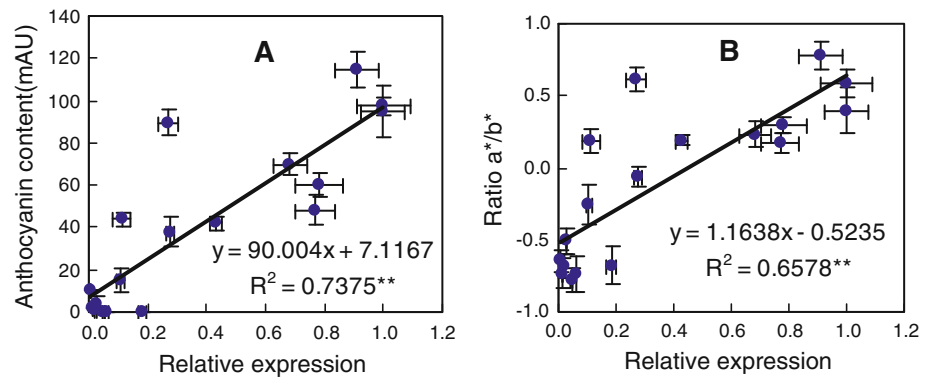
Fig. 4 Correlation analysis between *McF3H* expression and anthocyanin content (**a**) or color parameter (**b**) in crabapple leaves. *Double asterisks* indicate that the correlation was significant at $P \leq 0.01$. The *bars* represent SD

flavonol and anthocyanin (Wisman et al. 1998). It seems that *McF3H* gene is necessary for high accumulation level of anthocyanins to form red coloration in crabapple leaves.

In addition, peak period of *F3H* gene expression and its duration time also appeared to affect anthocyanin accumulation and red coloration in crabapple leaves, although it will need the further evidence to prove. Either 'Royalty' or 'Radiant' with red young leaves had an earlier peak of transcript levels compared to 'Flame' with green young leaves. 'Royalty' with red mature leaf showed a longer peak duration than 'Radiant' with green mature leaf. It suggests that the products of *F3H* gene are active in the biosynthesis of anthocyanin in crabapple leaves, especially at the early stage of leaf development. Research on apple also showed that high transcript levels of *F3H* gene accumulated in flower at an early stage associated with anthocyanin accumulation (Dong et al. 1998). It should be noticed that the maximum expression of *McF3H* gene in 'Royalty' and 'Radiant' preceded the peak of anthocyanin accumulation. This probably attributed to that either the anthocyanin biosynthesis enzymes or the anthocyanin pigments are stable for prolonged time (Kim et al. 2003).

However, the expression of *McF3H* gene can not necessarily guarantee the eventual production of anthocyanins and formation of red crabapple leaves, since the transcript of *McF3H* gene was also be detected in green crabapple leaves, which even had no detectable anthocyanin accumulation (for example, the leaves in 'Flame' at 24 and 30 DALE). Moreover, a small peak of transcript level at 18 DALE in 'Flame' did not induce a small increase in accumulation level of anthocyanins. The reason maybe lies in that the product of *F3H* can serve as an intermediate for the biosynthesis of anthocyanidins as well as flavonols (Honda et al. 2002). F3H seems not a key control point in the anthocyanin biosynthesis pathway to eventually determine the leaf color of crabapple.

Considering that the different enzymes before ANS take part in the production of a large amount of flavonoids rather than only anthocyanins (Nguyen and Cin 2009; Holton and Cornish 1995), it appears that other late step of

F3H gene in the anthocyanin pathway, such as *ANS* or *UFGT*, may control red pigmentation in crabapple leaves. But in our previous research, the expression of *McANS* gene was also detected in evergreen-leafed crabapple. It is possible that *UFGT* may play a key role in controlling the anthocyanins accumulation and red coloration in crabapple leaves. In grapes, it has been shown that UFGT induction during anthocyanin accumulation is a key regulatory step in the development of red coloration in fruit (Boss et al. 1996; Kobayashi et al. 2001). In apple, it also suggested the deficiency of anthocyanin in 'Golden Delicious' is attributed to a block in the last step in the anthocyanin biosynthesis pathway that is catalyzed by the UFGT enzyme (Han et al. 2010). A number of studies also proved that multiple flavonoid structural genes, not a specific gene, are coordinately regulated by regulatory genes for anthocyanin biosynthesis in fruits (Takos et al. 2006). In apple fruits, *MYB*s, transcription factors, have been shown to play an important role in transcriptional regulation of anthocyanins in apple fruit skin and flesh (Espley et al. 2007). Thus, the key gene(s) to control the anthocyanin synthesis may be different among species and sometimes among different tissues of the same species (Khlestkina et al. 2009).

Conclusion

We obtained some molecular information about leaf pigmentation in crabapple. *McF3H* is one of the important structure genes related to anthocyanin accumulation in crabapple leaves and the red coloration of crabapple leaf is associated with high expression level of this gene. Isolation of this gene provides a crucial step in understanding the molecular mechanism of anthocyanin biosynthesis in crabapple leaves. However, an unsolved problem still remains. Which gene(s), on earth, control the anthocyanin synthesis and determine the leaf pigmentation in crabapple? We are now isolating more structure genes, such as *CHI*, *DFR* and *UFGT*, and will try to clone some regulatory genes like *MYB*s from red leaves of crabapple. The

profiles of gene expression will progress understanding of the regulation of crabapple leaf pigmentation, and will help to contribute to the breeding of novel leaf colors in the future.

Author contributions Yuncong Yao and Junping Gao provided an idea, Hongxiang Shen, Jie Zhang, Ji Tian, Tingting Song and Jian Geng performed the experiments, Hongxiang Shen analyzed the data. All authors wrote the text and approved the manuscript.

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