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Analysis of Variation in *MYB1* and *MYB10* in Several Malus Plants

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Abstract For the study of the polymorphism and evolutionary characteristics of *MYB1* and its allele *MYB10* involved in the regulation of anthocyanin biosynthesis in genus *Malus*. The *MYB1* and *MYB10* gene sequences amplified by RT-PCR technology in several *Malus* plants were sequenced and predicted for their protein structures to detect the differences. Six *MYB1* sequences were obtained and the full-length CDS were 1 239 bp except for those of *Malus* cv. Eleyi and *Malus sieversii* (1 209 bp). While nine MYB10 were sequenced and the full-length CDS were 732 bp (*Malus micromalus* was 717 bp). The result from the comparison of protein sequences of *MYB1* and *MYB10* showed that the polymorphism of MYB1 was significantly higher than MYB10 and the polymorphism difference between the alleles may be related to the differentiation of gene function. Two polymorphism sites, the interchange of alkaline arginine and non-polar leucine, located on functional domain which may affect the function of MYB1 in different *Malus* plants. The result suggested that sequence conservatism of the allele *MYB10* and *MYB1* significantly differed from each other, and the genetic function responsible for evolution was differentiated. The result can provide further theoretical basis for the study of the gene function and regulation mechanism of apple fruit color.

Keywords *Malus*; *MYB1*; *MYB10*; Anthocyanin; Transcription factor

The plants from *Malus* were the fruit trees with important economic value and ornamental value, such as apple and begonia. Apple plays an irreplaceable role in fruit production and trade, while begonia is essential for the beautification of the landscape because of their fruit color. The color of the fruit is differential among *Malus* plants. The fruit coloration, an important appearance index for apple fruits, directly determines its commercial value and garden application value. The degree of red on apple peel is determined by the anthocyanin content in the peel, and anthocyanin biosynthesis is under the control of environmental and in vivo development signals, especially the pigmentation by responding to the changes of light (Jaakola, 2013). At present, at least three kinds of genes are known to be involved in the regulation of anthocyanin synthesis, including MYB transcription factor, bHLH transcription factor and WD40 protein (Albert et al., 2011).

MYB1 and its allele *MYB10* (Wang et al., 2010), located in the same locus of apple 9 linkage group (Wang et al., 2010), belong to MYB transcription factors, and their proteins all have a double DNA binding domain named R2R3 at the N-terminal (Allan et al., 2008). *MYB1* and *MYB10* play a key role in the regulation of anthocyanin accumulation in apple peel by binding the promoter region of gene in anthocyanin synthesis pathway (Takos et al, In 2006). MYB transcription factors involved in anthocyanin regulation have been identified in many fruit trees, including strawberry, grape, sweet cherry, peach and Litchi (Zhu Tingting et al., 2016).

In the previous study of *MYB1* in apple, the researchers found that there were at least three alleles with slight differences, namely *MdMYB1-1*, *MdMYB1-2* and *MdMYB1-3*. Among them, the coding regions of *MdMYB1-1* and *MdMYB1-2* were the same, but the difference sites were located in the promoter region. The differential sites of *MdMYB1-1* and *MdMYB1-3* were located in the third exon, causing the conversion of arginine and serine near the C-terminal of the protein, but no significant change in function was detected between the two alleles (Takos et al., 2006). A promoter mutation *MYB10* gene was found in red-fleshed apples (Espley et al., 2009; Mahmoudi et al.,

2012). Regarding the study of *MYB* gene in begonia, Cao Dongmei et al. (2006) cloned *MYB1* of *Malus xiaojinensis*, but the sequence was quite different from that of *MdMYB1*. *MYB10* gene of North American begonias was cloned, and the sequence was the same as that of *MdMYB10* (Tian et al., 2015).

Although a large number of studies have explored the *MYB1* and its allele *MYB10* which control anthocyanin synthesis, there were few studies on other plants in *Malus* except for cultivated apple, and little attention has been paid to the polymorphism of this gene in *Malus*. In this study, the method of homologous gene amplification was employed to analyze the polymorphism of *MYB1* and its allele *MYB10* among the research objects containing several cultivars and begonias, to explore the evolutionary characteristics of the allele of the locus and to speculate the possible influence of evolution on the allele of the locus to provide the technical theoretical basis for the pigmentation of apple peel.

1 Results

1.1 Cloning of *MYB1* and its allele *MYB10*

The PCR results of 9 *Malus* plants (Figure 1) showed that actin fragments (positive control) were successfully amplified, the PCR products were about 250 bp and the size was in accordance with the expected design size. MYB10 were amplified in 9 *Malus* plants and fragments were about 850 bp in size. However, *MYB1* were amplified in 6 *Malus* plants except *Malus*×*domestica* Borkh. cv. Golden Delicious, *Malus micromalus* Makino and *Malus*×*domestica* Borkh. cv. Delicious (The amplification did not achieve after refinement of multiple amplification conditions and primer). The fragments were about 1200 bp. These results suggested that *MYB10* gene may be widely existed in *Malus*, but its allele *MYB1* might not appear in every *Malus* plants.

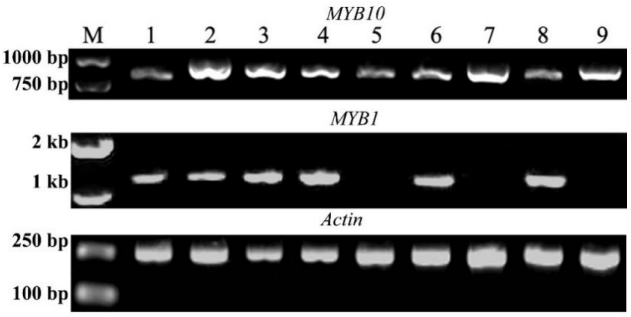

Figure 1 Amplifications of MYB1 and MYB10 in nine species of Malus

Note: Sample number corresponding to table 2; M: DL2000 DNA Maker

1.2 Obtaining sequences of *MYB1* and its allele *MYB10*

Sequencing results showed that the amplified fragments of *MYB10* in nine materials were 817 bp except for *Malus micromalus* Makino which was 802 bp. Among them, the open reading frame length of *Malus micromalus* Makino was 717 bp, encoding 238 amino acids, and the rest of the materials were 732 bp, encoding 243 amino acids. The comparison of the amplified *MYB10* with NCBI database showed that the sequences were highly similar to the *MdMYB10* from NCBI database with a similarity between 97% to 100%. The similarity between nine materials *MYB10* was high, with a sequence consistency of 99.63%. There were seven gene differential sites, and five differential sites were located in the open reading frame, including a 15 bp deletion in *Malus micromalus* Makino.

The amplification of *MYB1* showed that the fragments of *Malus domestica* subsp. chinesis var. binzi, *Malus domestica* Borkh., *Malus robusta* and *Malus* cv. Radiant were 1 241 bp in length, and 1 239 bp and 412 amino acids in open reading frame. While the amplified fragments of *Malus* cv. Eleyi and *Malus sieversii* were 1 211 bp in length, and 1 209 bp and 402 amino acids was in open reading frame. The similarity of *MYB1* from 6 *Malus* plants with the target gene *MYB1* in the NCBI database was between 90% and 98%, and the *MYB1* consistency of 6 *Malus* plants was 94.48%. And there were more polymorphism sites in *MYB1* compared with *MYB10*. Among the polymorphism sites, *MYB1* of *Malus* cv. Eleyi and *Malus sieversii* lost 48 bp and 9 bp fragments and inserted a 27 bp fragment. Sequence analysis showed that the obtained *MYB1* and *MYB10* gene sequences of different

Malus plants were highly similar to those of cultivated apples, but there were still some polymorphism loci in *MYB1* and *MYB10* in different *Malus* plants. The polymorphism of *MYB1* was higher than that of *MYB10*, and there were obvious differences in the conservation between the alleles.

1.3 Obtaining of amino acid sequences in *MYB1* and allele *MYB10*

The sequences were translated into amino acid sequences by machine, and clustering was carried out with the sequences of NCBI database. It was found that *MYB1* of *Malus robusta*, *Malus domestica* subsp. chinesis var. binzi and *Malus domestica* Borkh were similar to those of *Malus baccata* in the database, and *MYB1* of *Malus* cv. Radiant was highly similar to that of apple in the database, clustering into a small branch with *Pyrus bretschneideri* and *Pyrus usssuriensis*. The *MYB1* of *Malus* cv. Eleyi and *Malus sieversii* were similar to *Malus*×*domestica* Borkh. cv. Golden Delicious (Figure 2A). The translating results were highly similar to *MYB1* of plants such as *Malus*×*domestica* Borkh. cv. Golden Delicious and *Malus baccata* in the database, indicating that the amplifications were indeed *MYB1*. The *MYB10* of the nine *Malus* plants clustered together with the Royal Gala, red-fleshed apples and *Malus baccata* in the database and were also similar to those of the pear species (Figure 2B), indicating that the amplifications were indeed *MYB10*. Cluster analysis of *MYB1* and *MYB10* showed that the obtained sequences were clustered into one group with *Maloideae* plants and were more closely related to those of *Liliaceae*.

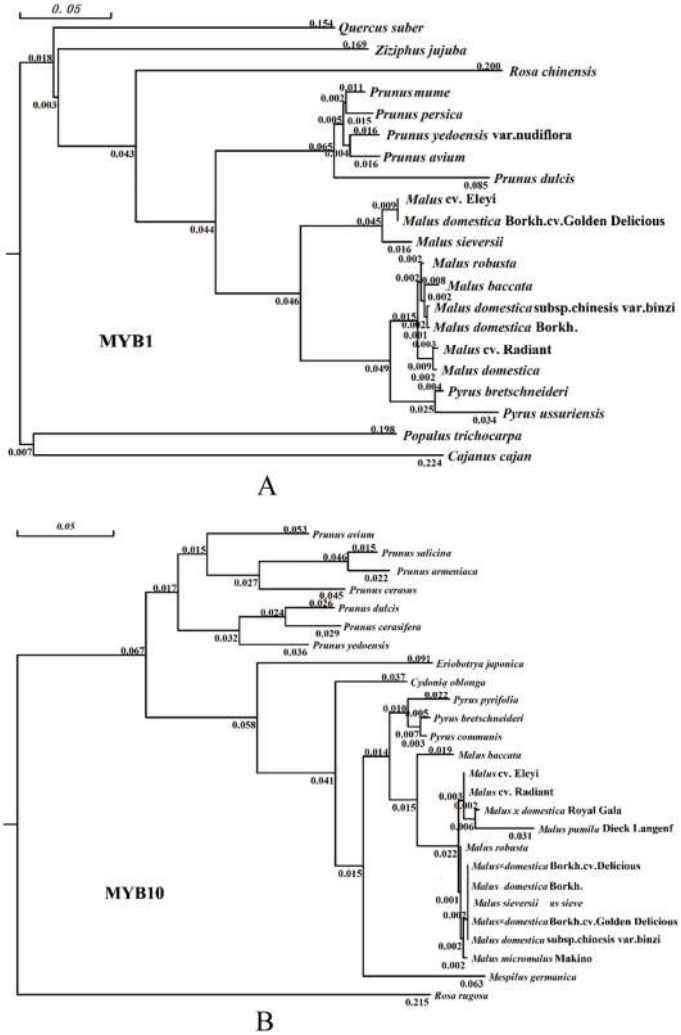

Figure 2 Phylogenetic analysis of MYB1 (A) and MYB10 (B) in nine species of Malus and other plants

Note: The number in figure were branch length

1.4 Obtaining of the protein physical and structural informations of MYB1 and its allele MYB10

According to the translated amino acid sequence, the protein prediction tool was used for analysis of isoelectric point, molecular weight, hydrophilicity and other parameters with the reference of three cultivated apple sequence in NCBI database. The results showed that the MYB1 protein isoelectric point and hydrophilicity of *Malus sieversii* and *Malus* cv. Eleyi decreased compared with other proteins (Table 1; Table 2). The basic properties of MYB10 protein showed that the protein properties of Binzi, Delicious, Golden Delicious, *Malus domestica* Borkh and *Malus sieversii* were the same, the isoelectric point and hydrophilicity of *Malus* cv. Eleyi and *Malus* cv. Radiant decreased, and the hydrophilicity of *Malus robusta* increased. The results suggested that the difference of amino acid sequence did affect the hydrophilicity of MYB1 and MYB10 in different materials. The isoelectric point CV (coefficient of variation) of MYB10 was 0.015 for 9 plants of *Malus*, and hydrophilicity standard deviation (SD) of MYB10 was 0.010. While isoelectric point CV and hydrophilicity SD of MYB10 were respectively 0.061 and 0.038 for 6 plants of *Malus*. From the numerical differences of the coefficient of variation and the standard deviation, it can be seen that the isoelectric point (the proportion of positive and negative residues) of MYB1 fluctuated more than MYB10, which may reflect the change of their function.

Table 1 Analysis of the basic properties of MYB1 protein in six plants of *Malus*

Name	Isoelectric point	Molecular weight (g/mol)	Negative residue	Positive residue	Grand average of hydropathicity
Malus domestica subsp. chinesis var. binzi	6.51	45483.3	51	48	-0.553
Malus robusta	6.37	45331.0	51	47	-0.541
Malus cv. Radiant	6.37	45238.9	51	47	-0.550
Malus domestica Borkh	6.51	45513.3	51	48	-0.559
Malus sieversii	5.54	43808.1	54	42	-0.509
Malus cv. Eleyi	5.80	43993.4	54	45	-0.510
Malus domestica (ABK20308.1)*	6.51	45251.9	50	47	-0.550
Malus×*domestica* cv. Golden Delicious (XP_008392894.1)*	5.80	43993.4	54	45	-0.510
Malus baccata (TQD84684.1)*	6.04	45547.2	54	47	-0.551

Note: * shows the sequence from the NCBI database

Table 2 Analysis of the basic properties of MYB10 protein in nine plants of *Malus*

Name	Isoelectric point	Molecular weight(g/mol)	Negative residue	Positive residue	Grand average of hydropathicity
Malus domestica subsp. chinesis var. binzi	8.82	28660.0	35	39	-1.015
Malus robusta	8.99	28730.0	35	40	-1.030
Malus cv. Radiant	8.61	28730.0	36	39	-1.028
Malus domestica Borkh	8.82	28660.0	35	39	-1.015
Malus×*domestica* cv. Delicious	8.82	28660.0	35	39	-1.015
Malus sieversii	8.82	28660.0	35	39	-1.015
Malus micromalus	8.62	27973.3	35	38	-0.998
Malus cv. Eleyi	8.61	28725.3	36	39	-1.028
Malus×*domestica* cv. Golden Delicious	8.82	28660.0	35	39	-1.015
Malus×*domestica* Royal GalaACD36023.1)*	8.61	28739.0	36	39	-1.027
Malus pumila Dieck Langenf (ADB22516.1)*	8.62	28720.2	37	40	-1.004
Malus baccata (TQD91033.1)*	8.94	28492.9	33	38	-0.921

Note: * shows the sequence from the NCBI

Further domain prediction of the protein was carried out to analyze the effect of polymorphism sites on the functional domain. The MYB1 had 6 amino acid polymorphism sites in the predicted major functional domains (Figure 3A). Among them, the 121st amino acid polymorphisms (130th for *Malus* cv. Eleyi and *Malus* sieversii) were located in the key sites of DNA binding domain, and the polymorphic amino acids were non-polar amino acids glycine and alanine. The polymorphic amino acids of 46th and 113rd (55th and 122nd for *Malus* cv. Eleyi and *Malus sieversii*) were basic arginine and non-polar leucine. The polymorphic amino acids of 39th and 90th (48th and 99th for *Malus* cv. Eleyi and *Malus sieversii*), 45th (54th for *Malus* cv. Eleyi and *Malus sieversii*) were polar neutral serine and threonine. The analysis showed that the protein sequence of MYB10 was more conservative than that of MYB1 (Figure 3B). The three polymorphic sites of MYB10 were all located at the downstream of the functional domain, but some of polymorphic sites were located at the functional domain of MYB1, which may affect the function of the gene, especially the two polymorphic sites of basic arginine and non-polar leucine. The two polymorphic sites were likely to lead to the change of protein spatial conformation to affect the recognition of downstream genes.

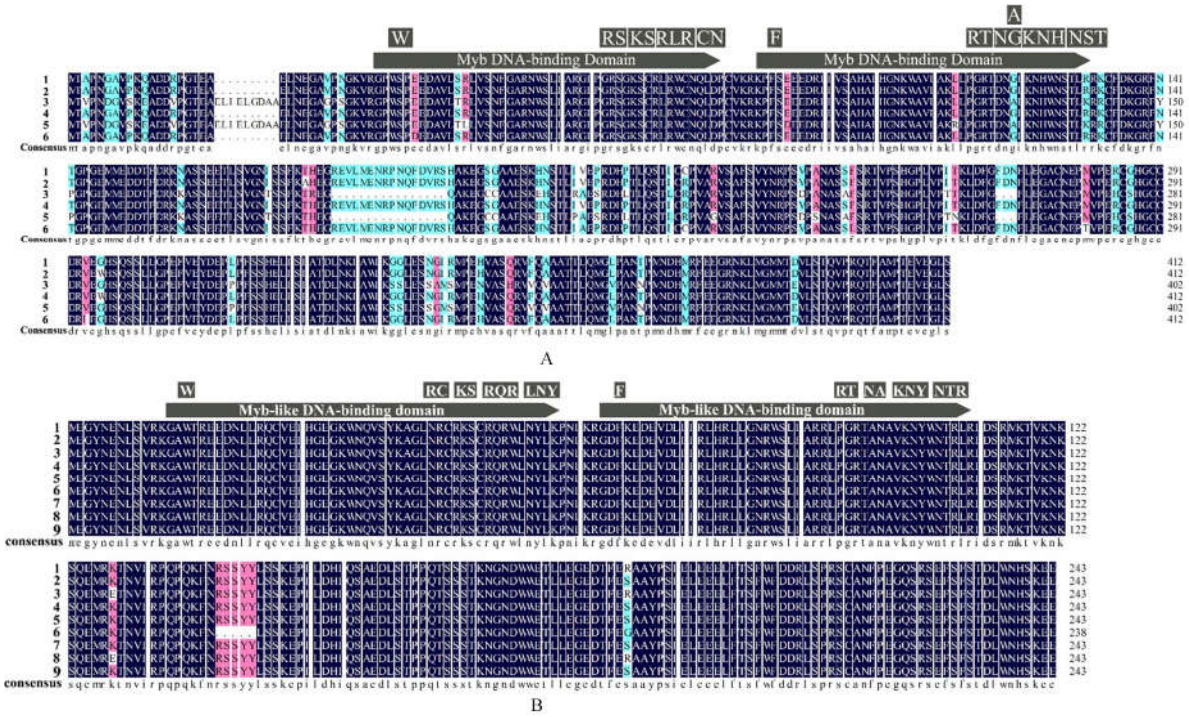

Figure 3 Alignment and functional domain analysis of MYB1 (A) and MYB10 (B)in six plants of Malus

Note: Sample number 1: Malus robusta; 2: Malus domestica subsp. chinesis var. binzi; 3: Malus cv. Eleyi; 4: Malus domestica Borkh.; 5: Malus sieversii; 6: Malus cv. Radiant; 7: Malus×domestica Borkh.cv. Golden Delicious; 8: Malus micromalus Makino; 9: Malus ×domestica Borkh. cv. Delicious; The amino acid residue at the top of the picture is the DNA binding site

2 Discussion

The fruit color of *Malus* depends on the base color and cover color. The base color is related to the content of chlorophyll and carotene, while the red color of cover color is mainly related to the pigmentation of anthocyanin (Jaakola, 2013). The accumulation of anthocyanin is affected not only by genetic factors, but also by environmental factors, especially the change of light, such as paper-bagging (Chang bo et al, 2018) can make the fruit red. At present, it has been confirmed that many R2R3-MYB transcription factors can participate in the regulation of anthocyanin synthesis in *Malus*, including MYB1 and MYB10 (Jaakola, 2013). However, in previous studies, most of the related MYB transcription factor genes were amplified from cultivated apples, such as Cripps' Red (Takos et al., 2006), Golden Delicious, Gala, Jonathan and Australian green apple (Mahmoudi et al., 2012). The amplification of the related MYB transcription factor genes in Begonia was mainly concentrated in

some varieties of Begonia in North America (Li et al., 2018). There was a lack of comparative study on genetic diversity among species and evolutionary characteristics of this locus gene.

In this study, the MYB10 sequence of 9 *Malus* plants and the MYB1 sequence of 6 *Malus* plants were obtained. The polymorphic level of MYB1 was significantly higher than that of MYB10, and the variation range of isoelectric point and hydrophilicity of MYB1 were larger than those of MYB10. In addition, two polymorphic sites in the functional domain of MYB1which were basic arginine and non-polar leucine were polar transformation of amino acid. The changes were likely to affect the function of MYB1 transcription factors. While the MYB10 polymorphism sites were only three, and all of them were located in the non-functional domain. This suggests that the MYB10 transcription factor was more conservative in evolution than the MYB1 on the same locus in the detected *Malus* plants. This indicated that the genes of the same locus of MYB1 and MYB10 may be subject to different selection pressures in evolution, and also suggests that MYB1 and MYB0 were involved in different gene regulation in fruit color control.

It has been shown in the previous study that MYB1 and MYB10 have different emphasis on the regulation of fruit color. A mutant of MYB10 gene, due to an insertional mutation of a 23 bp microsatellite sequence in its promoter region, caused the whole apple plant to turn red, including the peel, pulp and leaves (Espley et al., 2009). According to the phenotypic analysis, it can be inferred that the MYB10 gene was dominant and constitutively expressed (Espley et al., 2009; Wang et al., 2010). However, MYB1 was shown to be expressed only in the peel but not in the pulp (Mahmoudi et al., 2012), and MYB1 expression was regulated by light (Takos et al., 2006) and also by epigenetic regulation induced by paper-bagging (Bai et al., 2016; Ma et al., 2018), which indicates that MYB10 itself may have the potential to express in different tissue, but no similar expression pattern has been reported for MYB1 induced by a variety of in vitro factors. This tendency of different expression regulation patterns may be related to the differences in the conservativeness of MYB10 and MYB1 sequences in this study, and may be related to the functional differentiation of this locus among alleles in artificial cultivation and domestication. In addition to its involvement in the regulation of anthocyanin synthesis, MYB10 may also be involved in some other physiological and biochemical processes, while MYB1 may be more specifically responsive to environmental signals to regulate fruit color changes. The MYB1 sequence varied more in the genus *Malus*, but the expression tissue was single, while the MYB10 sequence is conserved and can express in more tissues.

Gene mutations, especially those caused by extensive bud mutation in fruit trees, are common phenomena of fruit trait-related variation in fruit trees, especially when mutations occur in transcription factor genes, which may lead to a series of trait changes. The polymorphic sites of MYB1 detected in this study, especially the polarity changes of amino acid in the differential sites of functional domains, may lead to the influence of transcription factors on the regulation of anthocyanin synthesis genes, thereby affecting the regulation of anthocyanin synthesis. Previous studies have demonstrated that there are indeed examples of gene mutations that alter fruit color, such as reddening of red meat umbilical orange which is thought to be associated with the accumulation of lycopene caused by a mutation of the lycopene β-cyclase gene (non-polar methionine changed into alkaline arginine) (Xu, 2002). Red-fleshed apples were associated with mutations in the MYB10 promoter region (Espley et al., 2009). However, the differences detected in MYB1 of functional domains or other non-functional domains, especially two polymorphic sites with changes between basic arginine and non-polar leucine in functional domains, were likely to cause changes in gene function. Although some of the samples collected from *Malus* plants have been studied, there may be a limitation of small coverage for the entire genus *Malus*. Whether the mutations in the polarity changes of the two amino acids do affect the function of transcription factors needs further validation and analysis. MYB1 was only amplified from six of the nine *Malus* plants except for Golden Delicious, *Malus micromalus* Makino and Delicious, even after several replacements of primer design sites (other primers were not listed in this paper). However, the amplification results of other materials were stable. It may be related to the variation of amplification sites or amplification conditions. MYB1 was not amplified from two cultivars and a

crabapple, and there was no obvious preference in the taxa. The specific reasons for the result need further analysis and verification.

In this study, the obvious differences in evolutional conservation were observed in the alleles MYB10 and MYB1 in *Malus* plants. There are many variations in the sequence of MYB1 within *Malus*, but the expression site is single, while the sequence of MYB10 is conserved, which can express in more tissues. The two polymorphism sites of MYB1 may affect the regulation of anthocyanin synthesis after domain prediction, thus affecting fruit pigmentation, and there is differentiation in the genes function responsible for allele evolution. The results can provide a certain data for further exploring the fine regulation of fruit color in *Malus* and provide theoretical reference for the study of the evolution of fruit color in *Malus*. There are many variants in this locus. Further study of the functional differentiation of these variants from an evolutionary view may be a new direction in the study of fruit color in *Malus*.

3 Materials and Methods

3.1 Experimental materials

The leaves of plants more than three years old entering the flowering and fruiting period were frozen and stored in a refrigerator at -80℃, *Malus robusta*, *Malus* cv. Radian, *Malus* cv. Eleyi and *Malus micromalus* Makino were collected from nursery of Tianjin Agricultural University. *Malus sieversii*, *Malus domestica* subsp. chinesis var. binzi, *Malus*×*domestica* Borkh. cv. Delicious, *Malus*×*domestica* Borkh. cv. Golden Delicious and *Malus domestica* Borkh. were collected from the nursery of research institute of pomology, Chinese academy of agricultural sciences.

3.2 RNA extraction

RNA was extracted by modified CTAB method (Kiefer et al., 2000). DNA was digested and removed by DNase I (Takara). The total RNA was detected by electrophoresis and then frozen in refrigerator at -80℃.

3.3 RT-PCR

The total RNA was reversed by the primer oligo(dT)18 and the reverse enzyme M-MLV (Promega). The protocol was followed by the instructions of M-MLV. The Premix Ex*Taq*™ HotStart Version (Takara) was employed to process the PCR with reverse transcripts. The amplification system and procedures were carried out according to the instructions. The amplification of actin gene was used to detect the quality of cDNA.

The amplification primer of *MYB1* was based on the sequence of EF016490.1 in NCBI database, and the primer of *MYB10* was based on NM_001328848.1. The primer premier 5.0 was used to design on the both sides of open reading frame, and actin primer was designed with sequence NM_001301116 (Table 3). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Table 3 Sequence of primer for amplification

Gene name	Primer name	Sequence (5'-3')
MYB1	MYB1-S	CAATGACGGCCCCAAAC
	MYB1-A	CTAGCTCAATCCCTCAACTTCAG
MYB10	MYB10-S	GATAAGAGATGGAGGGATATAACG
	MYB10-A	ATCCCACATTTACAAGCAAGG
Actin	Actin-S	GAACCCAAAGGCTAATCGG
	Actin-A	CATAAGGGCATCGGTGAGG

3.4 Cloning and sequencing

After the PCR products have been detected by electrophoresis, T-A clone is carried out by using the pEASY-T1 vector according to the instructions. After the confirmation by the original amplified PCR primers, the samples were sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing, and each gene was sequenced by two-ways, with three biological repetitions to eliminate the error.

3.5 Sequence analysis and alignment

The sequencing results were compared with the NCBI database by blast program, and then DNAman 6.0 software was used for cluster analysis (Non experimental sequence came from NCBI database) after machine translating. The Protparam was used for basic information analysis of protein chemistry (https://web.expasy.org/protparam/), and domain information collected from NCBI database.

Authors' contributions

Ma Chao, Bo Yifan were the experimental designers and the executors of the experimental research; Ma Chao, Bo Yifan and Zhang Chenghui completed the data analysis and the writing of the first draft of the paper; Ma Hongzheng participated in the experimental design and the analysis of the experimental results; Hu Baoquan was the designer and the person in charge of the project, guiding the experimental design, data analysis, and the writing and modification of the paper. All authors read and approved the final manuscript.

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