Independence of color intensity variation in red flesh apples from the number of repeat units in promoter region of the MdMYB10 gene as an allele to MdMYB1 and MdMYBA

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Abstract
MdMYB10 gene expression results in accumulation of anthocyanin in many tissues including flesh of apple fruit. The *MdMYB1* and *MdMYBA* genes are close homologues to MdMYB10 gene and both are responsible for red color phenotype in apple fruit skin. In the current study, an apple genome sequence draft analysis indicated that these three genes are located in a unique contig. Further analysis suggested that these homologues are alleles of a single locus and they differ in a repeated sequence of the promoter region. This repeated sequence ensures high expression level of *MdMYB10* in most of the plant tissues while *MdMYB1* and *MdMYBA* alleles lack such a repeated sequence in their promoters and their expression is confined to the fruit skin. Also, we suggest a tissue- and genome-specific expression pattern for these three alleles considering our data and other recent publications. No variation was detected in the sequence or in the number of repeats of *MdMYB10* promoter in Iranian red flesh apple geo-variants, pointing that the number of repeat is not related to flesh color intensity or variation, and the repeat elements have occurred once during the evolution.
Keywords: Allele; *Malus×domestica;* Color intensity; MdMYB; Red flesh; Tandem repeats

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INTRODUCTION

Anthocyanins are a group of secondary metabolites with diverse functions in plants. Anthocyanins along with carotenoids are responsible for the coloration of flowers, fruits, seeds and other plant tissues causing purple, blue and red color of organs (Schaefer *et al*., 2004). Coordinated with flavonoids, anthocyanins are also involved in plant resistance against insect pests (Makoi *et al*., 2010). Moreover, there is a direct relationship between anthocyanin consumption and reduction of cancer, heart disease and diabetes through its antioxidant and anti-inflammatory activities (Prior and Wu, 2006).

Apple (*Malus× domestica*) is one of the most widely cultivated and important fruit tree in temperate regions which represents diversity in color. Based on fruit flesh color, apples have either white or red flesh. Red flesh plants grow wildly and rarely. There is high anthocyanin accumulation in the flesh of red flesh apples. These anthocyanin originated apple features are useful in breeding programs and in market ability.

Former studies have shown that chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS) and UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT) enzymes are involved in antho-

cyanin biosynthesis and expressed in the red skin apple (Honda *et al*., 2002). Coordinated expression of these enzymes is ensured by plant MYB transcription factors (Allan *et al*., 2008). MYBs are one of the largest transcription factor families in plants that contain one to three conserved repeats (R1-R2-R3) in their DNA-binding domain (Jin and Martin, 1999). MYBs are mostly coordinated with bHLH transcription factors via their binding domains (Ramsay and Glover, 2005). About 125 R2R3-MYB genes have been annotated in Arabidopsis thaliana among which, *AtMYB75* and *AtMYB90* are characterized as regulators of anthocyanin level (Stracke *et al*., 2001). Other reports have indicated that R2R3-MYBs are involved in anthocyanin regulation in other plants including red apple as well (Takos *et al*., 2006; Romero *et al*., 1998). For example, in grape (Vitis venifera), skin color is determined by *VvMYBA1* and *VvMYBA2* which are located in a single locus (Walker *et al*., 2007; Kobayashi *et al*., 2004). Inactivation of these two genes, due to transposon insertion in *VvMYBA1* and point mutation in *VvMYBA2*, has made white berry phenotype (Walker *et al*., 2007).

Exposing of dark-reserved apples to light showed higher *MdMYB1* expression and anthocyanin accumulation in the skin (Takos *et al*., 2006). In another report, a tissue and cultivar-specific gene, *MdMYBA*, was found to be responsible for the red skin color in apple (Ban *et al*., 2007). *MdMYB10* gene was expressed in the fruit flesh and the leaves of "Red flesh" apple cultivar, resulted in anthocyanin accumulation (Espley *et al*., 2007). A new report indicated that a minisatellite located in the promoter of *MdMYB10* gene correlates with higher transcriptional level of this gene (Espley *et al*., 2009).

We aimed to investigate the coding region and the promoter of *MdMYB10* gene and its expression pattern in Iranian red and white flesh apple varieties, targeting possible geographical polymorphism. Furthermore, we present an analysis of the relationship among homologous genes that have been reported as red color regulators in apple.

MATERIALS AND METHODS

Plant materials: Samples of fruit (leaves skin, flesh and seed) of 16 red and white flesh apple (*Malus*×*domestica*) varieties were collected from "Seed and Plant Improvement Institute of Karaj", Karaj, Iran, during 2008 and 2009 (Table 1). For RNA isolation, pieces of flesh, skin, seed and leaves of a red variety (cv. B.9) and a white variety (var. Khansari) were collected in the ripening stage (102 DAF for B.9 and 120 DAF for Khansari). For DNA extraction at the beginning of growth, young leaves were collected from all the red and white flesh varieties. All samples were immediately frozen in liquid nitrogen and stored at -80°C.

Analysis of the upstream region of MdMYB10b gene: For promoter sequence analysis of the red and white flesh apples (*Malus*×*domestica*), 1.9 kb fragment of gene upstream was isolated using Mybprom-F2 (5-GGCCCGTTTGTAACCGACTGAGATA) and Mybexo1-R1 (5'-ACGCACTGCCTGAGAAGATT) primers. PCR products of red (GH1) and white (Arous) varieties were cloned in TA vector using Topo TA cloning kit (Fermentas Co.) and were sequenced. PCR was carried out in a 25 µl reaction volume containing 10x PCR buffer, 2.5 mM of each dNTP, 40 mM $MgCl_2$, 5 µM of each primer and 1U of Taq polymerase. The PCR reaction carried out as follows: predenaturation at 94°C for 5 min, followed by 37 cycles of 94°C (45 s), 59°C (30 s), and 72°C (2 min) and a final extension at 72°C for 10 min.

Amplification of repeated section: Genomic DNA

Table 1. Red and white flesh apple (*Malus*×*domestica*) varieties and their characteristics.

Variety	Flesh color	Skin color	Type
B.9	Red	Red	Wild type
T3	Red	Red	Wild type
H-GH	Red	Red	Wild type
Ardebil	Red	Red	Wild type
GH 1	Red	Red	Wild type
GHV 2	Red	Red	Wild type
GHV 3	Red	Red	Wild type
SH10	Red	Red	Wild type
Granny smith	White	Green	Commercial
Golden delicious	White	Yellow	Commercial
Golab	White	Yellow	Commercial
Arous	White	Red	Commercial
Gala	White	Red	Commercial
Fuji	White	Red	Commercial
Red delicious	White	Red	Commercial
Jonathan	White	Red	Commercial

All the red flesh varieties grow wildly in different areas of Iran and have red skin. Non-red apple varieties are commercial and show different skin colors.

was isolated from the leaves by modified CTAB (Cetyltrimethyl Ammonium bromide)-based method (Khan *et al*., 2007). About 120 ng of genomic DNA was used for each PCR reaction. In order to amplify the repeated area of the *MdMYB10b* gene, a pair of primers (mini-F; 5'-AGCAGCGAAAGCATGATAAAG-GTATCT and mini-R; 5'-AAGGCCAGTGACGTGCAT-GTCTG) located in two sides of the promoter repeated sequence were used. PCR program was initiated with pre- incubation at 94°C for 5 min followed by 35 cycles of 94°C (45 s), 60°C (40 s), and 72°C (40 s) with a final extension at 72°C for 10 min. The PCR products were separated on 1% agarose gel and stained with ethidium bromide.

Sequence analysis and primer design: Obtained sequences were blasted in NCBI website (http://www.ncbi.nlm.nih.org). The primers were designed using primer3 software and IDT website (http://eu.idtdna.com). Sequence alignments were performed using ClustalW.

Isolation of MdMYB10b gene: Total RNA was extracted from 500 mg of target tissues according to a modified method (Gasic *et al*., 2004). The first strand cDNA was synthesized from preheated and snap cold treated 350 ng of total RNA in a 20 µl reaction containing; 40 U M-Mulv reverse transcriptase, 5x reverse transcription buffer, 20 U "RiboLock™ RNase inhibitor", 10 mM each dNTPs and incubated in 42°C for 90 min. The enzymes were inactivated at 70°C for 10 min. The specific primers Myb10-F1 (5'-ATG-GAGGGATATAACGAAAACC) and Myb10-R1 (5'-TTCTTCTTTTGAATGATTCCA) were used for amplification of the ORF of the gene. The PCR conditions for degenerate primers were 4 min at 94°C then 35 cycles of 40 s at 95°C, 40 s at 57°C, and 1 min at 72°C, and a final extension of 10 min at 72°C.

RESULTS

Isolation of a new eco-variant for MdMYB10: *MdMYB10* gene is involved in anthocyanin synthesis in the red flesh apple. The first strand cDNA was prepared from total RNA of the flesh of a red flesh apple (*Malus× domestica*, cv. B.9) cultivar. Then, using MdMYB10 specific primers (Myb10-F1 and Myb10-R1), a 729 bp fragment encoding 243 amino acid residues was amplified by using RT-PCR. Sequencing of this clone showed 99% homology with reported *MdMYB10* gene ORF. This variant was named *MdMYB10b* (accession # AB592747.1), differed with sequence reported of MdMY10a and MdMYB10 by one and two amino acids, respectively. *MdMYB10b* clone showed %99 sequence identity to *MdMYB1* and *MdMYBA* as well (both are responsible for the red color in apple) but, showed only one amino acid difference.

Detection of the MdMYB10b gene expression: *MdMYB10b* allele expression was detected at the middle of flesh growth in red flesh apple cv. "B.9" using RT-PCR. *MdMYB10b* transcripts were also detectable in the flesh, skin fruit, seed and leave of red flesh apple (Fig. 1, lanes 1 to 4). *MdMYB10b* expression was not detectable in the tested tissues of "Khansari" white flesh apple variety (Fig. 1, lanes 5 to 7).

Sequencing of MdMYB10b Promoter: The promoter sequence of MYB genes, determined the level of transcription factor production and signaling. In order to find the regulatory sequences of the *MdMYB10b* promoter which affect transcriptional level of this gene and possible polymorphism in eco-variants, 1.9 kb DNA from the upstream region of this gene was amplified from red (GH1) and white (Arous) flesh apple (*Malus× domestica*) varieties. Sequencing of PCR products revealed two types of tandem repeats in the promoter; a short tandem repeat (Microsatellite) of "GT" dineucleotids composed of seven units located -252 to -265 upstream of the start codon. Another tandem repeat was composed of five identical units (23 bp each) which two of them were located in the middle of the sixth disrupted repeat, all located -220 to -391 bp upstream of the start codon. In the upstream region of *MdMYB10b* gene in the white flesh variety "Arous ",

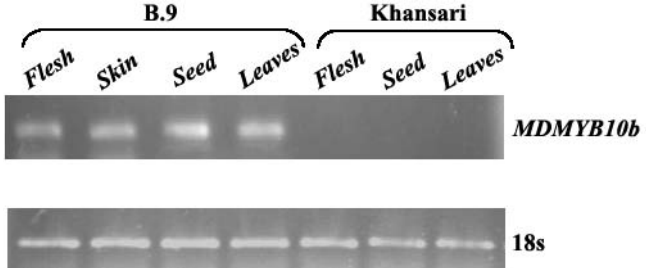

Figure 1. Analysis of *MdMYB10b* expression in the red and white flesh apple tissues using RT-PCR. Lanes 1 to 4 show expression of *MdMYB10b* in flesh, skin, seed, and leaves (700 bp) of red flesh "B.9" in the middle of ripening stage. There is no detectable expression in the white apple tissues of "Khansari" (lanes 5 to 7). Below is 18s (550 bp) targeted PCR product used as positive control in RT-PCR reactions.

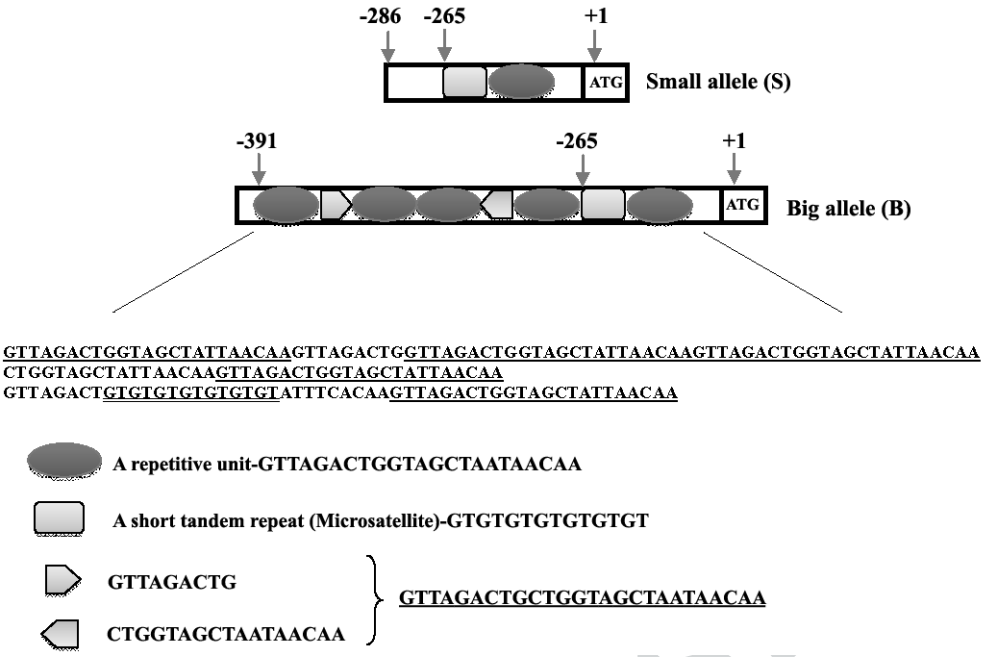

Figure 2. Location and sequence of *MdMYB10b* promoter tandem repeats. The first 23 bp unit located -220 bp upstream of the start codon followed by single microsatellite and 4+1 more 23 bp units in the big allele. The 23 bp unit and microsatellite are unique in the small allele.

the only 23 bp unit and "GT" dinucleotide repeat were unique (Fig. 2).

Association of MdMYB10b promoter tandem repeats with red phenotype: In order to investigate the possible *MdMYB10* promoter repeat polymorphism and its association with the intensity of red flesh color, eight red and eight white flesh apple varieties were gathered from various climate conditions of Iran. *MdMYB10b* promoter sequence in these geo-variants were PCR amplified and sequenced using specific primers for the flanking sequences surrounding repeated area of the promoter.

A unique 312 bp PCR product was amplified from all the white flesh geo-variants as expected (Fig. 3, lanes 1 to 8). On the other hand, two or three bands were amplified where the red flesh background variants (Fig. 3, lanes 8 to 16) were used as template. The smallest band (S) was exactly identical to the unique

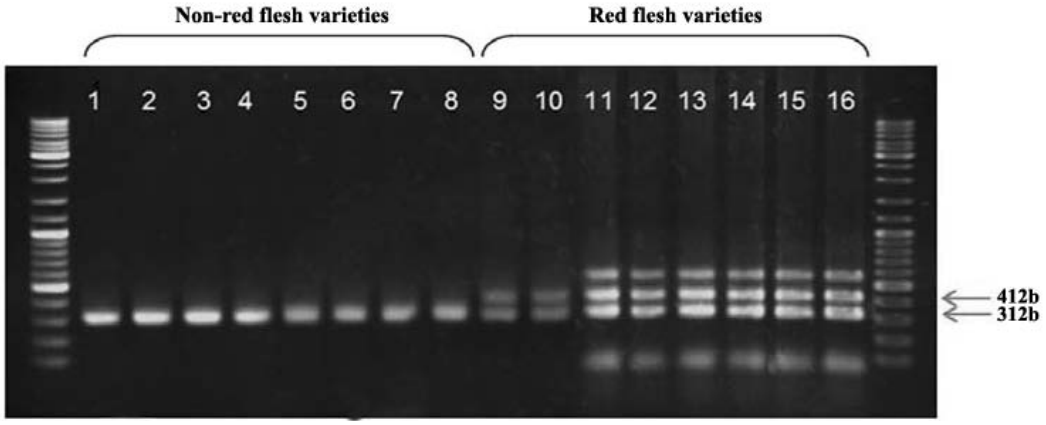

Figure 3. PCR products of *MdMYB10b* promoter area flanking its tandem repeat from red and white varieties. The 312 bp band exists in all red and white varieties (Lane 1 to 16). The band 412 exists just in red varieties (lane 9 to 16) and a ~550 bp amplified in the some red varieties (11 to 16). *Malus ×domestica* varieties were: 1) "Arous"; 2) "Granny smith"; 3) "Golden delicious"; 4) "Red delicious"; 5) "Gala"; 6) "Fuji"; 7) " Janathan"; 8) "Golab"; 9) "H-GH"; 10) "Ardebil"; 11) "B.9"; 12) "SH10"; 13) "GHV3"; 14) "GHV2"; 15) "GH1"; 16) "T3".

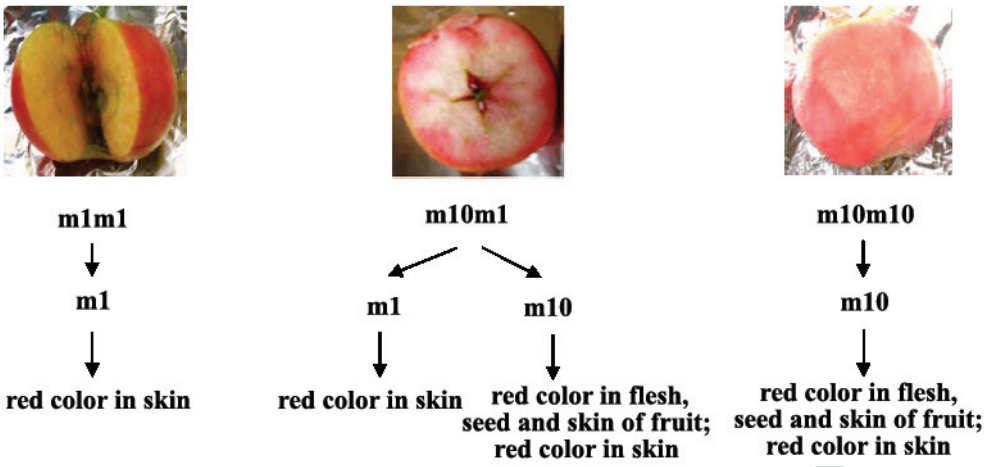

Figure 4. Deduced allelic relationship for the MdMYB genes which are involved in red colored apple plant tissues phenotype. In this model, MdMYB1 (MdMYBA) and MdMYB10 are alleles of the same locus. While MdMYB1 allele is only expressed in the fruit skin, MdMYB10 allele (containing tandem repeats in its promoter) is expressed in all the tested plant tissues, including the flesh of apple fruit. M1, represents MdMYB1 allele and M10, represents MdMYB10 allele in this model.

band of white flesh background. The 412 bp band (M), was similar to the S band but it had two extra stretches of tandem repeats. S and M bands were present in all tested red flesh variants (Fig. 3, lanes 1 to 16). In the white flesh background the S band was unique but simultaneous presence of S and M bands in all the red flesh backgrounds indicated that M allele was necessary and enough to make red flesh phenotype. In the other mean, tandem repeat sequence (5-repeat section) was associated with the red phenotype. In some cases though, a third band (L) was detected in red flesh background (Fig. 3 lanes 11 to 16). Since the L band was not detected in some of the red flesh apples (Fig. 3 lanes 9 and 10), it seems that this band is not necessary for red phenotype creation. Despite variable number of repeats (either 8 or 9) in few varieties, sequence of "GT" microsatellite was the same in nearly all the tested red and white flesh phenotypes. In terms of subtle geo-variation of repeats, no difference of 23 bp repeats was detected in the promoter of other geo-cultivars as well. Analysis of the banding patterns in these geo-variants indicated that only one allele (S band) existed in the white flesh apples while M band resulted in red phenotype along with heterozygote inherency in red apples.

MdMYB10 repeates and new phenotype on the gel: The L band as the longest (~550 bp) PCR product on the gel, was only originated from some tested red flesh apple background. Purification of L band from the gel and subsequent electrophoresis on a new gel resulted in B, M and S bands again. Pre-heating of the L band DNA did not make any difference in the bands pattern of this sample on the gel. Sequencing of the L band DNA revealed that its sequence was composed of S and M fragment. In other word, the L band DNA sequencing graph was a hybrid of two simple graphs that belong to M and S bands. Regarding to lack of the same fragment in PCR products of white flesh apple genome, this phenomenon could be described by chimeric structure of L band that is related to the repeat sequence. Previous reports indicated that this phenomenon might result from recombination of related sequences such as multiple family members, repetitive sequences, and heterozygote loci during the PCR reaction (Judo *et al*., 1998; Bradley *et al*., 1997; Meyerhans *et al*., 1990). This phenomenon was not the focus of our research and remained to be analyzed.

Testing for the adjacency of MdMYB10 homologues: Considering high DNA sequence homology among three MYB genes (*MYB10*, *MYB1*, *MYBA*) in apple, a PCR protocol was designed using LA Taq polymerase to check for the possible linkage of them. All the possible combinations of primers failed to amplify a PCR product (data not shown), suggesting that the *MdMYB10* and its homologous were not closely linked. Since August 2010, whole genome sequence of apple (*Malus× domestica* var. Golden delicious) has been sequenced (Velasco *et al*., 2010) and is publicly available (http://www.rosaceae.org). We used promoter or ORF and cDNA sequences of *MdMYB10* and its homologues as queries to search for the possible extra homologues and locations in the published draft

sequences. Our data surfing resulted in a common significant hit with contig number of MDCO13323.319. This 22 kb contig is located at the end of chromosome 9 (from nucleotide 29465538 to 29488265). There were no other contig showing acceptable homology and coverage with these genes. This result suggested that *MdMYB10*, *MdMYB1* and *MdMYBA* are alleles which located in a single locus.

DISCUSSION

Allelism relation of the MdMYB10, MdMYB1 and MdMYBA genes: Full length of a R2R3-MYB transcription factor was isolated and named *MdMYB10b* as a variant of *MdMYB10*. MdMYB10b showed %99 amino acid homology to MdMYB10, MdMYB1 and MdMYBA, all which are involved in anthocyanin biosynthesis (Ban *et al*., 2007; Espley *et al*., 2007; Takos *et al*., 2006). Both *MdMYB10* and *MdMYB10b* are highly expressed during the ripening stage of fruit; in leaves and fruit skin (Fig. 1). *MdMYBA* expression has been detected in the ripened red skin fruit of "Jonathan", "Niedzwetzkyana" and "Tsugaru" (Ban *et al*., 2007). Also, *MdMYB1* expression has been detected in the red skin of "Cripps Red" apple fruit after light treatment of dark reserved apples while has not been detected in its white flesh (Takos *et al*., 2006).

Several clues supported an allelic relationship model between these MdMYB genes. First, the level of amino acid sequences similarity and their related functions in anthocyanin production suggested such an allelic relationship between them. Later, an apple genome sequence draft was bioinformatically analyzed and all of these genes were mapped to the same locus. The promoter, full length or ORF of the MdMYB10 and MdMYB1 genes were used as queries in blastn program against apple genome sequence. The most significant hit for all of these queries was a common 22 kb contig (MDC013323.319) on the 9th chromosome of apple genome. This is consistent with the other reports locating MdMYB10 and its homologues at the end of 9th apple genome linkage group (Ban *et al*., 2007; Chagne *et al*., 2007). MdMYB10 ORF was also used as a query in a blastx search against the predicted proteins of apple proteome database. The same contig (MDC013323.319) was again found to carry the gene (ID # MDP0000259614). There were other less significant hits as commonly found with a big gene family like MYB transcription factors. In the single contig which hit by *MdMYB10* and *MdMYB1* promoters, there was only one 7 kb stretch of DNA related to *MdMYB10* gene (promoter and transcribed region) with no more homology. Long PCR using promoter or ORF specific primers (all the possible combinations) was performed in order to check the adjacency of two possible *MdMYB10*/*MdMYBA*/*MdMYB1* loci but no PCR product was amplified (data not shown).

All these analyses guided us to the fact that there is only one locus of *MdMYB10*/*MdMYBA*/*MdMYB1* in the apple genome. This suggestion is supported by previously published data pointing the allelism relationship among these genes (Sekido *et al*., 2010; Wing *et al*., 2010).

Despite the high homology between ORF and promoter regions of *MdMYB10* and *MdMYB1* (*MdMYBA*), only *MdMYB10* causes red color in the leaves and flesh. Over-expression of *MdMYB10* resulted in accumulation of anthocyanin in tobacco leaves (Espley *et al*., 2007). However, transformation of *MdMYBA* had no effect on tobacco leaves (Ban *et al*., 2007). It seems that all these possible alleles cause red color in apple in a tissue-specific and genome-specific pattern. It could be as a result of interaction with other transcription factors or presence of a regulatory element in the *MdMYB10* which specifies its expression in the leaves as well. Shuffling promoters and exon-intron fractions of *MdMYB10* and *MdMYB1* will help to find the main determinant of tissue specificity for *MdMYB10* expression. Hence we conclude that, in the red skin apple cultivars, only *MdMYB1*/*MdMYBA* is present (Fig. 4) and expressed in the skin as a result of light stimulation (Takos *et al*., 2006). MDMYB10 (b) allele is highly expressed in all of the plant tissues (flesh, skin, seed and leaves) regardless of light stimulation or the presence and the expression of MdMYB1 in skin (Fig. 4) suggesting that light stimulation is not necessary for the MdMYB10 gene expression.

Independence of color intensity variation from the number of repeat units: Two types of tandem repeat elements were discovered in the *MdMYB10b* promoter (Fig. 1); a short microsatellite of seven "GT" dinucleotide and a 23 bp unit that was repeated five times, of which two were located in the middle of the sixth disrupted unit (Fig. 2). This 5+1 section was present in all the eight tested red flesh apples and absent in all the white flesh geo-variants. The number of repeats for the 23 bp unit was the same in all the red variants but the number of "GT" varied either 8 or 9 in few verities. In the "Arous" white flesh apple variety, 23 bp unit as well as "GT" dinucleotide repeats were unique (Fig.

1). To search the other possible genes which have similar regulatory elements, the 23 bp sequence was used as query against apple genome database in a blast search. No significant hit was found in this search, suggesting that this repeat element might be unique for the *MdMYB10* gene promoter. This result indicated that not only the 23 bp sequence is specific regulatory element for *MdMYB10* gene but also the *MdMYB10* locus is unique in the apple genome.

All the tested red flesh geo-varieties were heterozygous for *MdMYB10* locus. This was documented through PCR amplification of *MdMYB10b* (with five 23 bp unit) and *MdMYB1* alleles (with one 23 bp unit), represented as M (412 bp) and S (312 bp) bands in Figure 3 respectively. The S band was unique in all the tested non-red flesh varieties indicating that all were homozygous for *MdMYB1* allele (Fig. 3). The presence of S and M bands in all the red flesh varieties indicated that M allele was necessary and enough to make red flesh phenotype. In other words, five-repeat sections of 23 bp unit were conserved and associated with red phenotype. In most cases, the third band (L) was detected in the red flesh background (Fig. 3). As the L band was not detected in some of the red flesh apples (Fig. 3, lanes 9 and 10), its presence is not necessary for the creation of red phenotype. In the white flesh background, the presence of only one 23 bp unit does not ensure up- regulation of *MdMYB1* gene. However, 5+1 repeat of this 23 bp unit in *MdMYB10* promoter ensures higher level of gene expression and anthocyanin accumulation. There are some studies indicating effects of such repeat units on transcript levels (Huda *et al*., 2009; Vinces *et al*., 2009). Esplely *et al*. (2009) reported that *MdMYB10* protein targets its own promoter (using a Dual luciferase transient tobacco assay) on a minisatellite, identical to 23 bp repeat in the promoter of *MdMYB10b*, resulting in a higher transcription of this gene in the transgenic tobaco. Other areas of the apple genome were searched and no other similar 23 bp repeat was found. Therefore, this element hardly could be considered as a real minisattelite which is used for the repeated elements scattered in the genome. *MdMYB10* could be the only factor that interacts to this 23 bp repeat element but present evidences do not exclude the possibility of other regulatory factors interacting to this element.

The intensity of red color in the flesh of tested apple geo-variants was divers from light pink to dark red. Also, the pattern of anthocyanin accumulation was different, as in some apples accumulated in the interior core while in other in the external flesh. Since repeated area of the promoter of *MdMYB10* gene has been linked to the red flesh color formation (Espley *et al*., 2007), we suggested that variation of flesh color phenotype might be linked to the number of repeat units in this area as well. All tested geo-variants had the same number and sequence of repeat units at the promoter. This lack of direct correlation between pattern of color or color intensity and number of repeat units indicates that, flesh color diversity of tested geo-variants which grow in different climates might be affected by genes other than *MdMYB10* and environmental effects. It has been indicated that two factors light and low temperature affect anthocyanin accumulation in apple fruit (Saure, 1990; Lancaster, 1992).

The same number of repeats and conserved sequences of repeat units among all tested geo-variants, by present and another study (Espley *et al*., 2007), indicated that this variation has happened only once from one repeat to the 5+1 repeat in the process of evolution. A sudden mutation might have occurred in a primary ancestor and selective pressure maintained it in the next generations and spread all around the world. Therefore, it is suggested that red flesh apples around the world have evolved from a common ancestor and this mutation has never changed during the course of evolution.

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