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

Ebrahim Mahmoudi, Bahram Mohammad Soltani, Abbas Yadollahi, Ebrahim Hosseini

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 x domestica; Color intensity; MdMYB; Red flesh; Tandem repeats

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 x 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-
cytochrome 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 vinifera), 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, resulting 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 Mybexo1-F2 (5-GGCCGTTTGTAACCGACTGAGATA) and Mybexo1-R1 (5'-ACGCACCTGCTGAGAAGATT) 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 MgCl2, 5 μM of each primer and 1U of Taq polymerase. The PCR reaction carried out as follows: pre-denaturation 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>
<thead>
<tr>
<th>Variety</th>
<th>Flesh color</th>
<th>Skin color</th>
<th>Type</th>
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<tbody>
<tr>
<td>B.9</td>
<td>Red</td>
<td>Red</td>
<td>Wild type</td>
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<tr>
<td>T3</td>
<td>Red</td>
<td>Red</td>
<td>Wild type</td>
</tr>
<tr>
<td>H-GH</td>
<td>Red</td>
<td>Red</td>
<td>Wild type</td>
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<tr>
<td>Ardebil</td>
<td>Red</td>
<td>Red</td>
<td>Wild type</td>
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<td>Red</td>
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<tr>
<td>GHV 2</td>
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<td>Red</td>
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<tr>
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<td>Red</td>
<td>Wild type</td>
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<tr>
<td>SH10</td>
<td>Red</td>
<td>Red</td>
<td>Wild type</td>
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<tr>
<td>Granny smith</td>
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<td>Green</td>
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<tr>
<td>Golab</td>
<td>White</td>
<td>Yellow</td>
<td>Commercial</td>
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<td>Arous</td>
<td>White</td>
<td>Red</td>
<td>Commercial</td>
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<tr>
<td>Gala</td>
<td>White</td>
<td>Red</td>
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<tr>
<td>Fuji</td>
<td>White</td>
<td>Red</td>
<td>Commercial</td>
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<tr>
<td>Red delicious</td>
<td>White</td>
<td>Red</td>
<td>Commercial</td>
</tr>
<tr>
<td>Jonathan</td>
<td>White</td>
<td>Red</td>
<td>Commercial</td>
</tr>
</tbody>
</table>

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’-ATGGAGGGATATAACGAAAACC) and Myb10-R1 (5’-TTCTTCTTTGGAATGATTCCA) 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, 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” dinucleotids 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”,
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

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**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.

**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 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) “Jonathan”; 8) “Golab”; 9) “H-GH”; 10) “Ardebiil”; 11) “B.9”; 12) “SH10”; 13) “GHV3”; 14) “GHV2”; 15) “GH1”; 16) “T3”.

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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-varients 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 repeats 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

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**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.
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). MdMYB1 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 (MDCO13323.319) 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 (MDCO13323.319) 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 MdMYB4 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/MdMYB4 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; Vincen et al., 2009). Espley 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 MdMYB10, resulting in a higher transcription of this gene in the transgenic tobacco. 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 minisatellite 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-varients 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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