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A and *MdMYB1* allele-specific markers controlling apple (*Malus* x *domestica* Borkh.) skin color and suitability for marker-assisted selection

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ABSTRACT. Pre-selection for fruit skin color at the seedling stage would be highly advantageous, with marker-assisted selection offering a potential method for apple pre-selection. A and *MdMYB1* alleles are allele-specific DNA markers that are potentially associated with apple skin color, and co-segregate with the *Rf* and *Rni* loci, respectively. Here, we assessed the potential application of these 2 alleles for marker-assisted breeding across 30 diverse cultivars and 2 apple seedling progenies. The red skin color phenotype was usually associated with the *MdMYB1-1* allele and A^1 allele, respectively, while the 2 molecular markers provided approximately 91% predictability in the 'Fuji' x 'Cripps Pink' and 'Fuji' x 'Gala' progenies. The results obtained from the 30 cultivars and 2 progenies were consistent for the 2 molecular markers. Hence, the results supported that *Rf* and *Rni* could be located in a gene cluster, or even correspond to alleles of the same gene. Our

results are consistent with the hypothesis that red/yellow dimorphism is controlled by a monogenic system, with the presence of the red anthocyanin pigmentation being dominant. In addition, our results supported that the practical utilization of the 2 function markers to efficiently and accurately select red-skinned apple cultivars in apple scion breeding programs.

Key words: Apple; Fruit coloration; Marker-assisted selection; A-alleles; *MdMYB1* marker

INTRODUCTION

Apple (*Malus x domestica* Borkh.) is one of the most important fruit crops in the temperate parts of the world. Fruit color is an important consideration for consumer choice, and significantly influences the market value of apple fruits (King and Cliff, 2002; Kim et al., 2003). Apple color is a blend of chlorophyll, carotenoids, and flavonoids. The composition and content of anthocyanins, which belong to a class of flavonoids, are responsible for the red skin color of apples (Lancaster and Dougall, 1992). The level of anthocyanin accumulation in apple is influenced by cultivar type, with red-skinned cultivars accumulating more anthocyanins compared to pale skinned ones, while non-red cultivars do not accumulate any anthocyanins (Honda et al., 2002). The high content of anthocyanins is considered to be valuable to human health, mainly because of their high antioxidant activity (Hou et al., 2004; de Pascual-Teresa and Sanchez-Ballesta, 2008). Hence, red skin color has become a quality marker for apple breeding, with breeding for strong red skin color representing an important objective of apple breeding programs.

With increasing of scientific and technological innovations, apple breeding methods have become diversified. However, cross breeding is still the main approach for apple breeding, and has been complemented by other techniques, such as chance seedlings and bud mutation. Apple is a self-incompatible and highly heterozygous species, with cross breeding resulting in diverse progeny, of which few are superior to their parents. Therefore, apple breeding usually involves backcrossing, to enhance traits of interest from high-quality parents into new cultivars (Rowan et al., 2009). Traditional cross breeding has generated numerous cultivars, such as ‘Gala’, ‘Fuji’, ‘Cripps Pink’, and ‘Qinguan’. However, many famous cultivars have arisen through chance seedlings, such as ‘Golden Delicious’, ‘Granny Smith’, and ‘Delicious’, which continue to be planted worldwide.

Modern plant-breeding methods are based on screening seedlings from breeding progenies with DNA-based markers associated with traits of interest, termed, marker-assisted selection (MAS). Such methods offer more efficient tools for cultivar improvement, particularly for fruit trees with long juvenile periods (Rowan et al., 2009). MAS has been widely implemented for plant breeding, and is particularly useful in cases where the traits of interest show recessive or polygenic inheritance, and/or are difficult or impossible to select directly (Yeam et al., 2005). Moreover, MAS holds particular promise for apple breeding, where the long juvenile period of this fruit-tree and the polygenic nature of fruit quality traits are major bottlenecks for conventional breeding programs. In other words, the progeny resulting from a cross have to be maintained for a long time before fruit quality traits may be evaluated (Eti-

enne et al., 2002). Furthermore, large numbers of progeny must be screened to identify promising genotypes. In comparison, MAS technology accelerates this selection process.

The application of MAS to apple-breeding programs has mainly focused on certain well-known traits of interest, including resistance to scab, flavor, firmness, columnar growth habit, and skin color (Takos et al., 2006; Bus et al., 2007; Zhu and Barritt, 2008; Moriya et al., 2012). For the skin color trait, it has been shown that a RAPD DNA marker derived from the red skin gene, *Rf*, is able to discriminate between most red and non-red apple cultivars, and has been used in advanced breeding selections (Cheng et al., 1996). A derived cleaved amplified polymorphic sequence (dCAPS) DNA marker based on a single nucleotide polymorphism (SNP) in the promoter of the *MdMYB1* gene was developed and found to be associated with skin color in most red and non-red apple tested cultivars, and was completely associated with skin color in a progeny population of 136 seedlings from the cross between a sibling of 'Cripps Pink' and 'Golden Delicious' (Takos et al., 2006).

In this study, 30 cultivars and 2 breeding progenies were genotyped using 2 allele-specific DNA markers (A alleles and *MdMYB1* alleles). The relationship between genotype and phenotype (fruit skin color) was analyzed at harvest. Our results supported the practical utilization of the 2 function markers to efficiently and accurately select red-skinned apple cultivars in apple scion breeding programs.

MATERIAL AND METHODS

Plant materials

Thirty apple cultivars and 2 seedling progenies were used for the analysis of A-alleles and *MdMYB1* alleles. The 2 crosses are listed, followed by the number of individuals examined in parentheses, as: 'Fuji' x 'Cripps Pink' (223); 'Fuji' x 'Gala' (202). Fruit from 'Fuji', which is a widely planted cultivar, has red skin. In addition, fruit from 'Cripps Pink', which is customarily named 'Pink Lady', also produces red-skinned fruit. 'Gala', which is an early-maturing variety, also has red skin. Both of the male parents, 'Cripps Pink' and 'Gala', are offspring of 'Golden Delicious', which is a yellow variety. Young leaves from 30 separate breeding parent cultivars and 425 seedlings of the 2 progenies were collected from an orchard at the Northwest A&F University Apple Experimental Station in Baishui, Shaanxi Province, China, in 2012. The fruit skin color phenotype of the seedlings was evaluated according to Zhu et al. (2011). All of the leaves were immediately frozen in liquid nitrogen, and stored at -80°C until use.

DNA isolation

Leaf tissue from 30 separate breeding parent cultivars and seedlings of the 2 progenies were used for DNA isolation. Genomic DNA was isolated according to Cullings (1992).

DNA markers

The markers and primer sequences used in this study are listed in Table 1. The A allele primer sequences were designed by Cheng et al. (1996). The *MdMYB1* allele primer

sequences were designed by Takos et al. (2006). The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd, China.

Table 1. Primers of DNA markers used.

DNA marker	Primer sequence	Restriction enzyme
A alleles	F 5'-GACAGGTTACGGTCCACTGCT-3' R 5'-ACGTAAGGTCAAAGATTCAGATC-3'	None
MdMYB1 alleles	F 5'-CCTGAACACGTGGGAACCGGCCCGTTGGTAAC-3' R 5'-GTGAAGGTTGTCTTTATTAGTGACGTG-3'	*Eco*O65 I

PCR amplification of *MdMYB1* marker analysis

PCR reactions were performed in a final mix of 25 µL, containing 50 ng genomic DNA, 0.3 mM of each dNTP, 1 mM $MgSO_4$, 0.3 µM of each primer (forward and reverse), 2.5 µL 10 X PCR buffer, and 1 U Platinum *Pfx* Taq DNA Polymerase (Invitrogen Biotechnology, Shanghai, China). Thermal cycling was performed on a Veriti 96-Well Thermal Cycler (Applied Biosystems, Foster, CA, USA), and included 1 cycle at 94°C for 2 min, 35 cycles at 94°C for 15 s, 58°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 5 min. After PCR reactions, 15 µL of the product was digested with 10 units of *Eco*O65 I (Takara Biotechnology, Dalian, China) at 37°C for 3 h. The digested DNA fragments were analyzed on 3% (w/v) NuSieve GTG agarose gel (Lonza, Rockland, ME, USA), and visualized by staining with ethidium bromide. PCR primers for the *MdMYB1* marker assay are listed in Table 1.

PCR amplification of A-allele analysis

The primers suggested by Cheng et al. (1996) (Table 1) were used for the detection of A-alleles analysis. Polymerase chain reactions (PCRs) were performed in a final mix of 25 µL, containing 50 ng genomic DNA, 0.25 mM of each dNTP, 1.5 mM $MgCl_2$, 0.3 µM of each primer (forward and reverse), 2.5 µL 10 X PCR buffer, and 1 U Taq polymerase (Takara Biotechnology). The Veriti 96-Well Thermal Cycler (Applied Biosystems) performed the following thermal profile: 94°C for 2 min, 40 cycles at 94°C for 30 s, 47°C for 1 min, 72°C for 1 min, followed by a final extension at 72°C for 7 min, and was then stored at 4°C. The co-dominant PCR products were electrophoresed on 2% agarose gel and visualized with ethidium bromide.

RESULTS

Skin color phenotype of apple progenies

The phenotype for the seedlings of the 'Fuji' x 'Cripps Pink' and 'Fuji' x 'Gala' crosses was classified according to Zhu et al. (2011). Apple skin color was visually recorded on a subjective scale of 5 categories: 0, non-red skin; 1, < 50% red skin, but low-intensity coloration; 2, 50-75% red skin, but low-intensity coloration; 3, 75-90% red skin, with intense coloration; 4, > 90%, with intense coloration. The 0 point scale represented non-red skin, and

the 1-4 point scale represented different levels of red coloration (Figure 1). Table 2 shows that in the 'Fuji' x 'Cripps Pink' cross combination the 0 score (non-red skin) contained 60 seedlings, and the 1-4 point scale contained 29, 31, 39, 64 seedlings, respectively. For the 'Fuji' x 'Gala' cross, there were 46 seedlings with non-red skin (0 point scale) fruit, and a total of 156 seedlings with red skin fruit (Table 2).

Figure 1. Skin color phenotypic categories used for apple progenies.

Table 2. Segregation of the *MdMYB1-1* allele in 'Fuji' x 'Cripps Pink' and 'Fuji' x 'Gala' progenies.

Cross combination	Skin color phenotype	Skin color score	No. of seedlings	Inheritance of *MdMYB1-1*	Association consistency (%)
'Fuji' x 'Cripps Pink'	Red	1	29	19:10	65.5
		2	31	31:0	100
		3	39	39:0	100
		4	64	64:0	100
	Non-red	0	60	6:54	90
	Total		223	159:64	93
'Fuji' x 'Gala'	Red	1	34	22:12	65
		2	46	46:0	100
		3	49	49:0	100
		4	27	27:0	100
	Non-red	0	46	6:40	87
	Total		202	150:52	91

Segregation of the *MdMYB1-1* allele in two progenies

The segregation of the *MdMYB1-1* allele with color inheritance was tested in the progenies from 'Fuji' x 'Cripps Pink' and 'Fuji' x 'Gala' crosses. For the 'Fuji' x 'Cripps Pink' progeny, 163 seedlings were observed with red skin color, while 60 seedlings had non-red skin color. In total, 153 seedlings with red skin fruit of this cross combination exhibited the 263-bp fragment, indicating that they contained the *MdMYB1-1* allele associating with red skin color, while the other 10 seedlings with a skin color score of 1 did not contain this allele (Figure 2, Table 2). In total, 54 of the non-red skinned seedlings only exhibited the undigested 291-bp fragment (*MdMYB1-2* and/or *MdMYB1-3* allele), which is usually associated with non-red skin. The 263-bp fragment was detected in the remaining 6 non-red skin seedlings, indicating that these seedlings also contained the *MdMYB1-1* allele. About 93% (207/223) of seedlings from this progeny showed the expected association between *MdMYB1-1* allele and red skin color (Figure 2, Table 2).

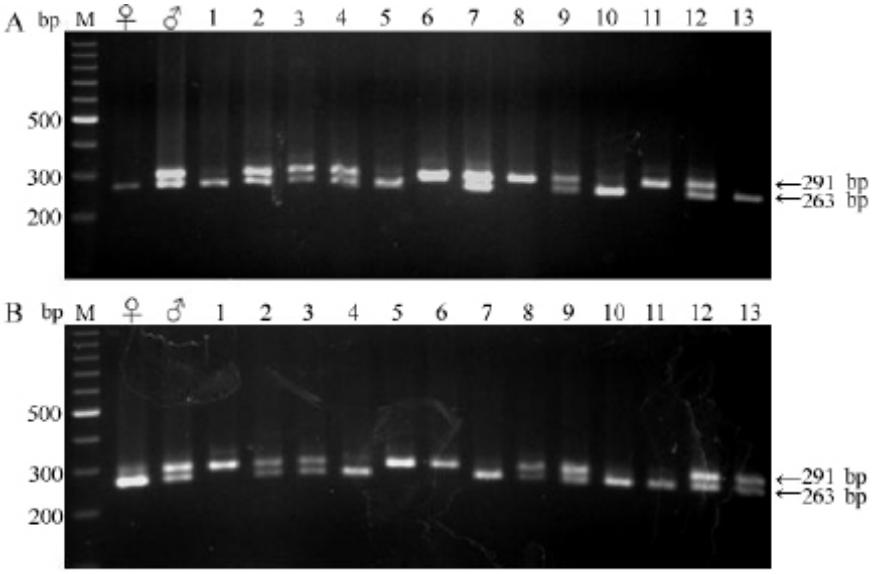

Figure 2. PCR analysis of the *MdMYB1* alleles in seedlings of the two apple progenies. **A.** 'Fuji' x 'Cripps Pink': ♀ = 'Fuji', ♂ = 'Cripps Pink', *lanes 1-13* = offspring. **B.** 'Fuji' x 'Gala': ♀ = 'Fuji', ♂ = 'Gala', *lanes 1-13* = offspring. Arrows: top 291-bp fragment, bottom 263-bp fragment. *Lane M* = 100-bp DNA molecular weight marker.

In the 'Fuji' x 'Gala' cross, red skin color was observed for 156 seedlings, while 46 seedlings had non-red skin color. Moreover, 91% (184/202) of seedlings showed the expected consistency between genotype and phenotype in this progeny, with the presence of *MdMYB1-1* allele for 92% (144/156) of the red skin color seedlings and the presence of only the *MdMYB1-2* or *MdMYB1-3* for 87% (40/46) of the non-red skin color seedlings. Twelve of the seedlings with a skin color score of 1 did not contain the *MdMYB1-1* allele, while the remaining 22 seedlings in this group and all seedlings with a skin color score of 2-4 had the *MdMYB1-1* allele. Only 6 seedlings from the non-red skin color group (with a skin color score of 0) contained the *MdMYB1-1* allele (Figure 2, Table 2). Overall, the *MdMYB1-1* allele was detected by the dCAPS assay in 150 seedlings of the 'Fuji' x 'Gala' progeny, and was not detected in the remaining 52 seedlings.

Genotypes of *MdMYB1* alleles with skin color for different cultivars

A 291-bp fragment was amplified using *MdMYB1* allele primers in 30 breeding parent cultivars. The PCR products were distinguishable after cleavage with the *Eco*O65 I restriction enzyme. Three banding patterns were observed in the 30 cultivars that were tested in this study (Figure 3). The 263-bp fragment appeared in all of the red cultivars, indicating that these cultivars contained the *MdMYB1-1* allele (Figure 3, Table 3). For 6 of these red cultivars (specifically, 'Anna', 'Honeycrisp', 'Empire', 'Jonathan', 'Qinyang', and 'Fuji'), only a 263-bp fragment (presumably homozygous for the *MdMYB1-1* allele) was detected. Another banding pattern, which was presumably heterozygous for the *MdMYB1-1* allele and *MdMYB1-2*/*MdMYB1-3* alleles, contained 263- and 291-bp fragments, respectively. This pattern was observed in the other 13 red cultivars (see Figure 3 and Table 3 for a list). Most of the non-red skinned cultivars in this study only

exhibited the 291-bp fragment, indicating that they did not contain the *MdMYB1-1* allele. Among these non-red skin cultivars, 2 green cultivars ('Granny Smith' and 'Newtown Pippin'), which sometimes develop a red blush on the exposed side of the fruit (Honda et al., 2002), appeared to have the same heterozygous genotype as some of the red cultivars (Figure 3, Table 3).

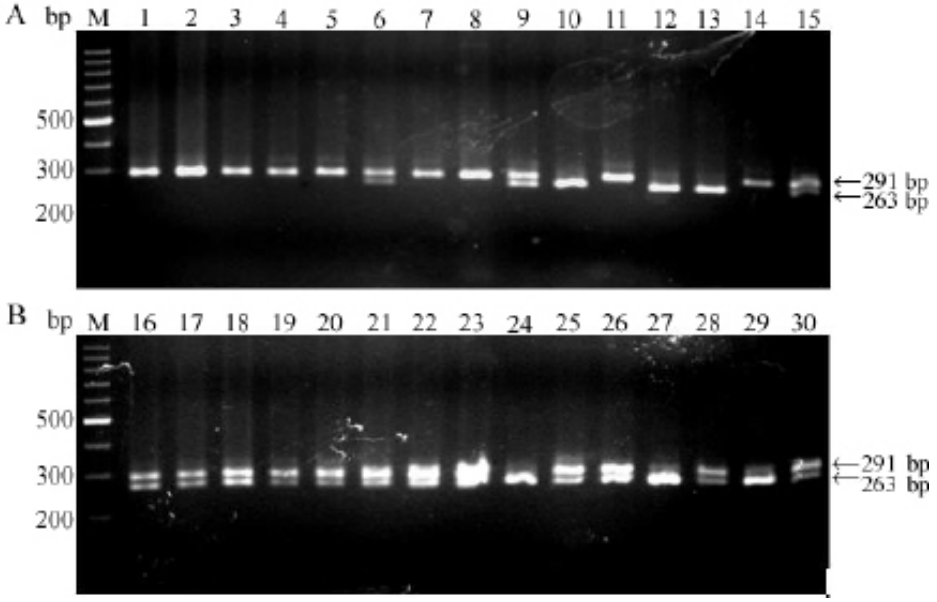

Figure 3. PCR analysis of the *MdMYB1* alleles in 30 apple cultivars. All numbering within this figure coincides with that in Table 3. Arrows: top 291-bp fragment, bottom 263-bp fragment. *Lane M* = 100 bp DNA molecular weight marker. For *lanes 1-30*, see Table 3.

Genotypes of the A alleles for different apple cultivars

As described by Cheng et al. (1996), 3 different fragments associated with apple skin color were amplified by using the universal primers (Figure 4). The A^1 fragment (1180 bp) was linked to red skin color, while the a^1 (1230 bp) and a^2 (1320 bp) fragments were linked to yellow skin color. The PCR products of A alleles for 30 apple cultivars are shown in Figure 4, while the phenotypes and genotypes of these cultivars are shown in Table 3.

Segregation of the A alleles in different progenies

The experimental results showed that both the 'Fuji' and 'Cripps Pink' genotypes were A^1a^1 (Table 3), indicating that both parents of the 'Fuji' x 'Cripps Pink' progeny were heterozygous. Three genotypes (A^1A^1, A^1a^1, and a^1a^1) were found in this progeny, with a genotypic ratio close to 1(A^1A^1): 2(A^1a^1): 1(a^1a^1). The population of the 'Fuji' x 'Cripps Pink' progeny had a 3:1 ratio for red and non-red skinned (yellow and green) fruit (Figure 5, Table 4). Trees with red skinned fruit had the A^1 fragment (presumed genotype A^1A^1 and A^1a^1), which was associated with red skin color, whereas non-red skinned fruit trees nearly always had only the a^1 fragment (presumed genotype a^1a^1).

Table 3. Genotypes of A and *MdMYB1* alleles for 30 apple cultivars.

No.	Cultivar	Parentage	Phenotype	Genotype	
				A alleles	*MdMYB1* alleles[a]
1	Golden Delicious	Unknown	Yellow	a^1a^2	2/2
2	Qrin	Seeding of Golden Delicious	Yellow	a^1a^2	2/2
3	Dailv	Seeding of Golden Delicious	Yellow-green	a^1a^2	2/2
4	Fucui	Rainier x Golden Delicious	Yellow	a^1a^1	2/2
5	Mutsu	Golden Delicious x Indo	Yellow	a^1a^2	2/2
6	Newtown Pippin	Unknown	Green	A^1a^2	1/2
7	Jinfu	Seeding of Golden Delicious	Yellow	a^1a^2	2/2
8	White Winter Pearmain	Unknown	Yellow-green	a^1a^2	2/2
9	Granny Smith	Unknown	Green	A^1a^2	1/2
10	Empire	McIntosh x Delicious	Red	A^1A^1	1/1
11	Indo	Unknown	Green	a^2a^2	2/2
12	Anna	Red Hadassiya x Golden Delicious	Red	A^1A^1	1/1
13	Honeycrisp	Macoun x Honeygold	Red	A^1A^1	1/1
14	Kuihua	Golden Delicious x Starking Delicious	Yellow	a^1a^1	2/2
15	Cameo	Red Delicious x Golden Delicious	Red	A^1a^1	1/2
16	Senshu	Toko x Fuji	Red	A^1a^1	1/2
17	Sekaiichi	Delicious x Golden Delicious	Red	A^1a^1	1/2
18	Yanguang	Golden Delicious x Delicious	Red	A^1a^1	1/2
19	Huahong	Golden Delicious x Megumi	Red	A^1a^2	1/2
20	Shengli	White Winter Pearmain x Ben Davis	Red	A^1a^2	1/2
21	Sunrise	(McIntosh x Golden Delicious) x PCF-3-120	Red	A^1a^2	1/2
22	Qinguan	Golden Delicious x Jiguan	Red	A^1a^1	1/2
23	Tsgaru	Golden Delicious x Jonathan	Red	A^1a^2	1/2
24	Jonathan	Seeding of Esopus Spitzenburg	Red	A^1A^1	1/1
25	Jonagold	Golden Delicious x Jonathan	Red	A^1a^2	1/2
26	Jiguan	Unknown	Red	A^1a^2	1/2
27	Qinyang	Seeding of Gala	Red	A^1A^1	1/1
28	Cripps Pink	Golden Delicious x Lady Williams	Red	A^1a^1	1/2
29	Fuji	Ralls x Delicious	Red	A^1a^1	1/1
30	Gala	Kidds Orange Red x Golden Delicious	Red	A^1a^2	1/2

[a]1 = *MdMYB1-1*; 2 = *MdMYB1-2* or *MdMYB1-3*.

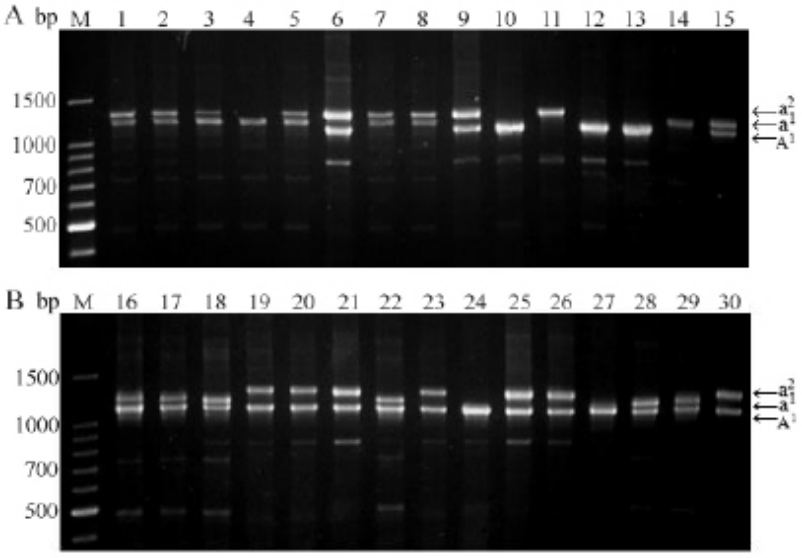

Figure 4. PCR analysis of the A alleles in 30 apple cultivars. All numbering within this figure coincides with that in Table 3. *Lane M* = 100-bp DNA molecular weight marker. For *lanes 1-30*, see Table 3.

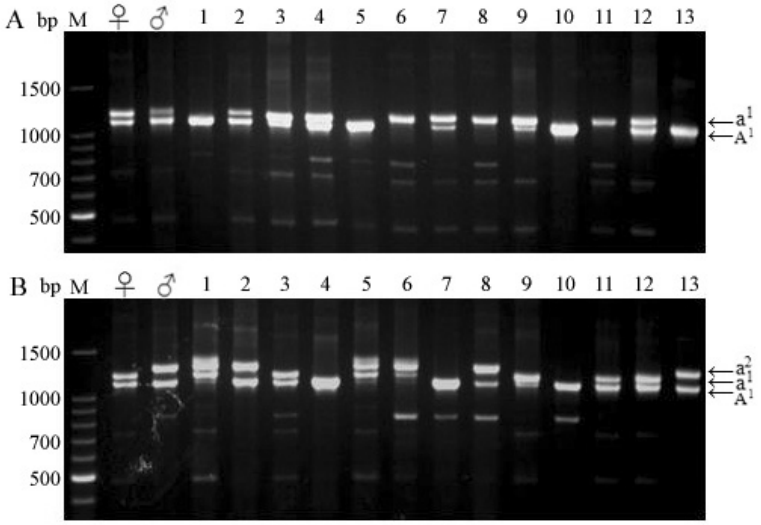

Figure 5. PCR analysis of the A alleles in seedlings of the two apple progenies. **A.** 'Fuji' x 'Cripps Pink': ♀ = 'Fuji', ♂ = 'Cripps Pink', *lanes 1-13* = offspring. **B.** 'Fuji' x 'Gala': ♀ = 'Fuji', ♂ = 'Gala', *lanes 1-13* = offspring. *Lane M* = 100 bp DNA molecular weight marker.

Table 4. Segregation of the A alleles in 'Fuji' x 'Cripps Pink' and 'Fuji' x 'Gala' progenies.

Cross combination	Phenotype or genotype	Segregation	Expected ratio	χ^2 value	P
'Fuji' x 'Cripps pink'	Fruit color	Red (163):Non-red (60)	3:1	0.432	0.511
	A alleles	A^1A^1 (58):A^1a^1 (101):a^1a^1 (64)	1:2:1	2.301	0.317
'Fuji' x 'Gala'	Fruit color	Red (156):Non-red (46)	3:1	0.5437	0.465
	A alleles	A^1A^1 (44):A^1a^1 (60):A^1a^2 (46):a^1a^2 (52)	1:1:1:1	3.0783	0.381

In the 'Fuji' x 'Gala' progeny, both parents were heterozygous. The genotype of 'Fuji' was A^1a^1, while that of 'Gala' was A^1a^2. There were 4 genotypes in this progeny, with the genotypic ratio of this progeny being close to 1(A^1A^1), 1(A^1a^1), 1(A^1a^2), or 1(a^1a^2) (Figure 5, Table 4). Like the 'Fuji' x 'Cripps Pink' progeny, the A1 fragment was also associated with red skin color in the 'Fuji' x 'Gala' progeny. However, there were 2 different fragments (a^1 and a^2), which were found to be linked to yellow or green skin color.

DISCUSSION

Fruit color is one of the characteristics that are important to the value of fruit, both commercially and aesthetically. A robust fruit skin color marker would be useful for a marker-assisted apple breeding program. In this paper, the utilities of 2 allele-specific DNA markers, A alleles and *MdMYB1* alleles, were evaluated in 30 cultivars and 2 breeding progenies with different genetic backgrounds. For the A alleles marker, 3 fragments (A^1, a^1, and a^2) linked to apple skin color in different apple cultivars were amplified using the universal primers described by Cheng et al. (1996). These genotypes were correlated with the skin color; marker A^1 was co-segregated with red skin color, while markers a^1 and a^2 were associated with non-red skin color (Figure 4).

The sequence information indicated that the a^1 and a^2 fragments were virtually identical to A^1 (except for their respective insertions), and were alleles of the red skin gene, *Rf*, which has been mapped to LG 09 (Cheng et al., 1996; Maliepaard et al., 1998). It is well known that *MdMYB1* plays an important role in the anthocyanin biosynthesis pathway for apples and has been shown to co-segregate with skin color (Takos et al., 2006). Red skin color was associated with the presence of the *MdMYB1-1* allele (263-bp fragment), with non-red-skinned cultivars only exhibiting the 291-bp fragment (*MdMYB1-2* and/or *MdMYB1-3* allele). In apple, 2 other genes (*MdMYB10* and *MdMYBA*), which are homologous to *MdMYB1*, have been isolated, and shown to regulate anthocyanin biosynthesis in apple skin or flesh (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). Chagné et al. (2007) derived a molecular marker (NZms_*MdMYB10*) from *MdMYB10*, which is able to control red color in apple. This marker co-segregates with the *Rni* locus, which is positioned on LG 09, a region that has been previously implicated in the control of red skin color. Previous research has suggested that *Rf* and *Rni* might be located in a gene cluster, or even correspond to alleles of the same gene, because they are both located at the bottom of LG 09 on the apple genome (Maliepaard et al., 1998; Chagné et al., 2007). Moreover, in our study, the evaluation of 30 cultivars and two progenies produced consistent results for the 2 DNA markers (Table 2, Table 4). These results supported that *Rf* and *Rni* could be alleles from the same gene.

A large number of researchers have studied the inheritance of apple fruit skin color, with 3 main hypotheses being available about the control of fruit skin color, because of contradictory results. Based on the analysis of anthocyanin in 'Red Delicious' apple, Lespinasse et al. (1988) suggested that anthocyanin synthesis in apple skin is controlled by 3 dominant genes. White and Lespinasse (1986) proposed that red fruit color is determined by 2 dominant complementary genes (A and B), with yellow fruit being produced by A or B alone, while yellow-green phenotypes result from the expression of a homozygous recessive genotype. It is well known that apples are complex polyploids in nature. Consequently, many important traits, including red color skin, have been shown to be controlled by single gene with diploid segregation. Crane and Lawrence (1933) proposed that a single-dominant gene controlled anthocyanin synthesis, while modifier genes might override the effect of *Rf* (red skin color gene) in certain cases (Schmidt, 1988). The research by Cheng et al. (1996) provided strong support for this hypothesis. The authors used a RAPD molecular marker that was co-segregated with the *Rf* locus in 56 cultivars or strains, and demonstrated that red/yellow color is controlled by a monogenic system, with red being dominant and yellow recessive in apple. However, this system was not applicable to green cultivars (Cheng et al., 1996). Our results supported those reported by Cheng et al. (1996).

In our study, all of the tested 19 red-skinned apple cultivars contained a 263-bp fragment (*MdMYB1-1* allele) and an A1 fragment, respectively, for the 2 DNA markers. For the non-red skinned cultivars, 'Granny Smith' and 'Newtown Pippin' with green skin color had the red genotype, while all others had the non-red genotype (Figure 3, Table 3). Our previous study showed that 'Granny Smith' apple skin rapidly turns red when the fruits are re-exposed to light after bag removal (Liu et al., 2013). It might not be an accidental phenomenon that anthocyanins are synthesized in the green cultivar 'Granny Smith'. Ju et al. (1997) suggested that it was also possible that non-red skinned cultivars contain the 'red' gene, because the fruit also produces anthocyanin under certain conditions. This finding indicates that the expression of anthocyanin biosynthetic genes in some non-red skinned apple cultivars might be blocked

in some way (Kim et al., 2003), with the bagging treatment reversing this blockage. Other researchers suggested that there may be a delayed ripening response that prevents pigment synthesis and accumulation in green-skinned apples (Cheng et al., 1996). Moreover, we also analyzed the key anthocyanin biosynthesis genes in the red skin of 'Granny Smith' fruit, and found that the expression of these genes was similar to those of the red-skinned cultivars during fruit coloration (data not shown). Therefore, we hypothesize that the coloring mechanism of 'Granny Smith' might be similar to, or the same as that of red-skinned cultivars.

In the study by Takos et al. (2006), a perfect (100%) association between the *MdMYB1-1* allele and red skin in the progeny from the cross between a sibling of 'Cripps Pink' and 'Golden Delicious' was obtained. However, this dCAPS marker provided approximately 93% and 91% predictability in 'Fuji' x 'Cripps Pink' and 'Fuji' x 'Gala' progeny, respectively. There are several explanations for this discrepancy. In addition to genetic factors, a number of external factors also affect the color intensity of apple fruit skin, including light, temperature, mineral nutrition, and orchard management practices (Saure, 1990).

In conclusion, we used 2 co-dominant DNA markers that co-segregated with the *Rf* and *Rni* loci, respectively, to test different theories for the inheritance of apple fruit skin color. Our results for the 2 populations segregated by fruit color demonstrated that the same markers showed linkage to the locus responsible for fruit skin color variation. Therefore, we concluded that the same locus was segregated in the 2 populations. The analyses of 30 cultivars and 2 breeding progenies further supported the single-gene hypothesis. Both the A and *MdMYB1* allele-specific DNA markers used here enabled us to predict the future fruit color by creating a variety at the beginning of the breeding process. It is evident that these molecular markers will reduce time, cost, and considerable effort, in addition to being expected to improve accuracy.

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