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# Identification of co-dominant RAPD markers tightly linked to fruit skin color in apple

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**Abstract** A simple genetic basis for the red/yellow skin-color polymorphism in apple was verified using DNA markers. Bulked segregant analysis identified one 10-base oligomer that generated different fragments in each of the bulks. After testing the primer in four populations, two fragments were found to be associated with red skin color and another two fragments associated with yellow skin color. Three of the fragments (1160, 1180, and 1230 bp) were partly sequenced and found to share high sequence homology, suggesting these were generated from the same locus. A pair of universal primers were designed to amplify the fragments. In the 'Rome Beauty' × 'White Angel' population, two fragments were associated with red skin color; one fragment designated as $A^1$ (1160 bp) was from 'Rome Beauty' and another fragment ($A^2$, 1180 bp) was from 'White Angel'. Progeny possessing both fragments, or either one, had red fruit. Both parents displayed an alternate fragment, $a^1$ (1230 bp), associated with yellow-skinned fruit. In three other crosses tested, only fragment $A^1$ co-segregated with red skin color; two fragments, $a^1$ and $a^2$ (1230 bp and 1320 bp), were associated with yellow skin color. Our results are consistent with the hypothesis that the red/yellow dimorphism is controlled by a monogenic system with the presence of the red anthocyanin pigmentation being dominant. There was no indication that other modifier genes could reverse the effect of the locus ($R_f$) linked to the markers. Examination of amplification products in 56 apple cultivars and advanced breeding selections demonstrated that the universal primers could be used to correctly predict fruit skin color in most cases.

**Key words** *Malus* × *domestica* · Anthocyanin biosynthesis · Marker-assisted selection · Sequence-tagged site

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## Introduction

Fruit skin color in apple is produced by a blend of carotenoids, chlorophyll, and anthocyanins (Lancaster 1992). Anthocyanins are responsible for the red color in the skin, and thus formation of anthocyanin in the skin is a critical character for apple breeders. Pre-selection for fruit skin color at the seedling stage would be highly advantageous. Though many researchers have studied the inheritance of apple fruit skin color, the results are contradictory (Brown 1992). Crane and Lawrence (1933) suggested single-dominant gene control of anthocyanin on apple, yet Schmidt (1988) postulated that in certain cases modifier genes could override the effect of $R_f$ (red skin color gene). White and Lespinasse (1986) proposed that red fruit color was determined by two dominant complementary genes (*A* and *B*), with yellow fruit produced by *A* or *B* alone, and yellow-green phenotypes resulting from the expression of a homozygous recessive genotype. A single dominant gene for blush was reported to be linked to *A* or *B*.

Molecular markers provide a powerful tool to help resolve our understanding of the inheritance of certain traits, and they offer a potential method for pre-selection. Co-dominant markers are valuable because they can be used to determine the exact genetic constitution of an individual plant and to distinguish homozygotes from heterozygotes, thereby increasing selection efficiency. Weeden et al. (1994) found the isozyme marker *Idh*-2 to be closely linked to apple fruit color in a progeny derived from 'Rome Beauty' × 'White Angel'. However, the enzyme activity of this isozyme is best detected in young leaves, and it may be used as a marker only in the populations segregating for *Idh*-2.

Randomly amplified polymorphic DNA (RAPD) markers have been useful in genetic analysis (Williams et al. 1990). RAPD markers, combined with bulked segregant analysis (BSA), have been used to identify markers closely linked to economically important traits (Michelmore et al. 1991). Conversion of such markers to sequence-tagged sites (Olson et al. 1989) further facilitates their use in map-

ping and marker-assisted selection applications. The objectives of the present study were to identify DNA markers linked to the gene(s) for fruit skin color in apple and to investigate the inheritance of this trait.

## Materials and methods

### Plant materials

Four progenies segregating for fruit skin color were studied. These crosses are listed, followed by the number of individuals examined in parentheses, as: 'Rome Beauty'×'White Angel' (72); NY 489× NY 61343-1 (22); 'Esopus Spitzenburg'×NY 75441-67 (23); 'Wijcik McIntosh'×NY 75441-23 (61). Fruit from 'Rome Beauty', a widely planted cultivar, has red skin; 'White Angel' is an ornamental crab apple with *Malus sieboldii* or *Malus sargentii* in its pedigree and also produces red-skinned fruit. NY 489, NY 61343-1, NY 75441-67, and NY 75441-23 are advanced breeding selections developed in the apple breeding program at Geneva, New York. NY489 is a hybrid of 'Northwest Greening'×'Golden Delicious;' NY 61341-1 is 'Spencer'×[Esopus Spitzenburg×PRI 14-145]; and NY 75441-23 and -67 are 'Prima'×'Spartan.' Of these NY 489 has fruit with yellow skin, whereas the others have red-skinned fruits. 'Esopus Spitzenburg' (red-skinned fruit) is of unknown parentage and 'Wijcik McIntosh' (red-skinned fruit) is a sport of 'McIntosh'. Fifty six cultivars and advanced breeding selections representing different types of fruit skin color were collected from the apple breeding program at Cornell University (Table 1).

### DNA extraction

Either young or mature leaves were used for DNA extraction. The extraction procedure was as described by Cheng and Roose (1995).

### PCR amplification

The PCR reactions were carried out in 16-µl volumes containing 10 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 40 mM of each dNTP, 1 unit of *Taq* polymerase (Promega), 1 µl of primer (10 µM), and 25 ng of genomic DNA. Amplification was performed in a PTC-100 Programmable Thermal Controller (MJ Research, Inc.) programmed for 45 cycles of 5 s at 94°C, 30 s at 36°C, and 60 s at 72°C. Amplification products were separated on 2% agarose gels. After staining with ethidium bromide, gels were observed and photographed under UV light.

### Bulked segregant analysis

Two DNA samples were bulked from ten individuals with red-skinned fruit and ten individuals with yellow-skinned fruit, respectively, from the 'Rome Beauty'×'White Angel' population and used to screen random primers (10-mer, University of British Columbia, Canada). Amplifications of DNA from the other populations were conducted after markers were found.

### Cloning and sequencing of the RAPD fragments

RAPD bands were excised from the gel and purified using GeneClean (BIO101 Inc.). The fragments were ligated directly into pGEM-T using the pGEM-T Cloning System (Promega). Plasmid DNAs were prepared according to the ABI 373A sequencing manual and the fragments were sequenced using a ABI 373A Sequencer at the Cornell University Biotechnology Program.

**Table 1** Fruit color phenotypes and corresponding marker genotypes as amplified by the universal primers

| Cultivar | Fruit color[a] | Genotype[b] |
|---|---|---|
| Granny Smith | G | $A^1a^2$ |
| Greening Rhode Island | G | $A^1a^2$ |
| Lodi | G | $a^1a^{1c}$ |
| Shamrock | G | $A^1a^1$ |
| Akane | R | $A^1A^{1c}$ |
| Baskatong | R | $A^1A^1$ |
| Britemac | R | $A^1a^1$ |
| Burgundy | R | $A^1A^1$ |
| Cortland | R | $A^1a^1$ |
| Delicious Idaho Spur | R | $A^1a^1$ |
| Earliblaze Stark | R | $A^1A^1$ |
| Empire | R | $A^1A^1$ |
| Empire Royal | R | $A^1A^1$ |
| Empress | R | $A^1A^1$ |
| Esopus Spitzenburg | R | $A^1a^1$ |
| Fuji | R | $A^1a^1$ |
| Gala | R | $A^1a^2$ |
| Idared | R | $A^1a^2$ |
| Jerseymac | R | $A^1A^1$ |
| Jonamac | R | $A^1a^1$ |
| Jonathan | R | $A^1A^1$ |
| Julyred | R | $A^1A^1$ |
| Liberty | R | $A^1A^1$ |
| Macoun | R | $A^1A^1$ |
| McIntosh | R | $A^1n^d$ |
| Monroe | R | $A^1A^1$ |
| N.Y. 429 | R | $A^1A^1$ |
| N.Y.711 | R | $A^1A^1$ |
| Newfane | R | $A^1A^1$ |
| Novaspy | R | $A^1a^1$ |
| Red Delicious | R | $A^1a^1$ |
| Rome Law | R | $A^1a^1$ |
| Rome Beauty | R | $A^1a^1$ |
| Spartan | R | $A^1a^1$ |
| Spy Northern | R | $A^1a^2$ |
| State Fair | R | $A^1a^1$ |
| Stayman Imperial | R | $A^1a^1$ |
| Tydeman's Early | R | $A^1A^1$ |
| White Angel | R | $A^2a^1$ |
| Blushing Golden | Y | $a^1a^2$ |
| Earligold | Y | $a^1a^2$ |
| Echo | Y | $a^1a^2$ |
| Firmgold | Y | $a^1a^2$ |
| Ginger Gold | Y | $a^1a^2$ |
| Golden Delicious | Y | $a^1a^2$ |
| Golden Delicious Compact | Y | $a^1a^2$ |
| Golden Delicious Smoothee | Y | $a^1a^2$ |
| Honeygold | Y | $a^1a^1$ |
| Hudson Golden Gem | Y | $a^2a^2$ |
| N.Y. 347 | Y | $a^1a^2$ |
| N.Y.489 | Y | $a^2a^2$ |
| Orin | Y | $a^1a^2$ |
| Ozark Gold | Y | $a^1a^2$ |
| Ultragold Stark | Y | $a^1a^2$ |
| Golden Supreme | Y | $a^1a^1$ |
| Yellow Newtown | Y | $a^2a^2$ |

[a] R=red; Y=yellow; G=green fruit color
[b] $A^1$, linked to red fruit color; $A^2$, linked to red fruit color; $a^1$, linked to yellow fruit color; and $a^2$, linked to yellow fruit color
[c] Some cultivars and selections shown with homozygous genotypes may have a 'null' allele for the marker sequence
[d] Absent fragment linked to the allele for yellow fruit color

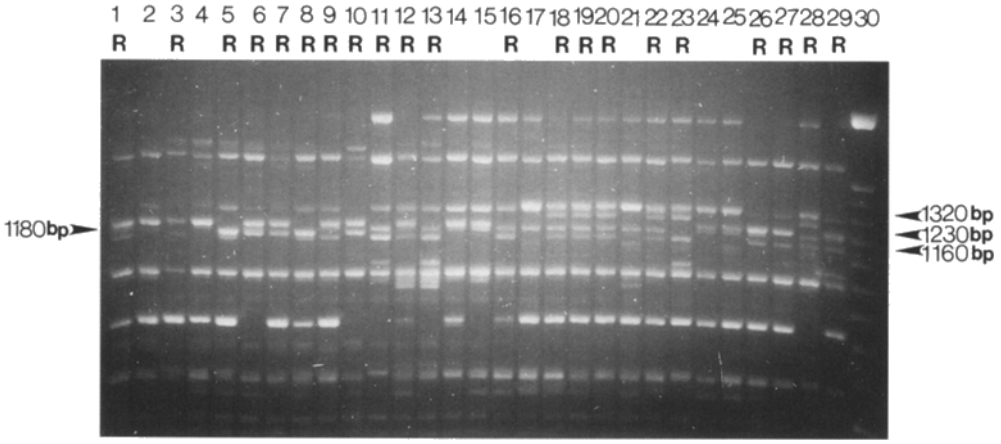


**Fig. 1** Amplification of genomic DNA from different apple progenies and their parents using primer BC 226. Key to individuals: *lanes 1 to 8* progeny of 'Rome Beauty'×'White Angel', *lane 9* 'Rome Beauty', *lane 10* 'White Angel'; *lanes 11–15* progeny of 'Wijcik McIntosh'×NY 75441-23, *lane 16* 'Wijcik McIntosh'; *lanes 17–20* progeny of NY 489×NY 61343-1, *lane 21* NY 489, *lane 22* NY 61343-1; *lanes 23–27* progeny of 'Esopus Spitzenburg'×NY 75441-67, *lane 28* 'Esopus Spitzenburg', *lane 29* NY 75441-67; *lane 30* molecular-weight marker. R=red-fruited progeny

Amplification of target fragments using one pair of specific primers

Universal primers were designed to amplify the fragments associated with fruit skin color. Based on sequencing data, the following primers were synthesized: 5′ GACAGGYTACGGTCCACTGCT 3′ and 5′ ACGTAAGGTYAAAGATTCAGATC 3′, where Y=T or C. The amplification reaction contained 20 ng of genomic DNA, 13.2 µl of $H_2O$, 2.5 µl 10x buffer, 1.5 µl of 2.5 mM $MgCl_2$, 1.25 µl of 2.5 mM of each dNTP, 0.4 µl of 20 µM primers each, and 0.5 units of *Taq* DNA polymerase (Promega). The total volume was 25 µl. The amplification was performed on an MJ Research Thermocycler. Cycling parameters consisted of 30 s at 94°C, 1 min at 45°C, 1 min at 72°C for 40 cycles.

## Results and discussion

### Identification of fragments linked to fruit skin color

About 350 random primers were screened against the two DNA bulks from the 'Rome Beauty'×'White Angel' population. An average of 11 to 12 bands were amplified per primer, although about 10% of the primers gave no amplification products. One primer (BC226, primer sequence 5′ GGGCCTCTAT 3′) amplified two fragments, which were present in the red fruit bulk and absent in the yellow fruit bulk. This primer was tested further on four populations. Several DNA fragments were found to be associated with red and yellow skin color (Fig. 1). In the 'Rome Beauty'×'White Angel' population, two fragments (1160 bp and 1180 bp) were associated with red skin color and one fragment (1230 bp) was associated with yellow skin color. In the 'Wijcik McIntosh'×NY 75441-23 population, two fragments (1160 bp and 1230 bp) co-segregated with red and yellow skin color, respectively. In the NY 489×NY 61343-1 and 'Esopus Spitzenburg'×NY 75441-67 populations, one fragment (1160 bp) and two fragments (1230 bp and 1320 bp) were associated with red and yellow skin color, respectively (Fig. 1).

### Amplification of the fragments using one pair of universal primers

Three DNA fragments (1160, 1180 and 1230 bp) that segregated in the four populations were cloned and sequenced. The sequencing information indicated that these fragments are highly homologous except for small deletions, insertions, and mutations (data not shown). A pair of specific primers were designed based on the sequence data of the 1160-bp and 1230-bp fragments.

Different annealing temperatures were tested to amplify all fragments linked to fruit skin color in the same reaction. At 55°C, fragments of the expected size were amplified except for the fragment (1180 bp) linked to red skin color from 'White Angel'. All fragments were amplified when the annealing temperature was lowered to 45°C. Three base differences were found in the primer regions between the 1160 bp and 1180 bp fragments (data not shown). Therefore, it was possible to amplify certain regions of the plant genome from distantly related species using RAPDs. We believe that the different sized fragments represent true homologous sequences because they segregate as a single locus. If they were different repeats of a multicopy family, it would be very difficult to explain why only one or two fragments were amplified in each variety tested.

### Segregation of the fragments in different populations

Different fragments amplified using the universal primers appeared to co-segregate with fruit skin color (Table 2 and Fig. 2). The different amplification products were designed as $A^1$, 1160 bp, linked to red skin color from 'Rome Beauty'; $A^2$, 1180 bp, linked to red skin color from 'White Angel'; $a^1$, 1230 bp, linked to yellow skin color; and $a^2$, 1320 bp, linked to yellow skin color.

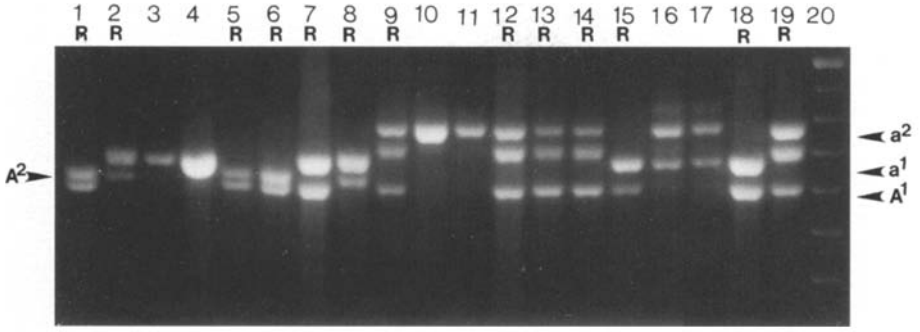


**Fig. 2** Segregation of the co-dominant locus amplified using a pair of universal primers. Key to individuals: *lanes 1–6* progeny of 'Rome Beauty' × 'White Angel', *lane 7* 'Rome Beauty', *lane 8* 'White Angel'; *lanes 9, 10* progeny of NY 489 × NY 61343-1, *lane 11* NY 489, *lane 12* NY 61343-1; *lanes 13–17* progeny of 'Esopus Spitzenburg' × NY 75441-67, *lane 18* NY 75441-67, *lane 19* 'Esopus Spitzenburg'; *lane 20* molecular-weight marker. R = red-fruited progeny

The 'Rome Beauty' × 'White Angel' population segregated 3:1 for red- and yellow-skinned fruit (Table 2) with fragments $A^1$ and $A^2$ associated with red skin color (Figs. 1 and 2). Trees with red-skinned fruit possessed either one or both fragments, whereas trees with yellow-skinned fruit nearly always displayed only the $a^1$ fragment (presumed genotype $a^1a^1$). These results indicated that both parents were heterozygous ($A^1a^1$ and $A^2a^1$). The genotypic ratio in the progeny was $1(A^1A^2):1(A^1a^1):1(A^2a^1):1(a^1a^1)$ (Table 2).

In 'Esopus Spitzenburg' × NY 75441 -67, both parents were heterozygous. The genotype of 'Esopus Spitzenburg' was $A^1a^1$ and that for NY 75441-67 was $A^1a^2$. Two different fragments were found to be linked to yellow fruit color. The genotypic ratio in the progeny was close to $1(A^1A^1):1(A^1a^1):1(A^1a^2):1(a^1a^2)$. In NY 489 × NY 61343-1, NY 489 was homozygous ($a^2a^2$) and NY 61343-1 was heterozygous ($A^1a^2$), with a $1(A^1a^2):1(a^2a^2)$ segregation ratio observed in the progeny (Table 2).

## Absence of fragment in 'McIntosh'

In the 'Wijcik McIntosh' × NY 75441-23 population, the $A^1$ and $a^1$ fragments co-segregated with red and yellow skin color, respectively, and no recombinants were observed (Table 3). However, progeny phenotypes segregated $A^1\_\_$ (29):$A^1a^1$(17):$a^1\_\_$ (15). 'Wijcik McIntosh' displays only the $A^1$ fragment, and if it were homozygous $A^1A^1$ we would expect a genotypic segregation ratio of 1:1 ($A^1A^1$:$A^1a^1$) in the progeny. The 2:1:1 ratio actually observed suggests that 'McIntosh' may contain a DNA sequence linked to yellow skin color that is not amplified by the universal primers. Careful analysis of the RAPD products produced by the original 10-mer primer indicated that no fragment linked to yellow skin color was amplified in 'McIntosh'. Thus, 'McIntosh' acts as though it possesses a null allele for the marker.

## Inheritance of fruit color

We used the DNA markers identified to test the different theories for the inheritance of fruit skin color (reviewed in Brown 1992). Our results for the four populations segregating for fruit color demonstrated that the same markers show linkage to the locus responsible for fruit skin-color variation (Table 3), and we concluded that the same locus is segregating in all four populations. As the cultivars used as parents in these crosses are of diverse genetic backgrounds, we suggest that red skin color in domestic apple is primarily controlled by one or more dominant genes at a single locus, $R_f$. This locus is within 2 cM of both *Idh-2* and the DNA fragments generated by the universal primers described in this paper (Table 3).

The analysis of 56 cultivars and advanced breeding selections further supports the single-gene hypothesis. Of the 17 cultivars analyzed with yellow-skinned fruit, two displayed only the $a^1$ fragment, 12 contained both $a^1$ and $a^2$, and three showed only the $a^2$ fragment (Table 1). If two complementary dominant genes were required for expression of red-skinned fruit, some of the yellow-fruited cultivars we examined would be expected to possess the dominant allele at $R_f$ and, hence, the $A^1$ or $A^2$ DNA fragment.

Of 35 cultivars and selections with red-skinned fruit, only 'White Angel' had fragment $A^2$, and all others were either the phenotypically indistinguishable genotypes $A^1A^1$ and $A^1n$ (18) or were heterozygous (13 $A^1a^1$ and 3 $A^1a^2$). 'White Angel' is a crabapple which appears to contain an Asian species in its pedigree (Simon and Weeden 1991). If fragment $A^2$ is derived from this Asian species, it suggests that red color is determined by the same locus in two distantly related subsections of the genus. We also found two fragments associated with yellow fruit color. The several size variants of the DNA marker may indicate the existence of two or more alleles at the $R_f$ locus.

It is important to note that the red/yellow skin-color polymorphism is usually defined as the skin being primarily (>50%) red or primarily (80–90%) yellow. Many yellow apples display a distinctive 'blush' of anthocyanin pigmentation in a limited region of the fruit, often that exposed to direct sunlight (Schmidt 1988). Thus, many cultivars producing fruit with 'yellow' skin are capable of anthocyanin synthesis. The basis of 'blush' formation may be the activation of different genes in the anthocyanin biosynthetic pathway or else a result of the $r_f$ allele being 'leaky' in certain environments. Many of the steps in the

**Table 2** Segregation analysis of fruit color and DNA fragments generated by a pair of universal primers

| Cross | Trait or marker | Segregation | Expected ratio | *P* |
|---|---|---|---|---|
| 'Rome Beauty'×'White Angel' | Fruit color | Red (55): yellow (17) | 3:1 | ns |
| | DNA marker | $A^1A^2$(18):$A^1a^1$(19): $A^2a^1$(19):$a^1a^1$(16) | 1:1:1:1 | ns |
| 'Wijcik McIntosh'×NY 75441-23 | Fruit color | Red (46): yellow (15) | 3:1 | ns |
| | DNA marker | $A^1$–(29):$A^1a^1$(17):$a^1$–(15) | 1:1 | s*[a] |
| 'Esopus Spitzenburg'×NY 75441-67 | Fruit color | Red (15): yellow (8) | 3:1 | ns |
| | DNA marker | $A^1A^1$(3):$A^1a^1$(7): $A^1a^2$(6):$a^1a^2$(7) | 1:1:1:1 | ns |
| NY 489×NY 61343-1 | Fruit color | Yellow (12): red (10) | 1:1 | ns |
| | DNA marker | $a^2a^2$(11):$A^1a^2$(11) | 1:1 | ns |

[a] See discussion of possible absent fragment in Results and discussion section

**Table 3** Joint segregation analysis for fruit color and DNA fragments in four apple progenies

| Cross | Progeny phenotypes[a] | | | | | | $\chi^{2b}$ | Recombination freq.±SE |
|---|---|---|---|---|---|---|---|---|
| | Red fruit | | | Yellow fruit | | | | |
| | AA | Aa | aa | AA | Aa | aa | | |
| 'Rome Beauty'×'White Angel' | 18 | 37 | 0 | 0 | 1 | 16 | 66.6 | 1.4 ± 12 |
| 'Wijcik McIntosh'×NY 75441-23 | 29 | 17 | 0 | 0 | 0 | 15 | 61.0 | no recomb |
| 'Esopus Spitzenburg'×NY 75441-67 | 3 | 12 | 0 | 0 | 1 | 7 | 19.8 | 3.9 ± 20 |
| NY489×NY 61343-1 | 0 | 10 | 0 | 0 | 1 | 11 | 18.3 | 4.6 ± 4.4 |
| Pooled estimate ($n$=178) | | | | | | | 139 | 1.7± 7.2 |

[a] AA includes fragment genotypes $A^1$__ and $A^1A^2$; Aa includes $A^1a^1$, $A^2a^1$ and $A^1a^2$; and aa includes $a^1$__, $a^1a^2$, and $a^2$__
[b] $P<10^{-4}$

biosynthetic pathway leading to anthocyanins are known to be catalyzed by enzymes coded by multigene families (Lancaster 1992), providing a plausible mechanism for the first hypothesis. The second hypothesis remains unsupported by direct evidence.

Our marker also failed to predict the incidence of green-skinned phenotypes. Of the four cultivars tested with green-skinned fruit, three had DNA marker genotypes for red-skinned fruit and the remaining one had a DNA marker genotypes for yellow-skinned fruit (Table 1). We suggest that an entirely different mechanism, such as a delayed ripening response, is involved in the production of green-skinned fruit. Similarly, our marker does not correlate with the presence or absence of stripes on red-skinned fruit or with the intensity of the anthocyanin pigmentation.

Although the precise biochemical function of $R_f$ has yet to be elucidated, so that several aspects regarding the pattern and hue of pigmentation cannot be addressed, our results do not justify the need for a more complex genetic model to explain the red/yellow polymorphism in apple. We have found no evidence for a second modifier gene that produces either red-skinned fruit in the absence of the $R_f$ allele or yellow-skinned fruit in the presence of the $R_f$ allele. We suggest that the appearance of a small fraction of trees with red fruit in a population produced from a yellow-by-yellow cross, as reported by Schmidt (1988), are probably the result of a low level of pollen contamination which is often observed in controlled crosses in apple. Similarly, we feel we can reject the hypothesis of White and Lespinasse (1986) and Lespinasse et al. (1988), at least for the several cultivars examined by both the Lespinasse group and ourselves, that red skin color is produced by a two-gene system. If two genes were involved, we would expect that skin color would segregate independently of our marker in some crosses and that our marker would not correctly correlate with fruit skin color in a significant proportion of the cultivars surveyed.

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