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Efficient Breeding System for Red-fleshed Apple Based on Linkage with S_3-RNase Allele in 'Pink Pearl'

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***Abstract*. We have used a red-fleshed apple cultivar, *Malus ×domestica* Pink Pearl, and its progeny, 'JPP 35', as paternal parents for producing new red-fleshed cultivars suitable for fresh use or processing such as pie fillings, dried apple, apple juice, or cider. In this process, we found that the S_3-RNase allele of 'Pink Pearl' was linked to its red flesh trait. It was suggested that this trait might be controlled by a new gene apart from the *MYB10* (*MdMYB10*) gene. Using 'JPP 35' (*S*-RNase allele genotype; S_3S_7) produced by 'Jonathan' (S_7S_9) × 'Pink Pearl' (S_3S_x) as a paternal parent, we developed a system for producing red-fleshed progenies suitable for fresh use. That is, 96% and 86% of progenies from 'Shinano Sweet' (S_1S_7) × 'JPP35' (S_3S_7) and 'Orin' (S_2S_7) × 'JPP35' (S_3S_7) containing the S_3-RNase allele, respectively, showed the red flesh trait. Similarly, red-fleshed progenies suitable for apple pie or natural red juice could be produced by 'Jonathan' (S_7S_9) × 'JPP35' (S_3S_7).**

Apples (*Malus ×domestica* Borkh.) are produced commercially in most temperate countries not only for fresh use, but also for processed goods such as juice and in slices as an ingredient for cakes, pies, and tarts. 'Fuji', 'Shinano Sweet', 'Orin', and so on are popular apple cultivars for fresh use, whereas 'Jonathan' is preferred for processing in Japan. There is a wide range of skin color variation in apple cultivars from red such as 'Jonathan', yellow such as 'Orin', and green such as 'Granny Smith'. Generally, red color is important not only as an indicator of ripening, but also for its health benefits in the form of flavonoids and anthocyanins, which are the main components of red pigments in apple, and possess antioxidant activity (Eberhardt et al., 2000; Wolfe et al., 2003). Because most Japanese consumers peel apples before eating them, we have been engaged in a project for the breeding of new apple cultivars with red flesh since 1989. Red-fleshed apples should prove attractive to consumers given their novel color and health benefits. Fertilization in apple is controlled by a gametophytic self-incompatibility (GSI) system in which not only self pollen tube growth, but also pollen tube growth from a different cultivar having the same *S*-haplotype might be arrested in the style (de Nettancourt, 1977; Kobel et al., 1939). The *S*-RNase and SFB (*S*-locus F-box) genes, which are functional in pistils and pollen, respectively, are located within the *S*-locus responsible for GSI in apple (Broothaerts et al., 1995; Cheng et al., 2006) and from the nucleotide sequences of the *S*-RNases, the polymerase chain reaction (PCR)-based *S*-RNase allele genotype analysis method was developed (Broothaerts, 2003; Kitahara and Matsumoto, 2002a, 2002b; Kitahara et al., 2000; Matsumoto and Kitahara, 2000; Matsumoto et al., 1999a, 1999b, 2001, 2003a, 2009, 2010; Morita et al., 2009). Using the PCR method, we have investigated the *S*-RNase allele composition of more than 430 apple cultivars, lineages, and species in Japan (Matsumoto et al., 2003b, 2003c, 2007).

MdMYBA or *MdMYB1*, members of the *MYB* transcription factor family responsible for apple fruit skin anthocyanin accumulation, were isolated from 'Tsugaru' or 'Cripps Pink' (Ban et al., 2007; Takos et al., 2006). *MdMYBA* expression correlated with the skin anthocyanin accumulation induced by ultraviolet B irradiation and low-temperature treatment (Ban et al., 2007). To produce red flesh and foliage colors, another *MYB* gene or allele, *MdMYB10*, was isolated and has been shown to cosegregate with the red flesh and foliage phenotypes (Chagné et al., 2007; Espley et al., 2007), particularly after modification of its upstream regulatory region. Espley et al. (2009) found that red foliage and red-fleshed apples contained five direct tandem repeats of a 23-bp sequence in the *MYB10* promoter region (the R6 promoter), whereas white-fleshed apples contained no tandem repeats (the R1 promoter). They devised a model showing the autoregulation of the R6 promoter by *MYB10* and also suggested a mechanism for upregulation of the anthocyanin pathway leading to red foliage and flesh in apple.

Apples normally require several years from germination to fruiting as well as considerable field space if they are to be healthy. For instance, breeding of the major cultivar Fuji ('Ralls Janet' was crossed with 'Delicious' in 1939, selected in 1958, registered in 1962), 787 seedlings from 2004 seeds were grown healthily and took 12 years for first fruiting. In this article, a red-fleshed apple cultivar, Pink Pearl ('Surprise' × unknown pollen parent, selected in 1944), was used as a pollen parent for producing new red-fleshed cultivars suitable mainly for processed products such as natural red apple juice or pies. To efficiently produce new red-fleshed apple cultivars, we began by selecting 'JPP 35' ('Jonathan' × 'Pink Pearl') as the mother plant. We then developed breeding systems to produce mainly red-fleshed progenies for eating or processing.

Materials and Methods

Plant material. Malus plants used in this study were from collections at either the Nagano Fruit Tree Experiment Station, Japan, or from the Apple Research Center of National Station, Institute of Fruit Tree Science, NARO, Suzaka, Japan. Young leaves were collected and stored at –80 °C until use.

S-*RNase allele-specific polymerase chain reaction digestion analysis.* Total DNA from the leaves of individual plants was isolated as described by Thomas et al. (1993). The primers and conditions used for the *S*-RNase allele-specific PCR amplification and digestion were essentially those described by Broothaerts (2003) (S_2-, S_3-, and S_7-RNase allele), Kitahara and Matsumoto (2002b) (S_3- and S_{10}-RNase allele), Matsumoto et al. (1999a) (S_7-RNase allele), and Matsumoto et al. (1999b) (S_1-RNase allele).

Measurement of sugar content and acidity of apple. The sugar content of the juice squeezed from five equally weighted apple flesh blocks (10 g block from the sun and shade side, respectively, per fruit) was measured by a digital counter, PR-101alpha (Brix 0 to 45°; ATAGO Co., Ltd., Itabashi, Tokyo, Japan). Fruit acidity was calculated as the contents of anhydrous malic acid converted

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from a formula: T/S × 0.671 × F, in which T, titration amount (mL) of 0.1 mol·L^{-1} NaOH, S, vol. of sample (mL); and F, titers of 0.1 mol·L^{-1} NaOH.

MYB10 (MdMYB10) *promoter region-specific polymerase chain reaction amplification.* Total DNA from leaves of individual plants was isolated as described. The primers and conditions used for the *MYB10* (*MdMYB10*) promoter analysis were from Espley et al. (2009). A 496-bp fragment corresponds to the R6 promoter type and a 394-bp fragment to the R1 promoter type.

Results and Discussion

Selection of 'JPP 35' as a mother plant for production of red-fleshed cultivars. As shown in Table 1, three of four cross combinations ('Jonathan' × 'Pink Pearl', 'Himekami' × 'Pink Pearl', 'Megumi' × 'Pink Pearl') exhibited an ≈1:1 segregation ratio of red:white flesh, suggesting that the red flesh trait of progenies seemed to be inherited by mainly a single dominant gene from 'Pink Pearl'. The reason why white seedlings slightly outnumber red seedlings in all these crosses remains unknown, but the red flesh trait varied from white pink to dark red at the evaluation time from September to October (white is white, but red includes a white–pink to dark red color) so that some white seedlings may have turned white–pink after maturity. In contrast, all the progenies of 'Kizashi' × 'Pink Pearl' showed white flesh. Although we do not deny that some show white–pink after maturity, dark red seedlings were never observed. At that time, we had surmised that different white flesh cultivars were accidentally pollinated. Among these combinations, we selected 'JPP 35' ('Jonathan' crossed with 'Pink Pearl' in 1989 and selected in 2000) as the mother plant for further breeding of red-fleshed cultivars as a result of the stability of its dark red color (Fig. 1).

Breeding of red-fleshed apple cultivars suitable for processing. We backcrossed 'JPP 35' to 'Pink Pearl' to produce new red-fleshed cultivars homozygous for the red-fleshed allele, which we considered useful for producing stable and darker red-fleshed cultivars for processing. We expected the 3:1 segregation ratio of red:white flesh, because a single dominant gene, R^{pp}, was present as heterozygous in both 'JPP 35' and 'Pink Pearl'. However, contrary to that expectation, the segregation of red:white flesh did not deviate significantly from the 1:1 ratios, not 3:1 ratios (Table 2). At this stage, we assumed that the S_3-RNase allele in 'Pink Pearl' was tightly linked to its red flesh allele. The *S*-RNase allele genotype of 'JPP 35' was identified as S_3S_7, of which S_3 and S_7 were inherited by its paternal ('Pink Pearl' identified as S_3S_x) and maternal parents ('Jonathan' previously identified by us as S_7S_9), respectively. Expected *S* genotypes of progenies from 'Pink Pearl' (S_3S_x) × 'JPP 35' (S_3S_7) are either S_3S_7 or S_7S_x, because the S_3 in 'JPP 35' must be rejected by the S_3 in 'Pink Pearl'. We investigated whether the cosegregation of S_3-RNase and the red flesh allele was observed by *S*-RNase allele-specific PCR analyses. As shown in Table 3, 12 of 13 red-fleshed progenies from 'Pink Pearl' × 'JPP 35' contained S_3-RNase allele, but none of the 17 white progenies did, suggesting that S_3-RNase and the red flesh allele in 'Pink Pearl' must be linked. To further confirm this, we investigated five red-fleshed progenies of 'Fuji' (S_1S_9) × 'Pink Pearl' (S_3S_x) kindly provided by Dr. K. Abe and found that all the red-fleshed progenies contained the S_3-RNase allele (results not shown). On the other hand, the S_3-RNase allele was identified in seven red skin progenies and five yellow skin progenies, suggesting that the skin colors of the progenies were not cosegregating with the S_3-RNase allele (Table 3). Backcrossing of 'JPP 35' (S_3S_7) to 'Jonathan' (S_7S_9) will be the subject of our next project for producing red-fleshed cultivars suitable for processing, because the progenies (expected *S* genotypes S_3S_7 or S_3S_9) might show that red flesh trait if the S_3-RNase in 'Pink Pearl' and the red flesh trait turn out to be tightly linked.

Breeding of red-fleshed apple cultivars suitable for fresh use. As shown in Table 4, 'Pink Pearl' is higher in fruit acidity (1.27 g/100 mL) and lower in sugar content (11.0 °Brix) than those in cultivars for fresh market such as 'Shinano Sweet', 'Orin', or 'Fuji'. 'JPP 35' also exhibits a similar tendency with 'Pink Pearl'. We backcrossed 'JPP 35' to 'Shinano Sweet' or 'Orin' for producing new cultivars suitable for fresh use, i.e., by reducing fruit acidity and increasing sugar content. Because the *S*-RNase allele genotypes of 'Shinano Sweet' and 'Orin' were S_1S_7 and S_2S_7, respectively, all the progenies of 'Shinano Sweet' (S_1S_7) × 'JPP 35' (S_3S_7) and 'Orin' (S_2S_7) × 'JPP 35' (S_3S_7) were either S_1S_3 or S_3S_7 and S_2S_3 or S_3S_7, respectively. Because all of the *S* genotypes contain the S_3-RNase allele of 'JPP 35', it can be expected that all the progenies would exhibit the red flesh trait should the S_3-RNase in 'Pink Pearl' and red flesh allele prove to be tightly linked. As shown in Table 2, 67 of 70 and 51 of 59 progenies from 'Shinano Sweet' (S_1S_7) × 'JPP 35' (S_3S_7) and 'Orin' (S_2S_7) × 'JPP 35' (S_3S_7), respectively, showed the red flesh color. In the case of 'Orin' (S_2S_7) × 'JPP 35' (S_3S_7), we are inclined to think that some progenies with the white flesh color will turn white–pink after maturity. Because the genetic recombination around the *S*-locus must be maintained at a low level for the protection of *S*-haplotypes, we think the gene-related red flesh trait in 'Pink Pearl' might cosegregate with S_3 at a high level. As for 'Orin' × 'JPP 35', the segregation ratio of red:yellow skin color was 30:29 (Table 2), again

Table 1. Segregation and χ^2 results of *Malus* progenies from 'Jonathan' × 'Pink Pearl', 'Himekami' × 'Pink Pearl', 'Megumi' × 'Pink Pearl', and 'Kizashi' × 'Pink Pearl'.

		Segregation by flesh color		
		Expected	Observed	
Ovule cultivar	Pollen cultivar	Red:white	Red:white	χ^2
Jonathan (S_7S_9)	Pink Pearl (S_3S_x)	1:1	13:20	1.48 NS
Himekami (S_7S_9)	Pink Pearl (S_3S_x)	1:1	12:13	0.04 NS
Megumi (S_2S_9)	Pink Pearl (S_3S_x)	1:1	15:20	0.71 NS
Kizashi (S_2S_3)	Pink Pearl (S_3S_x)	1:1	0:26	26.0
		0:1[z]	0:26	—

[z]In case of S_3-RNase allele linked to red flesh trait in 'Pink Pearl'.
NS = nonsignificant.

Fig. 1. Flesh and skin of *Malus* ×*domestica* 'JPP 35'.

Table 2. Segregation and χ^2 results of *Malus* progenies from 'Pink Pearl' × 'JPP 35', 'Shinano Sweet' × 'JPP 35', and 'Orin' × 'JPP 35'.

		Segregation by flesh color		
		Expected	Observed	
Ovule cultivar	Pollen cultivar	Red:white	Red:white	χ^2
Pink Pearl (S_3S_x)	JPP 35 (S_3S_7)	3:1	13:17	16.04
		1:1[z]	13:17	0.53 NS
Shinano Sweet (S_1S_7)	JPP 35 (S_3S_7)	1:1	67:3	58.51
		1:0[z]	67:3	—
Orin (S_2S_7)	JPP 35 (S_3S_7)	1:1	51:8	31.33
		1:0[z]	51:8	—

[z]In case of S_3-RNase allele linked to red flesh trait in 'Pink Pearl'.
NS = nonsignificant.

Table 3. Cosegregation of S_3-RNase and red flesh allele from 'Pink Pearl'.

Cultivar	Color		Number	*S* genotype			
	Skin	Flesh					
Pink Pearl	Yellow	Red		S_3S_x			
JPP 35	Red	Red		S_3S_7			
Pink Pearl × JPP 35	Red	Red	7	S_3S_7			
	Yellow	Red	5	S_3S_7			
	Yellow	Red	1	S_7S_x			
	Red	White	9	S_7S_x			
	Yellow	White	8	S_7S_x			
Shinano Sweet	Red	White		S_1S_7			
Shinano Sweet × JPP 35	Red	Red	67	30	S_1S_3	37	S_3S_7
	Red	White	3	S_3S_7			
Orin	Yellow	White		S_2S_7			
Orin × JPP 35	Red	Red	25	11	S_2S_3	14	S_3S_7
	Red	White	5	4	S_2S_3	1	S_3S_7
	Yellow	Red	26	17	S_2S_3	9	S_3S_7
	Yellow	White	3	1	S_2S_3	2	S_3S_7

Table 4. Fruit acidity and sugar content of *Malus* cultivars.

Cultivar	Suitable for	Fruit acidity (g/100 mL)	Soluble solids (°Brix)
Pink Pearl	Processing	1.27	11.0
Jonathan	Processing	0.57	14.5
Himekami	Processing	0.50	15.2
Megumi	Processing	0.44	15.8
Kizashi	Processing	0.74	13.5
JPP 35	Processing	1.43	12.5
Shinano Sweet	Fresh use	0.31	15.8
Orin	Fresh use	0.24	14.9
Fuji	Fresh use	0.34	14.9

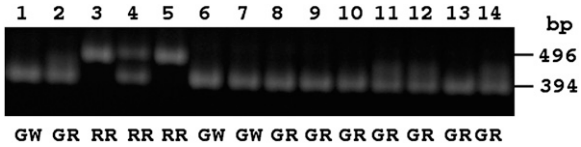

Fig. 2. Polymerase chain reaction analysis of the *MYB10* promoter region from *Malus* species, cultivars, and progenies of 'Shinano Sweet' × 'JPP 35'. The 496-bp band corresponds to the R6 promoter type and the 394-bp band to the R1 promoter type. Lane 1, 'Shinano Sweet' (GW = green foliage and white flesh); 2, 'JPP 35' (GR = green foliage and red flesh); 3, 'Tomiko' (RR = red foliage and red flesh); 4, 'Maypole' (RR); 5, 'Makamik' (RR); 6, '*M. sieversii*' (w1-10-49) (GW); 7, 'Jonathan' (GW); 8, 'Pink Pearl' (GR); 9-14 'Shinano Sweet' × 'JPP 35' (GR) progenies (No. 38, 44, 18, 55, 40, and 4, respectively).

suggesting that the red skin trait is regulated by a factor other than the R^{pp} allele. We have selected Nos. 29, 38, and 41 from 'Shinano Sweet' (S_1S_7) × 'JPP 35' (S_3S_7) as promising candidates for red-fleshed cultivars suitable for fresh use.

Recently, Espley et al. (2009) found that only red foliage and red flesh apples contained a 23-bp repeat motif in the *MYB10* (*MdMYB10*) promoter region (the R6 promoter). We investigated whether the R6 promoter existed in 'Pink Pearl', 'JPP 35', and their progenies showing the red flesh trait. As shown in Figure 2, only the R1 type promoter (394 bp) existing in white flesh cultivars and 'Maypole' (red foliage and red flesh phenotype, and R6/R1 promoter hetero type), but not the R6 promoter (496 bp), was observed in all red flesh and green foliage investigated. 'Pink Pearl', 'JPP 35', and all their progenies were of the red flesh and green foliage type. This was coincident with the observation on the cosegregation of *MYB10* (*MdMYB10*) with the inheritance of red flesh and red foliage, but not with that of the red flesh and green foliage (Chagné et al., 2007). Because the flesh color trait *Rni* locus (possibly the site of *MYB10*) (Chagné et al., 2007) and the self-incompatibility *S*-locus have been attributed to linkage groups 9 and 17, respectively (Chagné et al., 2007; Maliepaard et al., 1998), the red flesh trait in 'Pink Pearl' might be controlled by a second gene close to the S_3-RNase allele in the absence of a red foliage trait.

In conclusion, using the S_3-RNase allele tightly linked to the red flesh trait in 'Pink Pearl', we developed breeding systems for producing progenies mainly consisting of red flesh.

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