Practical Breeding of Red-fleshed Apple: Cultivar Combination for Efficient Red-fleshed Progeny Production

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Abstract. We re-investigated the flesh color and *S*-genotypes of progenies of red-fleshed apple cultivar JPP35, which was produced by ‘Jonathan’ × ‘Pink Pearl’, and clarified that 100% and 96% of progenies from ‘Shinano Sweet’ (*S*₁*S*₇) × ‘JPP35’ (*S*₃*S*₇) and ‘Orin’ (*S*₂*S*₇) × ‘JPP35’ (*S*₃*S*₇) containing *S*₃-RNase allele, respectively, showed the red flesh trait. Using this tight linkage between red flesh trait and self- and cross-compatibility relating allele such as *S*₃-RNase allele, we showed suitable cultivar combinations for efficient production of various red-fleshed apples. We also identified an unknown *S*-RNase allele in ‘Pink Pearl’ as *S*₁₁ and determined its partial genomic sequence, including a complete intron with its known *S*₃-RNase allele.

Variation of flesh color in apple (*Malus ×domestica* Borkh.) such as white or yellowish white is less than that of skin color with various shades of red, yellow, and green. Recently, the red-fleshed apple has attracted attention for its novel color, and new cultivars, Weirouge, Redlove Era, and Redlove Sirena, are planned for release within a few years (Warner, 2010). Red-fleshed cultivars are expected to have physiological functions such as antioxidative activity that were observed in the red skin cultivars (Eberhardt et al., 2000; Wolfe et al., 2003). One of the anthocyanins, cyanidin 3-galactoside, is mainly responsible for apple red coloration, and R2R3-MYB transcription factors have been shown to play an important role in transcriptional regulation of enzymes in the anthocyanin biosynthetic pathway of apple (Allan et al., 2007; Tsao et al., 2003). To date, two *MdMYB* alleles, *MdMYB1* and *10*, which are responsible for apple skin and flesh color, respectively, have been identified (Ban et al., 2007; Chagné et al., 2007; Espley et al., 2007; Takos et al., 2006). Espley et al. (2009) found that the modification of the *MdMYB10* upstream region, i.e., five direct tandem repeats

of a 23-bp sequence in the promoter region (the R6 promoter) in red-fleshed apples, was not present in white-fleshed apples (no tandem repeats, the R1 promoter). They showed the repeat number of the 23-bp sequence correlated to a mechanism for upregulation of the anthocyanin pathway leading to red flesh through the autoregulation of the R6 promoter by *MdMYB10*.

Recently, we found that the R6 promoter was not observed at the promoter region of *MdMYB* in red-fleshed apple cultivars Pink Pearl (‘Surprise’ × unknown pollen parent, selected in 1944), JPP35 (‘Jonathan’ × ‘Pink Pearl’), and any of their progenies with the red flesh trait (Sekido et al., 2010a). Although the molecular mechanism of their red coloration is largely unknown, we indicated that the red flesh trait in ‘Pink Pearl’ is tightly linked with its *S*₃-RNase allele (Sekido et al., 2010a). Because the flesh and skin color trait within the Rn1 locus (the site of *MdMYB1* and *10*) located in linkage group 9, not 17 of the *S*-allele location, and which is called the *S*-locus (Chagné et al., 2007; Maliepaard et al., 1998), the red flesh trait in ‘Pink Pearl’ seemed to be controlled by a different type of MYB transcription factor close to the *S*₃-RNase allele. Self-incompatibility in apple is gametophytically controlled by the *S*-locus and not only self pollen-tube growth, but also pollen tube growth from a different cultivar having the same *S*-haplotype can be arrested in the style (de Nettancourt, 1977; Kobel et al., 1939). For instance, ‘Shinano Sweet’ (*S*₁*S*₇) × ‘JPP35’ (*S*₃*S*₇) results in either *S*₁*S*₃ or *S*₃*S*₇ progenies because the *S*₇-allele in ‘JPP35’ is rejected by that in ‘Shinano Sweet’. The *S*-RNase and SFB (*S*-locus F-box) genes, which are functional in pistils and pollen,

respectively, are located within the *S*-locus (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; Kim et al., 2009; Kitahara and Matsumoto, 2002a, 2002b; Matsumoto and Kitahara, 2000; Matsumoto et al., 2009a; Morita et al., 2009). Using the PCR method, we have investigated the *S*-RNase content of more than 500 apple cultivars, lineages, and species in Japan (Kitahara and Matsumoto, 2002a, 2002b; Kitahara et al., 1999, 2000; Matsumoto and Kitahara, 2000; Matsumoto et al., 1999a, 1999b, 2001, 2003a, 2003b, 2003c, 2007, 2009b; unpublished results).

In this article, we show a strategy for efficient production of red-fleshed apple progenies. This will be useful for producing new red-fleshed cultivars because the genetic background of the red flesh trait in ‘Pink Pearl’ is quite different from that of cultivars to be released in the near future.

Materials and Methods

Plant material. *Malus* plants used in this study were from collections at the Nagano Fruit Tree Experiment Station, Japan. Young leaves were collected and stored at –80 °C until use.

Measurement of anthocyanin in apple. Ten to 20 fruits of ‘JPP35’, ‘Maypole’, and ‘Fuji’ were picked monthly from May to October. Half of the fruit on the tree was placed in light-impermeable two-layered double bags (Kobayashi Bag Mfg., Nagano, Japan) after ≈1 (‘Maypole’) or 2 (‘JPP35’ and ‘Fuji’) months of full bloom. The two-layered double bag blocks 99.6% to 99.7% of light corresponding to a wavelength range of 200 nm to 1100 nm. Anthocyanin measurement has been done essentially according to the method of Dong et al. (1995). A 1.0-g core or cortex of apple fruit was freeze-dried for 24 h, total anthocyanins were extracted overnight with 1% HCl-methanol (5 mL), and the absorbance of the extracts were measured at 530 nm and 657 nm. The difference between A₅₃₀ and A₆₅₇ was used to determine the concentration of total anthocyanin and eliminate the contribution of chlorophyll and its degradation products. Measurements were repeated three times, and the average value (mean ± SD) is plotted in Figure 1.

***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).

Sequence analysis of *S*₃- and *S*₁₁-RNase alleles. The ca 1500-bp and 370-bp fragments were amplified from ‘Pink Pearl’

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genomic DNA using the sense ('FTQQYQ') and antisense ('anti-¹/_MIWPNV') primers (Matsumoto and Kitahara, 2000). The reaction to DNA amplification was conducted in a 20- μ L mixture containing 50 ng of genomic DNA, 200 μ M of each deoxynucleotide, 300 nM of each primer, and 0.4 unit of KOD-Plus-DNA polymerase (TOYOBO Inc.). The analysis was programmed in a thermal Cycler (GeneAmp 2720 apparatus; Applied Biosystems) and conducted under the following conditions: 2 min preheating at 94 °C, 10 s at 98 °C, 30 s at 48 °C, and 2 min at 68 °C for 30 cycles. The sequences of the amplified fragments were directly determined by performing dideoxy chain termination on an Applied Biosystems 3130 Genetic Analyzer (Life Technologies Japan Co., Ltd.) using a Big Dye Terminator Version 3.1 Cycle Sequencing Kit (Life Technologies Japan Co., Ltd.).

Results and Discussion

Accurate linkage between red flesh trait and S_3 -RNase in 'Pink Pearl'. Previously, we indicated that 67 of 70 (96%) and 51 of 58 (88%) progenies from 'Shinano Sweet' (S_1S_7) $\times$ 'JPP 35' (S_3S_7) and 'Orin' (S_2S_7) $\times$ 'JPP 35' (S_3S_7), respectively, showed the red flesh color because the S_3 -RNase allele of 'Pink Pearl' was linked to its red flesh trait (Sekido et al., 2010a). At that time, we proposed that some progenies with white flesh color would turn white-pink after full maturity. As shown in Figure 1, red pigmentation in 'JPP35' flesh cortex increased according to fruit maturity in contrast to the cultivar Maypole in which red skin, flesh, and leaf color might be controlled by the *MdMYB10* (Sekido et al., 2010b). The red pigmentation in the cortex of 'Maypole' decreased according to fruit maturity (Fig. 1). Moreover, the red pigmentation of the flesh of 'JPP35' progressed without ultraviolet rays, which are essential for the development of red skin color, suggesting that harvest time, not light quality, is important for development of flesh color. The white-fleshed cultivar Fuji used as a control did not change its color and no red pigmentation was observed regardless of ultraviolet irradiation (Fig. 1). We re-investigated the flesh color and *S*-genotypes of the progenies, including some additional progenies at full maturity. As shown in Table 1, all 75 (100%) and 60 of 64 (94%) progenies having S_3 from 'Shinano Sweet' (S_1S_7) $\times$ 'JPP 35' (S_3S_7) and 'Orin' (S_2S_7) $\times$ 'JPP 35' (S_3S_7), respectively, showed the red flesh color. These higher rates of red-fleshed color than previously seemed to be caused by the development of published pink-red flesh after full maturity.

Cross-combination for efficient production of red-fleshed apple progenies. We have selected 'JPP 35' (S_3S_7) as a mother plant for production of red-fleshed cultivars (Sekido et al., 2010a). We have advanced the breeding of red-fleshed apple and selected Nos. 3, 41, and 42 as suitable for flesh use; Nos. 29, 37, 38, 44, 50, and 69 as suitable for processed goods from 'Shinano Sweet' (S_1S_7) $\times$ 'JPP 35' (S_3S_7);

Fig. 1. Changes in the anthocyanin concentration of apple cultivars JPP35, Maypole, and Fuji core and cortex. Fruits were placed in light-impermeable two-layered 'Fuji' wrapping bags (A) and in no wrapping bags (B).

Table 1. Rate of red-fleshed progenies of 'JPP35' with their *S*-RNase allele genotypes.

Cross	Yr	Flesh color (<i>S</i> -genotype)		Rate of red flesh (%)	
		Red	White	Expected	Observed
Shinano Sweet (S_1S_7)	2009 ^a	67 (30 S_1S_3 37 S_3S_7)	3 (S_3S_7)	100	96
$\times$ JPP 35 (S_3S_7)	2010	75 (35 S_1S_3 40 S_3S_7)	0	100	100
Orin (S_2S_7)	2009 ^a	51 (28 S_2S_3 23 S_3S_7)	8 (5 S_2S_3 3 S_3S_7)	100	86
$\times$ JPP 35 (S_3S_7)	2010	60 (35 S_2S_3 25 S_3S_7)	4 (2 S_2S_3 2 S_3S_7)	100	94
Pink Pearl (S_3S_{11})	2009 ^a	13 (12 S_3S_7 1 S_7S_{11})	17 (17 S_7S_{11})	50	43
$\times$ JPP 35 (S_3S_7)	2010	13 (12 S_3S_7 1 S_7S_{11})	19 (19 S_7S_{11})	50	41

^aData from Sekido et al. (2010a).

^bThe progeny was classified as No. 25.

and confirmed their *S*-RNase genotypes (Table 2). From the *S*-genotypes, new red-fleshed cultivars having variable taste and texture could be obtained efficiently by crossing S_3S_7 genotypes of 'JPP 35', Nos. 3, 42, 37, 50, and 69 with S_3 -absence and S_7 -presence cultivars such as 'Jonathan' (S_7S_9), 'Shinano Sweet' (S_1S_7), 'Orin' (S_2S_7), etc. Also, in the case of S_1S_3 genotypes of Nos. 41, 29, 38, and 44, S_3 -absence and S_7 -presence cultivars such as 'Fuji' (S_1S_9), 'Ralls Janet' (S_1S_2), and 'Senshu' (S_1S_7) could be used as a cross hybridization partner for efficient production of new red-fleshed apples. In this case, new red-fleshed cultivars incorporating characteristics of the major cultivar Fuji (S_1S_9) will be obtained efficiently.

Table 2. *S*-RNase allele genotypes of 'Shinano Sweet' $\times$ 'JPP35' progenies.

Lineage	Fresh color	Suitable for	<i>S</i> -genotype
No. 3	Pink-red	Fresh use	S_3S_7
No. 41	Red	Fresh use	S_1S_3
No. 42	Pink-red	Fresh use	S_3S_7
No. 29	Red	Processing	S_1S_3
No. 37	Pink-red	Processing	S_3S_7
No. 38	Red	Processing	S_1S_3
No. 44	Red	Processing	S_1S_3
No. 50	Red	Processing	S_3S_7
No. 69	Red	Processing	S_3S_7

Molecular structure of S_3 - and S_{11} -RNase allele in 'Pink Pearl'. Only one progeny, No. 25 (S_7S_x ; S_x is unknown *S*-RNase allele) from 'Pink Pearl' (S_3S_x) $\times$ 'JPP 35' (S_3S_7), showed

the red-fleshed trait despite its absence of S_3 (Table 1). In this case, the gene responsible for the red-fleshed trait seems to be present near the S_7 - or S_X -RNase allele by genetic recombination. To clarify the S_X -RNase allele structure, we investigated the S -RNase allele in 'Pink Pearl' using primers 'FTQQYQ' and 'anti-1/4 IWPNV'. It was clarified that 'Pink Pearl' had S_{11} -RNase allele in addition to the known S_3 -RNase allele (Fig. 2). The partial coding sequences with deduced intron sequences of S_{11} -RNase allele in 'Pink Pearl' were completely identical with those in 'Gravenstein' and 'Virginia crab' (Long et al., 2010; Matsumoto et al., 2003c). In contrast, we found a nucleotide substitution between the intron sequences of S_3 -RNase allele in 'Pink Pearl' and 'Mutsu' at position 343 (Fig. 2). We thought the polymorphism might be useful to discriminate the 'Pink Pearl' S_3 -RNase allele

with commercial cultivars in Japan having the S_3 -RNase allele. However, the polymorphism seems ambiguous because we could not identify the polymorphism in our 'Mutsu' ($S_2S_3S_{20}$), 'Golden Delicious' (S_2S_3), and 'Shinano Gold' (S_1S_3) samples. We have obtained a 124-bp fragment corresponding to the 'Pink Pearl' S_3 from the cultivars using the primers S3PF (5'-TCCTAACTAATTTT CAATTC-3'; position 324-343 in Fig. 2) and S3PR (5'-GGATTATATGAATGGCTACAT TTAAGA-3'; position 421-447 in Fig. 2) (results not shown). Although we could not discriminate the S_3 of 'Pink Pearl' from those of commercial cultivars using the identified single nucleotide polymorphism, we used the primers to confirm the absence of S_3 in a newly arrived red-fleshed material from 'Hatsushiga' (S_2S_9) $\times$ 4-23 (S_1S_2) $\times$ 'Megumi' (S_2S_9). Contrary to expectations, S_3 seemed to be present

in the material because we were able to detect a 124-bp fragment (results not shown). Because 'JPP35' alone was a red-fleshed cultivar having S_3 within pollen parents in use, the material seemed to be produced by 'JPP35' pollen accidentally used somewhere in the breeding process.

Using the No. 25 (S_7S_{11}), it is possible to produce new red-flesh cultivars that are homozygous for the red flesh allele derived from 'Pink Pearl', which we considered useful for producing stable dark red-fleshed cultivars. Thus, either S_3S_7 or S_3S_{11} from 'Pink Pearl' (S_3S_{11}) $\times$ No. 25 (S_7S_{11}) or 'JPP 35' (S_3S_7) $\times$ No. 25 (S_7S_{11}), respectively, must be homozygous with the red flesh trait. In addition, if the red flesh trait in No. 25 is linked to the S_{11} , we can use a S_3 cultivar such as the major cultivar Tsugaru (S_3S_7) as a partner for the crossing of No. 25 (S_7S_{11}). S -genotypes of F_1 from 'Tsugaru' (S_3S_7) $\times$ No. 25 (S_7S_{11}) will be either S_3S_{11} or S_7S_{11} and must show the red flesh trait because all the F_1 have S_{11} .

In conclusion, breeding for new red-fleshed apple cultivars using the linkage between the red flesh trait and the S -allele is a genuine breakthrough not only for its contribution to efficient red-fleshed apple breeding, but also for its expansion of the breeding range. For instance, yellow skin and the red-fleshed cultivar could be obtained from the progenies of 'Orin' (yellow skin and white flesh) and 'JPP35' (red skin and flesh), a combination not possible from the progenies with the $MdMYB10$ allele because the fruit skin and leaves are also controlled by the $MdMYB10$.

(A)		
S_{11}	TTTACGCAGCAATATCACTGGCTGTCTGCAACTCTAATCGTGCTCTTGTAAAGGATCC	60
S_{11}	F T Q Q Y Q L A V C N S N R A P C K D P	20
C1		
S_{11}	TCCTGACAAGTTGTTTACGGTTCACGGTTTGTGGCCTTCAAGCATGGTAGACCTGACCC	120
S_{11}	P D K L F T V H G L W P S S M V G P D P	40
C2		
S_{11}	AAGTAATGCTCGATAAGGAATATTCGGAAGGTAATATTATTAGTAATCAGATCGTCAAT	180
S_{11}	S N C S I R N I R K	50
S_{11}	ATTGTTAATTTTCATTATGTACTTGTATGTGTGTTGTATATTCAATATATACATACTCA	240
S_{11}	AACATAGATTTTCATGCACGTGTGTACAAATATACAAATTAGTTTAAATATAATCATAA	300
S_{11}	TATTTTTTTTCTATTGTGCATACAGAGAGAAAATCTCGAAGCCAGCTGCAATTA	360
S_{11}	R E K L L E P Q L A I	
S_{11}	TTTGCCGAACGTA 374	
S_{11}	I W P N V	
(B)		
S_{3p}	AGGATCCTCCTGACAAGTTGTTTACGGTTCATGGTTTGTGGCCTTCAAATGTTAATGGAA	60
S_{3p}	D P P D K L F T V H G L W P S N V N G	19
C2		
S_{3p}	GTGACCCCAAGAAATGCAAACTACAATCTTGAACCCCTCAAACGGTAATATTATTCAGGC	120
S_{3p}	S D P K K C K T T I L N P Q T	34
S_{3p}	AAAATTAATATCCAGTACTTAGTTATAATGTTCAATTAAGACCTTATTAGTTTTCAG	180
S_{3p}	ATTTTGATCGATGTCCCTAGCAAAATTCAAATGTCAATTACATAATATTTTAAATAAAAA	240
S_{3p}	TTAGTAATTGAACTTACACCCCTTATTAATGAATAAGTTGTACCCATTAATTTTCAATT	300
S_{3p}	CAAACTTACATCCTTATTAACCTCTTCTTCAAAATTTTCAATTTCAATTTCCAAAAAC	360
S_{3m}	TAATAATTAGAATAAGTTGTACCCATTAATTTTATAAAATGATTTTCAATTTTAA	420
S_{3p}	TCCTAAATGTAGCCATTCATATAATCCTACATTTACCCATTCTTAAATATACCATTTTGT	480
S3PR		
S_{3p}	TATGTAAATGTACCCCTTTTAAATATATAAATGTAACCCCTGTTTATATAAAATACATTC	540
S_{3p}	AAATTTTATTTTAAACCATATATTTTAAATATCCATTTTAAATATAAAACGTAACCA	600
S_{3p}	TTTTTTATATTAATCAATTCAAATTTTTCGAATCCATTTTAACTTATATGGG	660
S_{3p}	TACATCTTATTTGTTGATTTGAGAGCTATCGATACCAAGTTTAATGGAAGAAATTTAA	720
S_{3p}	TAATGTAAGCCTAATACCTCTTTTCTTCTTCAAAATCTACCTATGTTTGTATACAATA	780
S_{3p}	ATTTTGTGTAGTTTAAATGAACGTACCCATGTTTCTTATATATATATATATATATATG	840
S_{3p}	CATGCATGTATGTATGTATATATATATAACATATTGGAGGGCATAAAAAGGTCGTACC	900
S_{3p}	CAGTGACACAAGGCTCCGCTTTACGCGAGGCTCGGAGAAAGTGAATGTCGGCTAGCCTTA	960
S_{3p}	CCCCATTATGAGAGGCTGCTCCCAAGTCTCGAACCCGAGACCTACCGCTCATGGGCG	1020
S_{3p}	AAGGCACTTGCCATAATTTTAAATTTTAAATCTTAAATGGAATTTTATTTAAAAA	1080
S_{3p}	TCCTATGATATAAACAACCATATAAATTTTCAATTTTAAATATAAATAATCTACACA	1140
S_{3p}	TAAAAATACATTTATATTAATATGTCAGAACTTTGATCAAAACTGAAATGAGTAAGA	1200
S_{3p}	TTTCAATTAAAGAACATTGATTATTAGTACTGTGCCAAATCTCCCTATTATTCATAAT	1260
S_{3p}	TAGATGGTCAAAATGTTTATCTTTTCAATATATACATGCTCGACATAGATTTTTCATGCA	1320
S_{3p}	CTCGTGAAGATATTACTATTAAATTTAAATTTAATCATAATTTTCTATTATAA	1380
S_{3p}	TATTGTAT-GTCAGATAACA 1399	
S_{3m}	T 1400	
S_{3p}	I T 36	

Fig. 2. Partial nucleotide and deduced amino acid sequences of S_{11} - (A) and S_3 -RNase alleles in 'Pink Pearl' (B). The conserved regions C1 and C2 are boxed. The deduced intron sequences are italicized. Sequences of S_{11} and S_3 from 'Pink Pearl' were deposited under the DDBJ accession numbers AB618093 and AB618094, respectively. The nucleotide sequence of S_{11} -RNase allele in 'Pink Pearl' was completely identical with that in 'Gravenstein' (AB105060) and 'Virginia crab' (FJ008669). In the case of the S_3 -RNase allele, a nucleotide substitution at position 343 from C (S_{3p} ; S_3 in 'Pink Pearl') to T (S_{3m} ; S_3 in 'Mutsu' from AB428425) and an insertion of T at position 1389 of S_{3m} were identified. Sequences used as primers to identify the S_3 (S3PF and S3PR) are underlined.

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