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

Hitomi Umemura, Katsuhiro Shiratake, and Shogo Matsumoto
Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

Abstract. We re-investigated the flesh color and S-locus 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’ (S3-S7) × JPP35 (S3-S7) and ‘Orin’ (S3-S7) × JPP35 (S3-S7) containing S3-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 S3-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 $S_{12}$ and determined its partial genomic sequence, including a complete intron with its known S3-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 antioxidant 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 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 antioxidant 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; Takas 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 the red coloration is largely unknown, we indicated that the red flesh trait in ‘Pink Pearl’ is tightly linked with its $S_{12}$-RNase allele (Sekido et al., 2008). Because the flesh and skin color trait within the Rau locus (the site of MdMYB 1 and 10) located in linkage group 9, not 17 of the $S$-locus (Chagné et al., 2007; Maliepaard and de Nettancourt, 1977; Kobel et al., 1939). For instance, ‘Shinano Sweet’ (S3-S7) × JPP35 (S3-S7) results in either S3-S7 or S7-S7 progenies because the S3 allele in ‘Pink Pearl’ is rejected by that in ‘Shinano Sweet’. The S-RNase alleles of SFB (S-fus Red-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 genotyping 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 killing the flowers with hydrogen cyanide. The other half was used for reactions-digestion analysis. 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 extract was 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 ± so) is plotted in Figure 1.
genomic DNA using the sense (‘FTQYQY’) and antisense (‘anti-ι2mIWPVNY’) primers (Matsumoto and Kitahara, 2000). The reaction to DNA amplification was conducted in a 20-μL mixture containing 50 ng of genomic DNA, 200 μM 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\textsubscript{7}-RNase in ‘Pink Pearl’. Previously, we indicated that 67 of 70 (96%) and 51 of 58 (88%) progenies from ‘Shinano Sweet’ (S\textsubscript{1}S\textsubscript{7}) × ‘JPP 35’ (S\textsubscript{3}S\textsubscript{7}) and ‘Orin’ (S\textsubscript{1}S\textsubscript{7}) × ‘JPP 35’ (S\textsubscript{3}S\textsubscript{7}), respectively, showed the red flesh color because the S\textsubscript{7}-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 from ‘Shinano Sweet’ (S\textsubscript{1}S\textsubscript{7}) × ‘JPP 35’ (S\textsubscript{3}S\textsubscript{7}) and ‘Orin’ (S\textsubscript{1}S\textsubscript{7}) × ‘JPP 35’ (S\textsubscript{3}S\textsubscript{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\textsubscript{3}S\textsubscript{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, 48, and 49 as suitable for processed goods from ‘Shinano Sweet’ (S\textsubscript{1}S\textsubscript{7}) × ‘JPP 35’ (S\textsubscript{3}S\textsubscript{7}); 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\textsubscript{3}S\textsubscript{7} genotypes of ‘JPP 35’, Nos. 3, 42, 37, 50, and 69 with S\textsubscript{7}-absence and S\textsubscript{3}-presence cultivars such as ‘Jonathan’ (S\textsubscript{1}S\textsubscript{3}), ‘Shinano Sweet’ (S\textsubscript{1}S\textsubscript{3}), ‘Orin’ (S\textsubscript{1}S\textsubscript{3}), etc. Also, in the case of S\textsubscript{3}S\textsubscript{7} genotypes of Nos. 41, 29, 38, and 44, S\textsubscript{7}-absence and S\textsubscript{3}-presence cultivars such as ‘Fuji’ (S\textsubscript{1}S\textsubscript{7}), ‘Ralls Janet’ (S\textsubscript{1}S\textsubscript{7}), and ‘Senshu’ (S\textsubscript{1}S\textsubscript{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\textsubscript{1}S\textsubscript{7}) will be obtained efficiently.

*Figure 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                | Flesh color (S-genotype) | Rate of red flesh (%) |
|----------------------|--------------------------|-----------------------|
|                      | Red          | White    | Expected | Observed |
| Shimano Sweet (S\textsubscript{1}S\textsubscript{7}) × JPP 35 (S\textsubscript{3}S\textsubscript{7}) | 2009\textsuperscript{a} | 67 (30 S\textsubscript{1}S\textsubscript{3}, 37 S\textsubscript{3}S\textsubscript{7}) | 3 (S\textsubscript{3}S\textsubscript{7}) | 100 | 96 |
|                      | 2010         | 75 (35 S\textsubscript{1}S\textsubscript{3}, 40 S\textsubscript{3}S\textsubscript{7}) | 0 (S\textsubscript{3}S\textsubscript{7}) | 100 | 100 |
| Orin (S\textsubscript{1}S\textsubscript{7}) × JPP 35 (S\textsubscript{3}S\textsubscript{7}) | 2009\textsuperscript{a} | 51 (28 S\textsubscript{1}S\textsubscript{3}, 23 S\textsubscript{3}S\textsubscript{7}) | 8 (5 S\textsubscript{1}S\textsubscript{3}, 3 S\textsubscript{3}S\textsubscript{7}) | 100 | 86 |
|                      | 2010         | 60 (35 S\textsubscript{1}S\textsubscript{3}, 25 S\textsubscript{3}S\textsubscript{7}) | 4 (2 S\textsubscript{1}S\textsubscript{3}, 2 S\textsubscript{3}S\textsubscript{7}) | 100 | 94 |
| Pink Pearl (S\textsubscript{1}S\textsubscript{7}) × JPP 35 (S\textsubscript{3}S\textsubscript{7}) | 2009\textsuperscript{a} | 13 (12 S\textsubscript{1}S\textsubscript{3}, 1 S\textsubscript{3}S\textsubscript{7}) | 17 (17 S\textsubscript{3}S\textsubscript{7}) | 50 | 43 |
|                      | 2010         | 13 (12 S\textsubscript{1}S\textsubscript{3}, 1 S\textsubscript{3}S\textsubscript{7}) | 19 (19 S\textsubscript{3}S\textsubscript{7}) | 50 | 41 |

\textsuperscript{a}Data from Sekido et al. (2010a).

\textsuperscript{b}The progeny was classified as No. 25.
the red-fleshed trait despite of $S_3$ (Table 1). In this case, the gene responsible for the red-fleshed trait seems to be present near the $S$- or $S_R$-RNase allele by genetic recombination. To clarify the $S_R$-RNase allele structure, we investigated the X-RNase allele in ‘Pink Pearl’ using primers ‘FTQQYQ’ and ‘anti-12RIVWPY’. It was clarified that ‘Pearl’ had $S_{P2}$-RNase allele in addition to the known $S_{Y_R}$-RNase allele (Fig. 2). The partial coding sequences with deduced intron sequences of $S_{P2}$-RNase allele in ‘Pink Pearl’ were completely identical with those in ‘Grav-enstein’ and ‘Virginia crab’ (Long et al., 2010; Matsumoto et al., 2003c). In contrast, we found a nucleotide substitution between the intron sequences of $S_{Y_R}$-RNase allele in ‘Pink Pearl’ and ‘Mutsu’ from AB428425) and an insertion of T at position 1389 of $S_Y$-RNA (Fig. 2). Although we could not discriminate the $S_Y$ of ‘Pink Pearl’ from those of commercial cultivars using the identified single nucleotide polymorphism, we used the primers to confirm the absence of $S_{Y_R}$ in a newly arrived red-fleshed material from ‘Hatsushiga’ ($S_{S3}\pi$) × ‘Megumi’ ($S_{S3\pi}$) (results not shown). Although we could not identify the polymorphism in our ‘Mutsu’ ($S_{S3}\pi$), ‘Golden Delicious’ ($S_{S3\pi}$), and ‘Shinshiro Gold’ ($S_{S3\pi}$) samples, we obtained a 124-bp fragment corresponding to the ‘Pink Pearl’ $S_Y$ from the cultivars using the primers S3PF (5’TCTCTAAGATTTTCAACTC3’; position 324-343 in Fig. 2) and S3PR (5’GGATTATAGAATGGCTACATTTAAGA3’; position 421-447 in Fig. 2) (results not shown). Thus, either $S_{S3\pi}$ or $S_{Y_R}$ from ‘Pink Pearl’ ($S_{S3\pi}$), respectively, must be homozygous with the red flesh trait. In addition, if the red flesh trait in No. 25 is linked to the $S_Y$, we can use a $S_Y$ cultivar such as the major cultivar Tsuchu in ‘Pink Pearl’ ($S_{S3\pi}$) as a partner for the crossing of No. 25 ($S_{S3\pi}$), $S$-genotypes of F1 from ‘Tsuchu’ ($S_{S3\pi}$) × No. 25 ($S_{S3\pi}$) will be either $S_{S3\pi}$ or $S_{Y_R}$ and must show the red flesh trait because all the F1 have $S_{S3\pi}$.

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 the 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.

### Literature Cited

Allan, A.C., R.P. Hellens, and W.A. Lang. 2007. MYB transcription factors that colour our fruit. Trends Plant Sci. 13:99–102.

Ban, Y., C. Honda, Y. Hatsuyama, M. Igarashi, H. Besho, and T. Moriguchi. 2007. Isolation and functional analysis of a MYB transcription factor gene that is a key regulator for the development of red coloration in apple skin. Plant Cell Physiol. 48:958–970.

Broothaerts, W. 2003. New findings in apple S-locus analysis resolve previous confusion and request the re-numbering of some S-alleles. Theor. Appl. Genet. 106:703–714.

Broothaerts, W., G.A. Janssens, P. Proost, and W.F. Broekaert. 2005. cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. Plant Mol. Biol. 57:499–511.

Chagné, D., C.M. Carlisle, C. Blond, R.K. Volz, C.J. Whitworth, N.C. Oraguzie, R.N. Crowhurst, A.C. Allan, R.V. Espley, R.P. Hellens, and S.E. Gardiner. 2007. Mapping a candidate gene for harvest stimulation of skin color in Royal Gala apple. J. Amer. Soc. Hort. Sci. 132:175–183.

de Nettancourt, D. 1977. Incompatibility in angiosperms, p. 28–57. In: Frankel, R. G.A.E. Gal, and H.F. Linkens (eds.). Monographs on theoretical and applied genetics. Springer-Verlag, Heidelberg, Germany.

Dong, Y.-a., D. Mitra, and A. Kootstra. 1995. Post-harvest stimulation of skin color in Royal Gala apple. J. Am. Soc. Hort. Sci. 120:95–100.
Kitahara, K. and S. Matsumoto. 2002a. Cloning of Kitahara, K., J. Soejima, H. Komatsu, H. Fukui, Kitahara, K. and S. Matsumoto. 2002b. Sequence Kobel, F., P. Steinegger, and J. Anliker. 1939. HORTSCIENCE VOL. 46(8) AUGUST 2011 1101 Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2009. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183. Espley, R.V., R.P. Hellens, J. Putterill, D.E. Stevenson, S. Kutty-Amma, and C.A. Allan. 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor auto-regulation in red apples. Plant Cell 21:168–183.