Partial Genomic Sequences of S_6^- , S_{12}^- , S_{13}^- , S_{14}^- , S_{17}^- , S_{19}^- , and S_{21}^- RNases of Apple and Their Allele Designations

Shogo MATSUMOTO^{1*}, Yui FURUSAWA¹, Kentaro KITAHARA¹ and Junichi SOEJIMA²

¹Department of Biology, Faculty of Education, Gifu University, Gifu 501-1193, Japan

²Apple Research Center, National Institute of Fruit Tree Science, Morioka 020-0123, Japan

*Corresponding author E-mail address: shmatsum@cc.gifu-u.ac.jp

Received 2 April 2003; accepted 4 August 2003

Abstract

We determined partial genomic sequences including a hypervariable HVa (RHV) region and introns of the S_6^- , S_{12}^- , S_{13}^- , S_{14}^- , S_{17}^- , S_{19}^- , and S_{21}^- RNases of apple. Previously, it was suggested by Broothaerts (2003) that the S_6 corresponded to S_{25} in ‘McIntosh’/‘Wijcik’ and ‘Tydemans Early Worcester’, and S_{19} corresponded to S_{28} . However, S_6 and S_{19} are different from the S_{25} and S_{28} , respectively. The determined sequences of the S_6 and S_{12} , and S_{17} and S_{19} are identical, and these four alleles seem to act as the same allele. We newly assigned S_{6a} in place of S_6 and S_{12} , and S_{6b} in place of S_{17} and S_{19} from the results of sequence analyses, not pollination tests. The sequences of S_{13} and S_{14} were also identical. We also newly assigned S_{11} in place of S_{13} and S_{14} . We had speculated that S_{21} would correspond to S_t by PCR-digestion analysis, however, the determined sequence of S_{21} was different from S_t .

Key words: apple, *Malus x domestica*, S^- allele, S^- RNase, self-incompatibility.

Abbreviations

RHV, Rosaceae-specific hypervariable region; PCR, polymerase chain reaction.

Apple (*Malus x domestica* Borkh.) exhibits gametophytic self-incompatibility controlled by the multi-allelic S^- locus (Kobel *et al.*, 1939; de Nettancourt, 1977). Self-incompatibility is a mechanism for preventing self-fertilization in flowering plants, and in gametophytic self-incompatibility, pollen-tube growth is inhibited when an S^- -haplotype of pollen matches one of the S^- -haplotypes of the pistil. Using cross pollination followed by analysis of pollen tube growth in the style or analysis of the fruit and seed, 11 (S_l to S_{11}) and 10 (S_a to S_i , S_e) S^- -haplotypes have been identified (Kobel *et al.*, 1939; Komori *et al.*, 1999, 2000a). A pistil-specific glycoprotein with ribonuclease activity (S^- RNase) encoded by the S^- locus has been identified using molecular method (Broothaerts *et al.*, 1995), and shown to be related to the self-incompatibility response (Sassa *et al.*, 1992, 1993, 1997). As the S^- RNase does not seem to act as a pollen factor other than a pistil factor, at least two kinds of polymorphic genes might be related to gametophytic self-incompatibility. Up to date, genes encoding S_c^- , S_d^- , S_e^- , S_f^- , S_g^- , $S_{g'}^-$, S_h^-

, S_{10} (equivalent to i)⁻, S_t^- , and S_{25} (equivalent to z)⁻ RNases have been cloned in Japan (Sassa *et al.*, 1996; Matsumoto *et al.*, 1999; Kitahara *et al.*, 2000; Matsumoto *et al.*, 2000; Matsumoto and Kitahara, 2000; Matsumoto *et al.*, 2001a; Kitahara and Matsumoto, 2002a, b), and genes encoding S_2^- , S_3^- , S_4^- , S_5^- , S_7^- , S_9^- , S_{24}^- , S_{26}^- , S_{27a}^- , S_{27b}^- , and $S_{30(28)}^-$ RNases in Europe (Broothaerts *et al.*, 1995; Janssens *et al.*, 1995; Verdoodt *et al.*, 1998; Schneider *et al.*, 2001; Van Nerum *et al.*, 2001). Recently, the nucleotide sequence of S_{29} -RNase was released on a database under the accession number AY039702. As described in previous reports (Kitahara and Matsumoto, 2002a, b), it was confirmed that the S_a^- , S_b^- , S_c^- , S_d^- , S_e^- , S_f^- , S_g^- , S_h^- , S_i^- , and S_e^- alleles established in Japan corresponded, respectively, to the S_2^- , S_3^- , S_9^- , S_7^- , $S_{30(=28)}^-$, S_1^- , S_{20}^- , S_{24}^- , S_{10}^- , and S_{25}^- alleles established in Europe. As the S^- alleles taken up in this study are not included in these alleles, we investigated partial genomic sequences of unknown S^- RNases as described below.

The alphabetical S^- allele (variants of a polymorphic S^- gene within the S^- locus) designation in Japan is confusing. To resolve this confusion, we proposed to use the European numerical designations (Kitahara and Matsumoto, 2002a, b). How-

MDS6a(6)	CCGGCCGATGCAACTCTAATCCTACTCCTTGTAAACGATCCTGCTGACAAGTTGTTTACG	60
MDS6a(12)	CCGGCNGTATGCAACTNTAATCCTACTCCTTGTAAACGATCCTGCTGACAAGTTGTTTACG	
MDS6b(17)	CCGNCNGTATGCAACTCTAATCCTACTCCTTGTAAACGATCCTGCTGACAAGTTGTTTACG	
MDS6b(19)	CCGGCCGATGCAACTCTAATCCTACTCCTTGTAAACGATCCTGCTGACAAGTTGTTTACG	
MDS28(30)	CCGGCCGATGCAACTCTAATCCTACTCCTTGTAAACGATCCTGCTGAAAAATTTTACG	
PSS4	CCGGCCGATGCAACTCTAATCCTACTCCTTGTAAACGATCCTGCTGACAAGTTGTTTACG	
MDS6a(6)	P A V C N S N P T P C N D P A D K L F T	20
MDS6a(12)	P A V C N X N P T P C N D P A D K L F T	
MDS6b(17)	P X V C N S N P T P C N D P P D K L F T	
MDS6b(19)	P A V C N S N P T P C N D P P D K L F T	
MDS28(30)	P A V C N S N P T P C N D P P E K L F T	
PSS4	P A V C N S N P T P C N D P T D K L F T	
PSS1	P A V C N S N P T P C N D P T D K L F T	
MDS25	P A V C N S N R T P C K D P P D K L F T	
C2		
MDS6a(6)	GTTTCACGGTTTGTGGCCTTCA AACAAGAATGGACCTGACCCAGAAAAATGCAAGACTACA	120
MDS6a(12)	GTTTCACGGTTTGTGGCCTTCA AACAAGAATGGACCTGACCCAGAAAAATGCAAGACTACA	
MDS6b(17)	GTTTCACGGTTTGTGGCCTTCA AACAAGAATGGACCTGACCCAGAAAAATGCAAGACTACA	
MDS6b(19)	GTTTCACGGTTTGTGGCCTTCA AACAAGAATGGACCTGACCCAGAAAAATGCAAGACTACA	
MDS28(30)	GTTTCACGGTTTGTGGCCTTCA AACAAGAATGGACCTGACCCAGAAAAATGCAAGAAATATA	
PSS4	GTTTCACGGTTTGTGGCCTTCA AACAAGAATGGACCTGACCCAGAAAAATGCAAGACTACA	
MDS6a(6)	V H G L W P S N K N G P D P E K C K T T	40
MDS6a(12)	V H G L W P S N K N G P D P E K C K T T	
MDS6b(17)	V H G L W P S N K N G P D P E K C K T T	
MDS6b(19)	V H G L W P S N K N G P D P E K C K T T	
MDS28(30)	V H G L W P S N K N G P D P E K C K N I	
PSS4	V H G L W P S N R N G P D P E K C K T T	
PSS1	V H G L W P S N R N G P D P E K C K T T	
MDS25	V H G L W P S N R N G P D P E Y C K N T	
HVa (RHV)		
MDS6a(6)	GCCCTGAATTCTCAGAAG GTAATATTATTAATAATGAGATAGTCAATATTGTTTATTTC	180
MDS6a(12)	GCCCTGAATTCTCAGAAG GTAATATTATTAATAATGAGATAGTCAATATTGTTTATTTC	
MDS6b(17)	GCCCTGAATTCTCAGAAG GTAATATTATTAATAATGAGATAGTCAATATTGTTTATTTC	
MDS6b(19)	GCCCTGAATTCTCAGAAG GTAATATTATTAATAATGAGATAGTCAATATTGTTTATTTC	
MDS28(30)	CAAAATGAATTCTCAGAAGGTAATATTATTAATAATGAGATAGTCAATATTGTTTATTTC	
PSS4	ACCATGAATTCTCAGAAGGTAATATTATTAATAATGAGATAGTCAATATTGTTTATTTC	
MDS6a(6)	A L N S Q K	46
MDS6a(12)	A L N S Q K	
MDS6b(17)	A L N S Q K	
MDS6b(19)	A L N S Q K	
MDS28(30)	Q M N S Q K	
PSS4	T M N S Q K	
PSS1	A L N S Q K	
MDS25	T L D V T K	
MDS6a(6)	TTTATGCACCTGTGTATA-TAGACATTACA-ATACTCAACATAGATTTTCATGCATGCCCT	240
MDS6a(12)	TTTATGCACCTGTGTATA-TAGACATTACA-ATACTCAACATAGATTTTCATGCATGCCCT	
MDS6b(17)	TTTATGCACCTGTGTATA-TATACATTACA-ATACTCAACATAGATTTTCATGCACGCCGT	
MDS6b(19)	TTTATGCACCTGTGTATA-TATACATTACA-ATACTCAACATAGATTTTCATGCACGCCGT	
MDS28(30)	TTTATGTACTGTGTATA AAATATATATATAT ACTCAACATAGATTTTCATGCACGCCCT	
PSS4	TTTATGCACCGTGTGTATA-TATACATTACA TATGCTCAGCATAGATTTTCATGCACGCCCT	
MDS6a(6)	GTGCAAAATATTACAATTAAATTTAAAAATTTAAACCATGAATTGTTTCTATTACATAAATTATA	300
MDS6a(12)	GTGCAAAATATTACAATTAAATTTAAAAATTTAAACCATGAATTGTTTCTATTACATAAATTATA	
MDS6b(17)	GTGCAAAATATTACAATTAAATTTAAAAATTTAAATCATGAATTGTTTCTATTATATAAATTATA	
MDS6b(19)	GTGCAAAATATTACAATTAAATTTAAAAATTTAAATCATGAATTGTTTCTATTATATAAATTATA	
MDS28(30)	GTGGAATATTACAATTAAATTTAAAAATTTAAATCATGAATTGTTTCTATTATATAAATTATA	
PSS4	GTGCAAAATATTACAATTAAATTTAAAAATTTAAATCATGAATTGTTTCTATTATATAAATTATA	
HVa (RHV)		
MDS6a(6)	TTGTCAGATAGGAAATATGACAGCCCAATTG	331
MDS6a(12)	TTGTCAGATAGGAAATATGACAGCCCAATTGGA	333
MDS6b(17)	TTGTCAGATAGGAAATATGACAGCCCAATTGGA	
MDS6b(19)	TTGTCAGATAGGAAATATGACAGCCCAATTGGA	
MDS28(30)	TTGTCAGATAGGAAATATGACAGCCCA AGTTGGA	
PSS4	TTGTCAGATAGGAAATATGACAGCCCA AGTTGGA	
MDS6a(6)	I G N M T A Q L	54
MDS6a(12)	I G N M T A Q L	
MDS6b(17)	I G N M T A Q L	
MDS6b(19)	I G N M T A Q L	
MDS28(30)	I G N M A A Q L	
PSS4	I G N M T A Q L	
PSS1	I G N M T A Q L	
MDS25	I G N L Q A Q L	

Fig. 1 Alignment of the partial nucleotide and deduced partial amino acid sequences of $S_{6a(=6)}^-$, $S_{6a(=12)}^-$, $S_{6b(=17)}^-$, $S_{6b(=19)}^-$, $S_{28(30)}^-$, and S_{25}^- (only amino acid sequence) RNase in apple and S_1^- (only amino acid sequence) and S_4^- RNase in Asian pear.

The conserved region C2 and hypervariable region HVa (RHV) are boxed. The sequences of $S_{6a(=6)}$ (*MDS6a(6)*), $S_{6a(=12)}$ (*MDS6a(12)*), $S_{6b(=17)}$ (*MDS6b(17)*) and $S_{6b(=19)}$ (*MDS6a(19)*) were deposited under the DDBJ accession numbers AB094495, AB105061, AB105062, and AB094493, respectively. Apple $S_{28(30)}$ (*MDS28(30)*) and apple S_{25} (*MDS25*), Asian pear S_1 (*PSS1*) and Asian pear S_4 (*PSS4*) were from studies by Matsumoto and Kitahara (2000) together with Matsumoto *et al.* (2001b), Kitahara and Matsumoto (2002b), Ishimizu *et al.* (1999) and Norioka *et al.* (1995), respectively. The different sites are shown in bold. Intron sequences are italicized.

ever, the European numerical designation is also confusing, mostly due to the 11 *S*-alleles (S_{12} to S_{19} , S_{21} to S_{23}) that identified only in one cultivar by stylar protein analyses (Bošković and Tobutt, 1999). For instance, S_{22} and S_{23} which identified only in 'Alkmene' and 'Delbard Jubile', respectively (Bošković and Tobutt, 1999), were classified as S_{27b} by *S*-allele specific PCR and sequence analysis, not stylar protein analysis (Van Nerum *et al.*, 2001). Afterward, the S_{22}^- , S_{23}^- , and S_{27b}^- alleles were re-

numbered as S_{22} (Broothaerts, 2003). Van Nerum *et al.* (2001) pointed out that the electrophoretic differences between several *S*-RNases identified by Bošković and Tobutt (1999) appeared to be small, and it is conceivable that some *S*-alleles do not exist. Mainly to verify this, we amplified the genomic DNA including a hypervariable HVa (RHV) and introns corresponding to S_6^- , S_{12}^- to S_{14}^- , S_{17}^- , S_{19}^- , and S_{21}^- alleles using primers derived from the amino acid sequences 'FTQQYQ' and 'anti-¹/_M

MDS11(13)	CTGGCTGTCTGCAACTCTAATCGTCTCCTTGTAAAGGATCCTCTGACAAGTTCTTTACG	60
MDS11(14)	CTGGCTGTCTGCAACTCTAATCGTCTCCTTGTAAAGGATCCTCTGACAAGTTCTTTACG	
MDS21	CTGGCTGTCTGCAACTCTAATCGTCTCCTTGTAAAGGATCCTCTGACAAGTTCTTTACG	
MDS30(t)	CTGGCTGTCTGCAACTCTAATCGTCTCCTTGTAAAGGATCCTCTGACAAGTTCTTTACG	
MDS11(13)	L A V C N S N R A P C K D P P D K L F T	20
MDS11(14)	L A V C N S N R A P C K D P P D K L F T	
MDS21	L A V C N S T R T P C K D P P D K L F T	
MDS30(t)	L A V C N S N R T L C K D P P D K L F T	
PSS3	L A V C N S N R T L C K D P P D K L F T	
PSS5	L A V C N S N R T P C K D P P D K L F T	
MDS20a	P A V C H S N P T P C K D P P D K L F T	
C2		
MDS11(13)	GTTCACGGTTTGTGGCCTTCAAGCATGGTAGGACCTGACCCAAGTAATTGCTCGATAAAGG	120
MDS11(14)	GTTCACGGTTTGTGGCCTTCAAGCATGGTAGGACCTGACCCAAGTAATTGCTCGATAAAGG	
MDS21	GTTCACGGTTTGTGGCCTTCAAGCATGGTAGGACCTGACCCAAGTAATTGCTCGATAAAGG	
MDS30(t)	GTTCACGGTTTGTGGCCTTCAAGCATGGTAGGACCTGACCCAAGTAATTGCTCGATAAAGG	
MDS11(13)	V H G L W P S S M V G P D P S N C S I R	40
MDS11(14)	V H G L W P S S M V G P D P S N C S I R	
MDS21	V H G L W P S S M V G P D P S N C P I R	
MDS30(t)	V H G L W P S S M V G P D P S K C P I K	
PSS3	V H G L W P S N M V G P D P S K C P I K	
PSS5	V H G L W P S S M A G P D P S N C P I R	
MDS20a	V H G L W P S D S N G N D P K Y C K A P	
HVa (RHV)		
MDS11(13)	AATATTCGGAAGGTAATATTATTAGTAATCAGATCGTCAATATTGTTAATTTTCATTATG	180
MDS11(14)	AATATTCGGAAGGTAATATTATTAGTAATCAGATCGTCAATATTGTTAATTTTCATTATG	
MDS21	AACATTCGGAAGGTAATATTATTAGTAATCAGATCGTCAATATTGTTAATTTTCATTATG	
MDS30(t)	AATATTCGGAAGGTAATATTATTAGTAATCAGATCGTCAATATTGTTAATTTTCATTATG	
MDS11(13)	N I R K	44
MDS11(14)	N I R K	
MDS21	N I R K	
MDS30(t)	N I R K	
PSS3	N I R K	
PSS5	N I R K	
MDS20a	P Y Q T	
MDS11(13)	TACTTGTATGTGTGTTGTATATA--TTCAATATATACATACTCAAACATAGATTTTCATGC	240
MDS11(14)	TACTTGTATGTGTGTTGTATATA--TTCAATATATACATACTCAAACATAGATTTTCATGC	
MDS21	CACCTGTGTGTGTTGTGTATATTCAATATATACATACTCAAACATAGATTTTCATGC	
MDS30(t)	TACTTGTGTGTGTTGTGTATATTCAATATATACATACTCAAACATAGATTTTCATGC	
MDS11(13)	ACGTGTGTACAAATATTACAATTAGTTTAAAAATATAATCATAAATTTTTTTTTT---CTAT	300
MDS11(14)	ACGTGTGTACAAATATTACAATTAGTTTAAAAATATAATCATAAATTTTTTTTTT---CTAT	
MDS21	ACGCGTGTACAAATATTACAATTAGTTTAAAAATATAATCATAAATTTTTTTTTT---CTAT	
MDS30(t)	ACATGTGTACAAATATTACAATTAGTTTAAAAATATAATCATAAATTTTTTTTTT---CTAT	
HVa (RHV)		
MDS11(13)	TGTGCATACCAGAGAGAAAAATTACTCGAACCCTCAGCTGGC	341
MDS11(14)	TGTGCATACCAGAGAGAAAAATTACTCGAACCCTCAGCTGGC	
MDS21	TGTGCATGCCAGAGAGAAAAATTACTCGAACCCTCAGCTGGA	
MDS30(t)	TCTGCATACCAGAGAGAAAAATTACTCGAACCCTCAGCTGGA	
MDS11(13)	R E K L L E P Q L A	54
MDS11(14)	R E K L L E P Q L A	
MDS21	R E K L L E P Q L	53
MDS30(t)	R E K L L E P Q L	
PSS3	R E K L L E H Q L	
PSS5	R E K L L E P Q L	
MDS20a	I - K I L E H Q L	

Fig. 2 Alignment of the partial nucleotide and deduced partial amino acid sequences of $S_{11(=13)}^-$, $S_{11(=14)}^-$, S_{21}^- , $S_{30(=t)}^-$, and S_{20a}^- (only amino acid sequence) RNase in apple and S_3^- and S_5^- RNase in Asian pear (only amino acid sequence).

The conserved region C2 and hypervariable region HVa (RHV) are boxed. The sequences of $S_{11(=13)}$ (*MDS11(13)*), $S_{11(=14)}$ (*MDS11(14)*) and S_{21} (*MDS21*) were deposited under the DDBJ accession numbers AB 105060, AB094492, and AB094494, respectively. Apple $S_{30(=t)}$ (*MDS30(t)*), apple S_{20a} (*MDS20a*), and Asian pear S_3 (*PSS3*) together with S_5 (*PSS5*) were from studies by Matsumoto *et al.* (2000, 2001b), Matsumoto *et al.* (1999) and Ishimizu *et al.* (1998), respectively. The different sites are shown in bold. Intron sequences are italicized.

Table 1 S-alleles of apple cultivars and a species.

Proposed S-allele	Former S-allele	Cultivar/Species	S-alleles			Database Accession #
6a	6	Oetwiler Reinette	3	6a		AB094495
	12	Citron d'Hiver	3	5	6a	AB105061
6b	17	Blenheim Orange	1	3	6b	AB105062
	19	Bohnappel	6b	9	16	AB094493
11	10/11,13	Gravenstein	4	11	20a	AB105060
	14	Jacques Lebel	1	3	11	AB094492
21	21	Ribston Pippin	1	9	21	AB094494
28	19,30,e,d,de,g	Delicious	9	28		AB035273
	28,30	Red Delicious	9	28		AF201748
30	t	<i>Malus transitoria</i>	20b	30	?	AB035928

Malus plants used in this study are in bold face.

IWPNV' (Ishimizu *et al.*, 1999; Matsumoto and Kitahara, 2000). The conditions for PCR were as described by Ishimizu *et al.* (1999). The amplified fragments were directly sequenced by dideoxy chain termination on an ABI PRISMTM 310 DNA sequencer (Perkin-Elmer) using dRhodamine Terminator Cycle Sequencing Kits (Perkin-Elmer).

The seven S-RNases were divided into three groups of four, two and one, respectively: S_6 , S_{12} , S_{17} , and S_{19} (**Fig. 1**); S_{13} and S_{14} (**Fig. 2**); and S_{21} (**Fig. 2**), based on sequence similarity. Genes encoding all S-RNases in apple contain one intron at the same location within the hypervariable HVa (RHV) region (Matsumoto *et al.*, 2001b), and the seven S-RNases also contain an intron deduced from the presence of plant 5 and 3 splice site consensus sequences at corresponding sites (**Fig. 1, 2**). As shown in **Fig. 1**, the determined sequence of S_6 and S_{12} , and S_{17} and S_{19} were completely identical, and the deduced amino acid sequence of S_6 (S_{12}) was identical to S_{17} (S_{19}) except for one amino acid located outside of a hypervariable region (position 15) as described below, where alanine was replaced by proline (**Fig. 1**). In closely related S-RNases in Solanaceae, the specificity of cell-cell recognition phenomenon has been shown to depend on the amino acid sequences at the two hypervariable regions (HV a and HV b) (Kao *et al.*, 1996; Matton *et al.*, 1997). HV a and HV b regions alone are sufficient for S-allele discrimination (Matton *et al.*, 1997). The rosaceous S-RNases have only one hypervariable region (RHV) located at a position corresponding to that of the solanaceous region HV a (Ushijima *et al.*, 1998). Since the HV a and HV b regions merely control allelic specificity (Matton *et al.*, 1997, 1999; Luu *et al.*, 2001), the RHV region must be sufficient for allele discrimination of closely related apple S-alleles. Up to date, functionally different alleles having identical

RHV regions are unknown not only in apple, but also in other plants with gametophytic self-incompatibility. Since the RHV regions of S_6 , S_{12} , S_{17} , and S_{19} were identical (**Fig. 1**), the four alleles were thought to be functionally identical. We designated S_6 and S_{12} as S_{6a} , and S_{17} and S_{19} as S_{6b} , respectively (**Table 1**).

Recently, Broothaerts (2003) re-numbered $S_{30(28)}$ to S_{19} . This re-numbering was based on the finding of the $S_{30(28)}$ -allele in 'Bohnappel' ($S_9S_{16}S_{19}$) by PCR-digestion analysis, not sequence analysis. Broothaerts (2003) detected both $S_{30(28)}$ and S_{19} using his $S_{30(28)}$ -allele specific primers; however, we could not detect the $S_{30(28)}$ -allele in 'Bohnappel' with our PCR-digestion method (Matsumoto and Kitahara, 2000; data not shown). Moreover, from our sequence analysis, the five amino acids in the HV a (RHA) region of the S_{19} -allele differed from those of the $S_{30(28)}$ -allele in 'Delicious' and 'Red Delicious' (**Fig. 1**), suggesting that S_{19} and $S_{30(28)}$ were different alleles.

Broothaerts (2003) also speculated from a PCR analysis that 'McIntosh'/'Wijcik' ($S_{10}S_{25}$ from stylar protein analysis by Bošković and Tobutt, 1999) and 'Tydeman's Early Worcester' ($S_{24}S_{25}$ from stylar protein analysis by Bošković and Tobutt, 1999) contained the S_6 -allele, instead of the S_{25} -allele. Using S_{20} -primers, he observed a weak PCR product of a different size from that of the true S_{20} -fragment in 'Oetwiler' (S_3S_6), 'McIntosh'/'Wijcik' and 'Tydeman's Early Worcester'. Within the cultivars of 'Merlijn', 'McIntosh'/'Wijcik', 'Telamon', 'Trajan', and 'Tydeman's Early Worcester', which were shown to possess the S_{25} -allele from stylar protein analysis (Bošković and Tobutt, 1999), S_{25} in 'Merlijn' and 'Telamon' were replaced by S_{27b} - and S_{10} -alleles, respectively (Van Nerum *et al.*, 2001). As a result, the presence of the S_{25} -allele was contradicted (Van Nerum *et al.*, 2001). However,

we recently sequenced the S_{25} -allele in 'McIntosh' (Kitahara and Matsumoto, 2002b). We determined the S -genotype of 'McIntosh' and 'Tydeman's Early Worcester' to be $S_{10}S_{25}$ and $S_{24}S_{25}$, respectively, by PCR-digestion, which were the same results as those from stylar protein analysis (Kitahara and Matsumoto, 2002b). The S -genotype of 'Trajan' (S_2S_{25}) was also confirmed by PCR-digestion (data not shown). Finally, from our sequence analysis, the deduced amino acid sequence of S_6 was different from that of the S_{25} -allele in 'McIntosh' (Fig. 1), suggesting that S_6 and S_{25} were different alleles. S_{6a} and S_{6b} showed a marked similarity to the Asian pear (*Pyrus pyrifolia* (Burm) Nak.) S_1 and S_4 (Fig. 1) (Norioka *et al.*, 1995; Ishimizu *et al.*, 1998).

As the sequences of the S_{13} and S_{14} were completely identical to those observed between S_6 and S_{12} or S_{17} and S_{19} (Fig. 2), we designated them as neither S_{13} nor S_{14} , but S_{11} (Table 1). We confirmed S_{11} corresponded to S_{13} ($=S_{14}$) from the following. The S_{11} -allele was identified in 'Adam's Pearmain' (Kobel *et al.*, 1939), but we identified the S_1 -allele instead of the S_{11} -allele (Matsumoto *et al.*, 2003). As the S_{11} -allele within 'Adam's Pearmain' was refuted, S_{11} was possibly left only in 'Gravenstein' (S_4S_{10} (or S_{11}) S_x by Kobel *et al.*, 1939). As we did not identify the S_{10} -alleles in 'Gravenstein' by PCR-digestion (data not shown), its S -genotype was thought to be $S_4S_{11}S_x$, not $S_4S_{10}S_x$. On the other hand, 'Gravenstein' is the only cultivar in which the S_{13} -allele has been identified, and its S -genotype was identified as $S_4S_{13}S_{20}$ by stylar protein analysis (Bošković and Tobutt, 1999). As the RHV region of S_{13} was different from that of S_{20} (Fig. 2), we unified S_{11} and S_{13} ($=S_{14}$) to S_{11} (Table 1).

The S_{21} -allele seemed to correspond to the S_t -allele in *Malus transitoria* (Matsumoto *et al.*, 2000) since a PCR product very close to 259 bp was obtained from *M. transitoria* ($S_{20b=g}S_{30=t}$) and 'Ribston Pippin' ($S_1S_9S_{21}$) using the S_t -allele-specific primers 'St-sense' (5'-CAATAGATAACGAGAACCAC-3') and 'St-antisense' (5'-CAATCTATGAAATGTTCTCC-3') (data not shown). Digestion of the 259 bp fragment by *RsaI* cleaved it into fragments of the expected size, 214 bp and 45 bp (data not shown). To confirm that S_{21} corresponded to S_t , we determined the partial genomic sequence of the S_{21} -allele. As shown in Fig. 2, the deduced amino acid sequence of S_{21} was slightly different from that of the S_t . In particular, one amino acid difference was observed within the HVa (RHV) region (Fig. 2). The $S_{11(13,14)}$ also seemed to be close to S_{21} and S_t since only one or two amino acid differences were detected within the HVa

(RHV) region (Fig. 2). $S_{11(13,14)}$, S_{21} and S_t showed a high similarity to the Asian pear [*Pyrus pyrifolia* (Burm) Nak.] S_3 and S_5 (Fig. 2) (Ishimizu *et al.*, 1998). The *P. pyrifolia* S_3 - and S_5 -alleles are functionally different, and differed by two amino acids in the HVa (RHV) region (Fig. 2) (Ishimizu *et al.*, 1998). In this case, the lysine and histidine residues of S_3 -RNase differed from the arginine and proline residues of S_5 -RNase, respectively (Fig. 2). Within the two amino acid differences, the site of the one amino acid difference between histidine and proline corresponded to that between S_9 and S_9 -allele in apple. Previously, we established that one amino acid difference of S_9 - and S_9 -RNase within the HVa (RHV) region was not enough to distinguish them as different alleles (Matsumoto *et al.*, 2001a). If the difference between histidine and proline is not enough to distinguish them as functionally different alleles, another difference between lysine and arginine must be essential to enable the S_3 - and S_5 -allele discrimination in the Asian pear. Such a difference was observed between $S_{11(13,14)}$ and S_t , and S_{21} and S_t . The difference was not observed between $S_{11(13,14)}$ and S_{21} , but the serine residue at the start of the HVa (RHV) region of $S_{11(13,14)}$ differed from the proline residue of S_{21} . In this case, it is unknown whether the two alleles are the same or not. We could not carry out cross-pollination tests among $S_{11(13,14)}$, S_{21} and S_t , because the alleles of all the identified cultivars are triploid. Consequently, we did not change the allele designation except for S_t , but did change the S_t to S_{30} when converting from the alphabetical to the numerical designation (Table 1). Since S_{30} has been designated as the $S_{30(28)}$, we proposed the unification of S_{30} and S_{28} as S_{28} (Table 1).

Finally, we concluded that the S_6 and S_{12} , and S_{17} and S_{19} should be re-numbered to S_{6a} and S_{6b} , respectively. These four alleles are thought to act as the same allele. Similarly, S_{13} and S_{14} are re-numbered as S_{11} . S_{21} was left unchanged, and S_t was re-numbered as S_{30} .

Acknowledgments

We wish to thank Dr. Wim Broothaerts for supplying the plant samples and DNAs. This research was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 14360019 and 15208004).

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