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

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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 'Tydeman's 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_{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 gametophitic 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 gametophitic 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_i to S_{ij}) and 10 (S_a to S_i , S_z) 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 Slocus 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_{q^-} , $S_{q^{\prime-}}$, S_h -, $S_{10 \text{ (equivalent to i)}}$ -, S_t -, and $S_{25 \text{ (equivalent to z)}}$ -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_{q-} , S_{h^-} , S_{i^-} , and S_{z^-} 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 descrived 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) MDS6a(12) MDS6b(17) MDS6b(19) MDS28(30)	CCGGCCGTATGCAACTCTAATCCTACTCCTTGTAACGATCCTGCTGACAAGTTGTTTACG CCGGCNGTATGCAACTNTAATCCTACTCCTTGTAACGATCCTGCTGACAAGTTGTTTACG CCGNCNGTATGCAACTCTAATCCTACTCCTTGTAACGATCCTCCTGACAAGTTGTTTACG CCGGCCGTATGCAACTCTAATCCTACTCCTTGTAACGATCCTCCTGACAAGTTGTTTACG CCGGCCGTATGCAACTCTAATCCTACTCCTTGTAACGATCCTCCTGACAAGTTGTTTACG											60									
PSS4	CCG	GCCG	TAT	rgci	AAC	FCT/	AAT	CCTA	CTC	CTT	FGTI	AAC	GAT	CCT	ACTO	GAC	AAG	TTG	TTT.	ACG	
MDS6a(6)	Ρ	А	v	С	N	s	N	Р	т	Р	C	N	D	Р	A	D	ĸ	L	F	т	20
MDS6a(12)	Р	A	v	С	N	х	N	Ρ	т	P	С	N	D	Р	A	D	к	L	F	т	
MDS6b(17)	Ρ	х	v	С	N	s	N	P	т	Р	С	N	D	Р	р	D	K	L	F	т	
MDS6b(19)	Р	А	v	С	N	s	N	Р	т	Р	С	N	D	Р	Ρ	D	K	L	F	т	
MDS28(30)	Р	А	v	с	N	s	N	Р	т	P	С	N	D	Ρ	Р	Е	ĸ	L	F	т	
PSS4	Р	А	v	С	N	s	Ν	Ρ	т	Ρ	С	N	D	Ρ	T	D	ĸ	L	F	T	
PSS1	P	Α	v	С	N	s	N	Ρ	т	Р	С	Ν	D	Ρ	т	D	K	L	F	т	
MDS25	Р	A	v	С	N	s	N	R	т	Р	С	ĸ	Ð	Р	Ρ	D	К	L	F	T	
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MDS6a(6)	GTT(CACG	GT	ľTG.	rgg(CT'	TCA	AACA	AGA	ATC	GGA	CCT	GAC	CCA	GAA	AAA'	тбq	AAG	ACT	ACA	120
MDS6a(12)	GTT(CACC	GT.	PTG:	rggo	CCT	TCA	AACA	AGA	ATC	GAG	CTC	GAC	CCA	SAA/	AAA'	rgq	AAG	ACT.	ACA	
MD56D(17)	GTTC	CACC	GT.	rrG.	rgg	CT.	TCA	AACA	AGA	AT	JGA(CTC	GAC	CCA	GAA	AAA'	rgq	AAG	ACT.	ACA	
MD56D(19)	GTTC	CACG	GT.	PTG.	rgg	CCT"	TCA	AACA	AGA	ATC	GAG	CTC	GAC	CCA	GAA	AAA'	rgq	AAG	ACT.	ACA	
MDS28(30)	GTTC	CACG	GT.	rrG.	rggo	CT.	TCA	AACA	AGA	ATC	3GA(CCTO	GAC	CCA	JAA	AAA'	rgq	AAG	AAT.	ATA	
P554	GTT	JACG	GT.	rrg.	T.G.G.(CT.	TCA	AACE	GGA	ATC	GAC	CT	GAC	CCA	SAA	AAA'	req	AAG	ACT	ACA	• •
MDS6a(6)	v	H	G	т Т	W	P	S	N	ĸ	N	G	P P	D	P	E	ĸ	C	ĸ	T	T	40
MD56a(12)	V	н	G	г	W	5	S	N	ĸ	N	G	P	D	Р	E	ĸ	C	ĸ	T	T	
MDS6D(17)	V	H	G	1	W	P	S	N	ĸ	N	G	P	0	P	E	ĸ	C	K	T	T	
MDS6D(19)	V	н	G	ь -	W	Р	S	N	ĸ	N	G	P	D	Р	Е	ĸ	c	ĸ	T	T	
MDS28(30)	V	Н	G	L	W	P	S	N	ĸ	N	G	P	D	P	E	ĸ	C	K	N	I	
PSS4	V	H	G	ь т	W	P	S	N	R	N	G	P	D	P	Е	ĸ	c	ĸ	T	T	
PSSI	v	н	G	L	W	P	s	N	R	N	G	P	D	P	E	ĸ	C	ĸ	T	T	
MDS25	V	н	G	<u>ь</u>	W	P	S	N	R	N	G	Р	D	Р	Е	¥	сГ	K	N	<u>T</u>	
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MDS6a(12)	Δ	ц Т.	N	s	ň	ĸ															40
MDS6b(17)	A	т.	N	s	õ	ĸ															
MDS6b(19)	A	τ.	N	s	ñ	ĸ															
MDS28(30)	ō	M	N	s	õ	ĸ															
PSS4	Ť	м	N	ŝ	õ	ĸ															
PSS1	Ā	т.	N	s	õ	ĸ															
MDS25	т	ĩ	D	v	Ť	ĸ															
MDS6a(6)	TTT	ATGO	CAC	TTG	TGT	ATA	-TA	GACA	TTA	ACA-	-AT	ACT	CAA	CAT.	AGA	TTT	TCA	TGC.	ATG	CCT	240
MDS6a(12)	TTTT	ATGO	CAC	TTG'	TGT	ATA	-TA	GACA	TTA	CA-	-ATZ	ACT	CAA	CAT	AGA	TTT	TCA	TGC.	ATG	ССТ	
MDS6b(17)	TTT	ATGO	AC	TTG	TGTZ	ATA	-TA	TACA	TTA	CA-	-ATZ	ACT	CAA	CAT.	AGA	TTT	TCA	TGC	ACG	C G T	
MDS6b(19)	TTTATGCACTTGTGTATA-TATACATTACA-ATACTCAACATAGATTTTCATGCACGCGT																				
MDS28(30)	TTTATGTACTTGTGTATAAAATATATTACATATACTCAACATAGATTTTCATGCACGCCT																				
PSS4	$TTTATGCAC{{\pmb G}} TGTGTATA-TATACATTACA{\pmb T} A T{\pmb G} CTCA{{\pmb G}} CATAGATTTTCATGCAC{{\pmb G}} CCT$																				
MDS6a(6)	GTG(CAAA	TA	TTA	CAA'	TTA.	ATT	TAAZ	ATT	TA	ACC2	ATG	AAT	TGT	TTC	TAT	TAC.	ATA	ATT.	ATA	300
MDS6a(12)	GTG	CAAA	TA	TTA	CAA!	TTA	ATT	TAAA	ATT	TA	ACC/	ATG	AAT	TGT	TTC:	TAT	TAC.	ATA	ATT	ATA	
MDS6b(17)	GTG	CAAA	TA?	TTA	CAA!	TTA	ATT	TAAA	ATI	TA	ATCI	ATG	AAT	TGT	TTC'	TAT	TAT.	ATA	ATT.	ATA	
MDS6b(19)	GTG(CAAA	TA'	TTA	CAA:	TTA.	ATT	TAAA	ATI	TA	ATC/	ATG	AAT	TGT	TTC	TAT	TAT.	ATA	ATT.	ATA	
MDS28(30)	GTG	GAAA	ATA?	TTA	CAA!	TTA.	ATT	TAAZ	ATT	TA	ATC	ATG,	AAT	TGT	TTC	TAT	TAT	ATA	ATT.	ATA	
PSS4	GTG	CAAA	TA?	TTA	CAA!	TTA	ATT	TAAZ	ATI	TA	ATC	ATG	AAT	TGT	TTC:	TAT	TAT.	ATA	ATT.	ATA	
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MDS6a(6)	TTG:	rcac	AT?	AGGI	AAA'	[ATO	GAC	AGCC	CAA	TTC	3	-	331								
MDS6a(12)	TTG:	I'CAG	ATZ	AGGI	AAA	rat(GAC	AGCC	CAA	TTC	GA		333								
MDS6D(17)	TTG.	ICAG	AT/	1GGI	AAA	TAT	GAC	AGCC	CAA	TTO	GA										
MDG20(19)	TTG:	CAC	117	46G/	AAP.	CAT'	GAC	AGCC	CAA	ATTO	-GA										
FW528(30)	TTG:	CAG	A17	4GGZ	AA!	rA'I'(GGC	AGCC	CAG	TTC .	GA										
roo4 Mngeole	TTG!	rCA6	1112 -	aGGl	AAA'	LAT(JACI	AGCC		TTC -	эGA		۰.								
MDG6a(12)				G	N	M	T	A		노			54								
MDS66(12)				G	N	M	T'	A		1.											
MDS65(10)				G	IN N	M.	T	A		ц т											
MDS28(20)			1+	6	EN NT	P1 M	T	A		ц т											
DEEN (30)				G	IN NT	M	A.	A		1. *											
1 007 DCC1			1 +	2	EN TAT	11 14	1	A *		+											
MDS25			T	G	N	L	ō	A	lõ	л. Т.											
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Fig. 1 Alignment of the partial nucleotide and deduced partial amino acid sequences of $S_{6a(=6)^-}$, $S_{6a(=12)^-}$, $S_{6b(=17)^-}$, $S_{2B(30)^-}$, 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 $S_{\delta a (=6)}$ (*MDS6a* (6)), $S_{\delta a (=12)}$ (*MDS6a* (12)), $S_{\delta b (=17)}$ (*MDS6b* (17)) and $S_{\delta b (=19)}$ (*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 renumbered 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) MDS11(14) MDS21	CTG CTG CTG	GCT GCT GCT	GTC GTC GTC	TGC. TGC. TGC.	AAC AAC AAC	TCT TCT TCT	AAT AAT A C T	CGT CGT CGT	GCT GCT ACT	CCT CCT CCT	TGT) TGT) TGT)	AAG AAG	GAT GAT GAT	CCT CCT CCT	CCT CCT CC G	GAC GAC GAC	AAG AAG AAG	TTG TTG TTG	TTT TTT TTT	ACG ACG ACG	60
MDS30(t)	CTG	GCT	GTC	TGC.	AAC	TCT	AAT	CGT	ACT	CTT	TGT	AAG	GAT	CCT	CCT	GAC.	AAG	TTG	TTC	ACG	
MDS11(13)	L	А	v	С	N	s	Ν	R	A	Р	С	к	D	P	Р	D	ĸ	Ľ	F	T	20
MDS11(14)	\mathbf{L}	Α	v	С	N	s	N	R	A	Р	С	ĸ	Ð	P	P	D	ĸ	L	F	Ŧ	
MDS21	\mathbf{r}	А	v	С	N	s	T	R	т	Ρ	С	ĸ	D	Р	Р	D	ĸ	L	F	T	
MDS30(t)	L	А	V	С	N	s	N	R	т	L	C	ĸ	D	Ρ	Ρ	D	ĸ	Г	F	т	
PSS3	\mathbf{L}	А	v	С	N	S	N	R	т	L	С	ĸ	D	Р	Р	D	ĸ	L	F	T .	
PSS5	\mathbf{L}	А	v	С	N	s	N	R	т	P	С	к	D	P	P	D	к	L	F	T	
MDS20a	P	А	v	С	н	s	N	Р	т	Ρ	С	ĸ	D	Ρ	Р	D	к	L	F	T	
			(22													,				
MDS11(13)	GTT	CAC	GGT	TTG	TGG	CCI	TCA	AGC	ATC	GTA	GGA	CCT	GAC	CCA	AGT	ААТ	тGQ	TCC	ATA	AGG	120
MDS11(14)	GTT	CAC	GGI	TTG	TGG	CCI	TCP	AGC	ATG	GTA	GGA	CCT	GAC	CCA	AGT	AAT	тGq	TCC	ATA	AGG	
MDS21	GTT	CAC	GGT	TTG	TGG	CCI	"TC#	AGC	ATG	GTA	GGA	CCT	GAC	CCA	AGT	AAT	TGQ	CCC	ATA	AGG	
MDS30(t)	GTT	CAC	GGT	TTG	TGG	CCI	TCA	AGC	ATC	GTA	GGA	CCT	GAC	CCA	AGT	AAA	TGQ	CCC	ATA	AAG	
MDS11(13)	v	Н	G	\mathbf{L}	W	\mathbf{P}	s	s	М	v	G	Ρ	D	Ρ	S	N	С	S	Ι	R	40
MDS11(14)	v	Н	G	L	W	P	s	S	М	v	G	Ρ	D	\mathbf{P}	s	N	С	S	I	R	
MDS21	v	H	G	\mathbf{L}	W	\mathbf{P}	s	S	М	v	G	Ρ	D	Ρ	s	N	С	Р	Ι	R	
MDS30(t)	v	H	G	L	W	Р	S	s	М	v	G	\mathbf{P}	D	Р	s	ĸ	С	Р	I	K	
PSS3	v	н	G	L	W	P	s	N	М	v	G	Ρ	D	Ρ	s	ĸ	С	Р	Ι	ĸ	
PSS5	v	H	G	L	W	Р	s	s	М	A	G	\mathbf{P}	D	Ρ	s	N	С	Р	Ι	R	
MDS20a	v	н	G	L	W	Ρ	s	D	s	N	G	N	D	Р	к	Y	С	ĸ	A	P	
	HV	a (RHV	0																	
MDS11(13)	ААТ	יאיי	CGG	AAG	GTZ	ATA	TTT	ATTZ	AGT2	ATC	'AGA	TCG	TCA	ATA	TTG	TTA	ATT	TC	ATTI	ATG	180
MDS11(14)	ААЛ	רידעי	CGG	AAG	GTA	ATZ	10777	4 <i>ͲͲ</i> Ϋ	AGT?	ATC	AGA	TCG	TCA	ATA	TTO	TTA	ATT	TC	ATTI	TATG	
MDG21	220	היידי	rcco	AAC	GTZ	ATZ	1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	ATT2	GTA	ATC	ATG	TCG	TCA	ATA	TTG	TT 1	ATT	TCC	TTTT	ATG	
MDS30(+)	224	ריייבי	rca	AAC	GTZ	ATA	1777	\$777	AGTA	ATC	CAA	TCC	TCF	ATA	TTG	TTT	ATT	TCA	ATT	ATG	
MDC11(12)	N	т.		v	011		** **														44
MDC11(13)	19	- -	R R	v																	
MDG21	N	- +	D 10	v																	
MDG20(+)	IN N	T	D	R. K.																	
MDSSU(L)	19	T	D D	N N																	
roos DCCE	IN N	T	D	R R																	
F333 MDS20a	מ	v	Ô	Ţ																	
MDB20a			¥		1																
MDC11(12)	<i>m</i>h (יוחחי	ימייר	rcre	man	n Turna Turna	ימידבי	τa		"a a 7	מידבי	TA	י <u>ה</u> מיזי	10770	'A A A	CAI	"AG	ΛTT	TTC	ATGC	240
MDSII(13)	17AC	אחותי	3124. 700 M	ncma	2002		SIA.	78	-110	~ <u>7</u> 227	272	TAC	נידמי	1010	72 4 2	CAT	AG	111	TTC	ATGC	210
MDG11(14)	C AC	~110	ann.	rcma	200/20		2002	7 2 47		"D D 7	71111 72 (77 Z	TTAC	7A772	1010 1077(TAAZ	CAT	AG	Δ <i>η</i> ιην	TTC	ATGC	
MDG20(+)	0710	,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,,	7 m Cl	ncm/	ma	מתייתים	310. 3702	7277		"D D 7	-Δ. <i>Π</i> ΙΖ	TAC	יקעי	CTC	ממי	CAT	AG	100	TTC	ATGC	
MD530(L)	IAC		510.	1010	9101		31.0.		ал <i>т</i> (1010							
MDS11(13)	ACO	GTG	<i>TGT</i>	ACA/	ATZ	ATT.	ACA	ATT.	AGT	rta#	1AA7	TAT	4AT(CATZ	1A T /	ATT	(TT	TTT	(CTAT	300
MDS11(14)	ACC	GTG!	TGT	ACAA	ATZ	ATT	ACA	ATT	AGT!	TTA#	1AA7	ATA	AATO	CATZ	AT/	TTT	TTT'	TTT	(CTAT	
MDS21	ACC	G C G!	TGT	ACA	ATZ	ATT.	ACA	ATT	AGT	TTA	laat	TAT?	AAT	CATI	AA	CTT?	CT:	$TTT \cdot$	(CTAT	
MDS30(t)	ACI	TG	TGT	ACA	ATZ	ATT	ACA	ATT	AGT	T CA /	IAA7	'ATZ	ATC	CATZ	1AA2	C TT	TTT	TTT	TTT (CTAT	
HVa (RHV)																					
MDC11(12)	тĊ	PCC	a ጥ አ	- CA		GD		2 mm	ACTO	CAL	ACCC	CAC	CTC	GC	34	11					
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MDS21					I R	15 17	К. 17	ىد T	ц. т	<u>г</u>	r D		т			55					
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Fig. 2 Alignment of the partial nucleotide and deduced partial amino acid sequences of $S_{II}(=13)$ -, $S_{II}(=14)^{-}$, S_{2I}^{-} , $S_{30}(=1)^{-}$, 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 $S_{11(=15)}$ (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 $S_{30(=0)}$ (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.

Proposed S-allele	Former S- allele	Cultivar/Species	S	- alleles	Database Accession #		
6a	6	Oetwiler Reinette	3	6a		AB094495	
0	12	Citron d'Hiver	3	5	6a	AB105061	
6b	17	Blenheim Orange	1	3	6b	AB105062	
	19	Bohnapfel	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	Malus transitoria	20b	30	?	AB035928	

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

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 (HVa and HVb) (Kao et al., 1996; Matton et al., 1997). HVa and HVb 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 HVa (Ushijima et al., 1998). Since the HVa and HVb 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 'Bohnapfel' ($S_9S_{16}S_{19}$) 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 'Bohnapfel' with our PCR-digestion method (Matsumoto and Kitahara, 2000; data not shown). Moreover, from our sequence analysis, the five amino acids in the HVa (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_{I3} nor S_{I4} , but S_{II} (**Table 1**). We confirmed S_{11} corresponded to S_{13} (= S_{14}) from the following. The S_{II} - 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_{II} -allele within 'Adam's Pearmain' was refuted, S_{II} was possibly left only in 'Gravenstein' $(S_4 S_{10} \text{ (or } S_{11}) S_x \text{ by Kobel et al., 1939})$. As we did not identify the S_{10} alleles in 'Gravenstein' by PCR -digestion (data not shown), its S-genotype was thought to be $S_4 S_{11} S_x$, not $S_4 S_{10} S_x$. 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 $S_4 S_{13} S_{20}$ 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_{2i} -allele seemed to correspond to the S_{t-1} allele in Malus transitoria (Matsumoto et al., 2000) since a PCR product very close to 259 bp was obtained from *M. transitoria* $(S_{20b=q}, S_{30=t})$ and 'Ribston Pippin' $(S_1 S_9 S_{21})$ using the S_t -allele-specific primers 'St-sense' (5'-CAATAGATAAC-GAGAACCAC-3') and 'St-antisense' (5'-CAAT-CTATGAAATGTTCTCC-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_{2l} 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 $S_{II(I3, I4)}$ also seemed to be close to S_{2l} 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_a and $S_{a'}$ allele in apple. Previously, we established that one amino acid difference of S_q - and S_q - 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 $S_{II(I3,I4)}$ and S_t , and S_{2I} and S_t . The difference was not observed between $S_{II(I3, 14)}$ and S_{2I} , 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 crosspollination tests among $S_{II(13,14)}$ S_{2I} 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_i was renumbered as S_{30} .

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