Journal of Plant Biology, May 2008, 51(3) : 202-208

Molecular Characterization of New *S-RNases* ('S_{31}' and 'S_{32}') in Apple (*Malus* × *domestica* Borkh.)

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Apple exhibits gametophytic self-incompatibility (GSI) that is controlled by the multiallelic *S*-locus. This *S*-locus encodes polymorphic *S* ribonuclease (S-RNase) for the pistil-part *S* determinant. Information about *S*-genotypes is important when selecting pollen donors for fruit production and breeding of new cultivars. We determined the *S*-genotypes of 'Charden' ($S_2S_3S_4$), 'Winesap' (S_1S_{28}), 'York Imperial' (S_2S_{31}), 'Stark Earliblaze' (S_1S_{28}), and 'Burgundy' ($S_{20}S_{32}$), by *S-RNase* sequencing and *S*-allele-specific PCR analysis. Two new *S-RNases*, S_{31} and S_{32}, were also identified from 'York Imperial' and 'Burgundy', respectively. These new *S*-alleles contained the conserved eight cysteine residues and two histidine residues essential for RNase activity. Whereas S_{31} showed high similarity to S_{20} (94%), S_{32} exhibited 58% (to S_{24}) to 76% (to S_{25}) similarity in the exon regions. We designed new *S*-allele-specific primers for amplifying S_{31}- and S_{32}-*RNase*-specific fragments; these can serve as specific gene markers. We also rearranged the apple *S*-allele numbers containing those new *S-RNases*. They should be useful, along with an *S-RNase*-based PCR system, in determining *S*-genotypes and analyzing new alleles from apple cultivars.

Keywords: apple, gametophytic self-incompatibility, *Malus domestica*, S-genotype, S-RNase

Self-incompatibility (SI), a genetic means for preventing self-fertilization in flowering plants, is controlled in many species by a single locus (the *S*-locus) with multiple alleles. When an *S*-allele from pollen matches that from the pistil, the pollen is recognized as 'self', and pollen tube growth is stopped in the style. The *S*-allele encodes a ribonuclease called S-RNase (de Nettancourt, 1977, 2001). Apple (*Malus* × *domestica* Borkh.), in the Rosaceae family, exhibits gametophytic SI (GSI). To ensure fruit set, artificial pollination with compatible pollen from other cultivars has been conducted in commercial apple operations. Thus, the determination of *S*-genotypes would be an important tool for selecting appropriate pollen donors in fruit production and breeding programs.

Analysis of pollen tube growth in the style, as well as fruit and seed formation, has revealed 11 different *S*-alleles (S_1 to S_{11}); the S-genotypes of 14 diploid and 12 triploid varieties have now been determined in apple (Kobel et al., 1939). Komori et al. (1999, 2000) have investigated incompatibility relationships among Japanese apple cultivars, assigning a letter symbol to 10 *S*-alleles (S_a to S_i, and S_z) and reporting their correspondence to four of those Kobel *S*-alleles.

The S_a- to S_f-RNases of apple have been identified via protein analyses, isoelectric focusing (IEF), and 2-dimensional polyacrylamide gel electrophoresis (Sassa et al., 1994, 1996). *S*-allele typing of 56 cultivars has been carried out by separating the stylar protein extracts, using IEF and non-equilibrium pH electrofocusing, and the staining of ribonucleases (Boškoviæ and Tobutt, 1999). From that research, the genotypes of 10 cultivars are now available, and 14 new *S*-alleles, S_{12} to S_{25}, have been proposed in European apple (Boškoviæ and Tobutt, 1999).

Molecular approaches, such as PCR, PCR-RFLP, sequencing, and Southern blot analysis, have been taken to identify the *S*-allele in apple cultivars (Janssens et al., 1995, 1996; Sassa et al., 1996; Sakurai et al., 1997, 2000; Kim et al., 2004). To determine the *S*-genotypes, 10 *S-RNase* alleles (S_c-, S_d-, S_e-, S_f-, S_g-, S_h-, S_i-, S_t-, and S_z-*RNase*) have been cloned and sequenced in Japan (Sassa et al., 1996; Matsumoto et al., 1999, 2000, 2001a, b; Matsumoto and Kitahara, 2000; Kitahara et al., 2000; Kitahara and Masumoto, 2002a, b) while 11 *S-RNase* alleles (S_2-, S_3-, S_4-, S_5-, S_7-, S_9-, S_{24}-, S_{26}-, S_{27a}-, S_{27b}-, and $S_{30(=28)}$-*RNase*) have been found in Europe (Broothaerts et al., 1995; Janssens et al., 1995; Schneider et al., 2001; Verdoodt et al., 1998; van Nerum et al., 2001). Furthermore, S_{29}-*RNase* was registered in the GenBank database under the Accession Number AY039702 by Matityahu et al. (2005).

Allele-specific PCR and RFLP methods have been developed with 17 *S*-allele-specific primer pairs and allele-specific restriction enzymes to identify apple *S*-genotypes (Broothaerts, 2003). Broothaerts also has proposed the renumbering of four *S*-alleles (S_{10b}, S_{27a}, S_{27b}, and S_{28} becoming S_{23}, S_{16}, S_{22}, and S_{19}, respectively). In addition, partial genomic sequences of the S_6-, S_{12}-, S_{13}-, S_{14}-, S_{17}-, S_{19}-, and S_{21}-*RNases* have been determined in apple (Matsumoto et al., 2003). However, the many different systems employed in naming those apple *S*-alleles have led to misunderstandings and confusion in their annotation.

Even though those *S*-allele-specific primers have been used in the genotyping system, new apple *S*-alleles are difficult to identify. Based on genomic PCR with common primers (ASPF3 and ASPR3) designed from 23 *S-RNase* sequences

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reported previously, the *S*-genotypes of Korean-bred cultivars and parents have been determined but no new apple *S*-allele has been found (Kim et al., 2006). Moreover, the *S*-genotypes of many apple cultivars are still unknown.

Here, we performed *S-RNase* PCR and sequence analyses to examine the *S*-genotypes of five apple cultivars -- 'Charden', 'Winesap', 'York Imperial', 'Stark Earliblaze', and 'Burgundy'. These were selected because, even though they provide important genetic material for breeding against fire blight and silverleaf resistance (Bus et al., 1996; Steiner et al., 2006) their *S*-genotypes have not yet been elucidated. We determined the nucleotide and amino acid sequences of two new *S-RNases* from 'York Imperial' and 'Burgundy' and rearranged the *S*-allele numbers in apple. We also developed an *S*-allele-specific PCR system to identify two new *S-RNases*.

MATERIALS AND METHODS

Plant Materials

Young leaves from five *S*-genotype-unknown apple cultivars ('Charden', 'Winesap', 'York Imperial', 'Stark Earliblaze', and 'Burgundy') were collected at the Department of Apple Research National Institute of Fruit Tree Science (NIFTS), Morioka, Japan. The tissues were frozen in liquid nitrogen immediately after harvest, and stored at −80°C.

DNA Isolation and *S-RNase* PCR Analysis

Total DNA was isolated with a DNeasy Plant Mini Kit (QIAGEN, Germany), and served as our template. PCR amplification was performed using two *S-RNase* common primers, ASPF3 (5'-CAA TTT ACG CAG CAR TAT CAG-3') and ASPR3 (5'-CAA AGA SHG ACC TCA ACY AAT TC-3') (Kim et al., 2006). The reaction mixture contained 2.5 mL of 10× *Ex* PCR buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, and 25 mM $MgCl_2$], 2.5 mM of each dNTP, 20 μM of each primer, 2.5 units of *Ex Taq* polymerase (TaKaRa, Kyoto, Japan), and 25 to 100 ng of template DNA, made up to a total volume of 25 μL. PCR reactions were conducted on an ASTEC PCR Thermal Cycler (PC-320; Astec Co., Fukuoka, Japan), with the program consisting of an initial denaturing step at 94°C for 1 min; then 30 cycles at 94°C for 30 s, 58°C for 45 s, and 72°C for 2 min; followed by a final extension step at 72°C for 7 min.

Two primers were also designed for amplifying the ORF regions of our two new *S-RNases* from the 5'- and 3'-flanking regions: ASR5'F1 (5'-GTA ATT RAT CTG CCT YGC-3') and ASR3'R1 (5'-TAG CYG YGM TCT TAA TAC HG-3'). This analysis was done under the following conditions: an initial denaturing step at 94°C for 3 min; then 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; and a final extension step at 72°C for 5 min.

S-RNase Sequence Analysis

The amplified *S-RNase* PCR products for those apple cultivars were purified with a Geneclean Kit (BIO 101 Co., CA, USA) and sub-cloned into the pGEM-T Easy Vector with TA cloning kits (Promega, Madison, WI, USA). DNA sequences of the inserts from several clones were determined with the ABI PRISM™ 377 DNA Sequencer (Applied Biosystems, Foster, CA, USA), using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, Piscataway, NJ, USA). Those sequences were aligned manually by GENETYX-WIN 5.0 (GENETYX Co., Tokyo, Japan) and analyzed using the BLAST and CLUSTAL W programs to search against a database for homology with other nucleotide and amino acid sequences.

S-allele-specific PCR Analysis

Primers and conditions for our *S*-allele-specific PCR amplifications were as described by Sakurai et al. (1997), with details of the method according to those of Matsumoto et al. (1999) (S_1-allele), Sakurai et al. (1997) (S_2-, S_3-, and S_9-alleles), Broothaerts (2003) (S_{10}- and S_{20}-alleles), and Matsumoto and Kitahara (2000) (S_{28}-allele). To identify the S_{31}- and S_{32}-*RNases*, we performed PCR amplifications with the S_{31}- (AS31-SPF1: 5'-ATG GGG ACG GGG ATG ATA TAT ATG-3', nucleotides 1 to 24; and AS31-SPR1: 5'-CAG TCT CCG GCT TTT CCT ACC-3', nucleotides 737 to 757) and the S_{32}-allele-specific primer pair (AS32-SPF1: 5'-AAC TTT TTA GGA CCT GAC CCA-3', nucleotides 196 to 216; and AS32-SPR1: 5'-TCT CTT CCG TGT CCA CTT TTT-3', nucleotides 626 to 646). The program comprised initial denaturing at 94°C for 1 min; then 30 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a final extension at 72°C for 7 min.

RESULTS AND DISCUSSION

Determination of *S*-genotypes from Apple Cultivars and Rearrangement of the *S*-alleles

Using the common primer pair (ASPF3 and ASPR3), we amplified *S-RNase* PCR fragments from five *S*-genotype-unknown apple cultivars (Fig. 1). Sizes ranged from 600 bp to 900 bp. In addition, a PCR fragment ca. 1.7 kb long corresponding to S_3-*RNase* was detected in 'Charden' (Fig. 1). However, the *S*-genotypes could not be learned by PCR analysis alone, so we used the pGEM T-vector to clone and sequence their *S-RNase* fragments. Thus, we were able to determine the sizes of the PCR fragments and introns for eight *S-RNases* (Table 1) and to discover two new *S-RNases*, 'S_{31}' and 'S_{32}', from 'York Imperial' (S_2, S_{31}) and 'Burgundy' (S_{20}, S_{32}), respectively. Difference in PCR fragment lengths followed the order of: S_4 (637 bp) < S_2 (647 bp) < S_{32} (651 bp) < S_{28} (672 bp) < S_{31} (777 bp) < S_{20} (818 bp) < S_1 (844 bp) < S_3 (1790 bp). Further, those eight *S-RNases* had introns of various sizes in their hypervariable (HV) region between adjacent exons with fairly high homology, i.e., S_1 = 344 bp, S_2 = 147 bp, S_3 = 1290 bp, S_4 = 164 bp, S_{20} = 318 bp, S_{28} = 169 bp, S_{31} = 277 bp, and S_{32} = 151 bp. Galli et al. (2005) have used five SSR markers to analyze the molecular identification of 66 apple cultivars. For 'Charden', three distinct alleles have been determined, indicating that this cultivar is a triploid. Our results also showed that 'Charden' ($S_2S_3S_4$) retained three *S-RNases*, which is consistent with those Galli et al. findings.

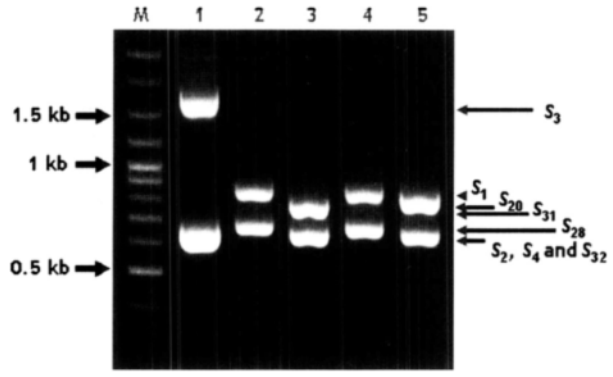

Figure 1. Electrophoretic separation of *S-RNase* fragments from 5 apple cultivars amplified by PCR using *S-RNase* common primer pair (ASPF3 and ASPR3). Lanes 1, 'Charden' ($S_2S_3S_4$); 2, 'Winesap' (S_1S_{28}); 3, 'York Imperial' (S_2S_{31}); 4, 'Stark Earliblaze' (S_1S_{28}); 5, 'Burgundy' ($S_{20}S_{32}$).

Table 1. The *S-RNases* and PCR fragments amplified with *S-RNase* common primers ASPF3 and ASPR3 for five apple cultivars.

S-RNase	PCR fragment (bp)	Intron (bp)	Sequence cloned from
S_1	844	344	'Winesap', 'Stark Earliblaze'
S_2	647	147	'Charden', 'York Imperial'
S_3	1790	1290	'Charden'
S_4	637	164	'Charden'
S_{20}	818	318	'Burgundy'
S_{28}	672	169	'Winesap', 'Stark Earliblaze'
S_{31}	777	277	'York Imperial'
S_{32}	651	151	'Burgundy'

Table 2. Arranged *S*-allele numbers for apple.

Arranged *S*-allele	Up-to-date in Japan		Up-to-date in Europe		Reference cultivar
	S-allele*	GenBank no.	*S*-allele*	GenBank no.	
S_1	S_f	D50837	S_1		'Fuji' (S_1S_9)
S_2	S_a		S_2	U12199	'Golden Delicious' (S_2S_3)
S_3	S_b		S_3	U12200	'Golden Delicious' (S_2S_3)
S_4			S_4	AF327223	'Gloster' (S_4S_{28})
S_5			S_5	U19791	'Gala' (S_2S_5)
S_{6a}	S_6	AB094495			'Oetwiler Reinette' (S_3S_{6a})
	S_{12}	AB105061			'Citron d'Hiver' ($S_3S_5S_{6a}$)
S_{6b}	S_{17}	AB105062			'Blenheim Orange' ($S_1S_3S_{6b}$)
	S_{19}	AB094493			'Bohnapfel' ($S_{6b}S_9S_{16c}$)
S_7	S_d	AB032246	S_7	U19792	'Idared' (S_3S_7)
S_8	S_8	AY744080			'Ontario' (S_1S_8)
S_9	S_c	D50836	S_9	U19793	'Fuji' (S_1S_9)
S_{10}	S_i	AB052683	S_{10}		'McIntosh' ($S_{10}S_{25}$)
S_{11}	S_{11}(=S_{13})	AB105060			'Gravenstein' ($S_4S_{11}S_{20}$)
	S_{14}	AB094492			'Jacques Lebel' ($S_1S_3S_{11}$)
S_{16a}			S_{27a}	AF016919	'Baskatong' ($S_{16a}S_{26}$)
S_{16b}			S_{27b} (=S_{22})	AF327222	'Alkmene' (S_5S_{22})
S_{16c}	S_{16c}	AB126322			'Bohnapfel' ($S_{6b}S_9S_{16c}$)
S_{20}	S_g	AB096138			'Inodo' (S_7S_{20})
S_{21}	S_{21}	AB094494			'Ribston Pippin' ($S_1S_9S_{21}$)
S_{23}			S_{10b}	AF239809	'Granny Smith' (S_3S_{23})
S_{24}	S_h	AB032247	S_{24}	AF016920	'Braeburn' (S_9S_{24})
S_{25}	S_z	AB062100			'McIntosh' ($S_{10}S_{25}$)
S_{26}			S_{26}	AF016918	'Baskatong' ($S_{16}S_{26}$)
S_{28}	S_e	AB035273	S_{28}	AF201748	'Delicious' (S_9S_{28})
	S-RNase I	AB017636			'Starking Delicious' (S_9S_{28})
S_{29}			S_{29}	AY039702	'Anna' (S_3S_{29})
S_{30}	S_t	AB035928			*Malus transitoria* ($S_{20}S_{30}$)
S_{31}	S_{31}	DQ135990			'York Imperial' (S_2S_{31})
S_{32}	S_{32}	DQ135991			'Burgundy' ($S_{20}S_{32}$)

*References: S_f, S_c: Sassa et al. (1996); S_3, S_5, S_7, S_9: Broothaerts et al. (1995); S_4, S_{10b}: van Nerum et al. (2001); S_d: Kitahara et al. (2000); S_6, S_{11}, S_{12}, S_{14}, S_{17}, S_{19}, S_{21}: Matsumoto et al. (2003); S_8: Li et al. (2004); S_i: Kitahara and Matsumoto (2002a); S_{27a}, S_{27b}: Broothaerts (2003); S_{16}c: Matsumoto and Furusawa (2005); S_g: Matsumoto et al. (2001a); S_h: Kitahara et al. (2000); S_{24}, S_{26}: Verdoodt et al. (1998); S_z: Kitahara and Matsumoto (2002b); S_e: Matsumoto and Kitahara (2000); S-RNase I: Okuno (2000); S_{28}: Schneider et al. (2001); S_{29}: Matityahu et al. (2005); S_t: Matsumoto et al. (2000).

We reviewed the *S*-alleles of apple registered in the GenBank of NCBI, referring to the report of *S-RNases* for assigning the *S*-allele numbers for these two new *S-RNases*. Recently, Broothaerts (2003) showed that S_6 and S_{19} correspond to S_{25} and S_{28}, respectively. However, their partial genomic sequences differ between those two pairings (Matsumoto et al., 2003). Nevertheless, the sequences for S_6 and S_{12}, S_{17} and S_{19}, and S_{13} and S_{14} are the same, and those pairings have now been re-numbered as S_{6a}, S_{6b}, and S_{11}, respectively. Although S_{21}-*RNase* seemed to correspond to S_t-*RNase* according to PCR-RFLP analysis, their amino acid sequences were slightly different, so they have re-numbered S_t as S_{30} (Matsumoto et al., 2003). Matsumoto and Furusawa (2005) have determined the genomic DNA sequence for $S_{16c(=16)}$-*RNase* in 'Bohnapfel' ($S_{6b}S_9S_{16c}$) apple and have re-numbered $S_{16(=S27a)}$ in 'Baskatong' ($S_{16=27a}S_{26}$) and $S_{22(=27b)}$ in 'Alkmene' ($S_5S_{22=27b}$) as S_{16a} and S_{16b}, respectively. However, the S_{15}- and S_{18}-alleles were reported by Boškoviæ and Tobutt (1999), but not yet registered in GenBank. Even though we re-checked the database of previously assigned *S*-alleles, we have now arranged the 32 *S*-allele numbers for apple (Table 2). This database can be relied upon when distinguishing the new alleles of the *S*-gene, and is expected to be useful for numbering.

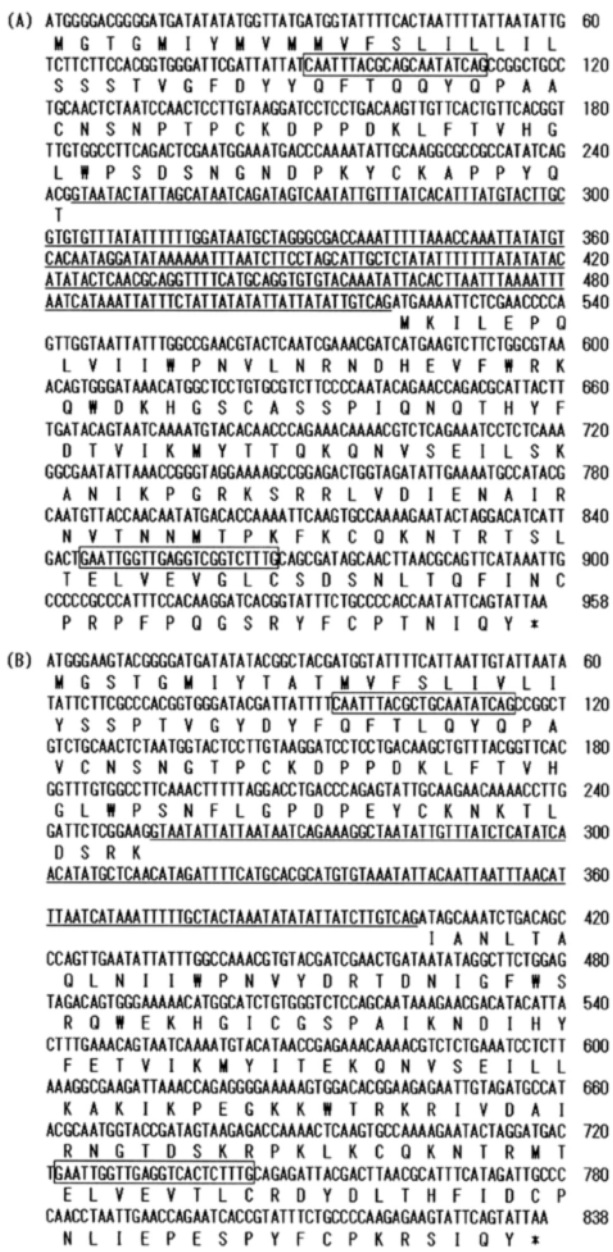

Figure 2. Nucleotide sequences of **A**, new S_{31}-*RNase* (GenBank Accession No. DQ135990) obtained from 'York Imperial' (S_2S_{31}), and **B**, new S_{32}-*RNase* (GenBank Accession No. DQ135991) from 'Burgundy' ($S_{20}S_{32}$). Deduced amino acid sequences are shown below nucleotide sequence; those of intron region are underlined. ASPF3 and ASPR3 primer positions are boxed.

Characterization of Two New *S-RNases*

The complete ORF regions of our two new *S-RNases* were amplified by primers ASR5'F1 and ASR3'R1, which were synthesized from the 5'- and 3'- flanking regions. Afterward, their fragments were cloned and sequenced for molecular characterization. Each ORF region contains two exons and one intron of the hypervariable region (RHV), the latter positioned between the two exons. The S_{31}-*RNase* fragment is 958 bp long, including the 277 bp intron present at positions 244 through 520 (Fig. 2A). The 958 bp nucleotide sequence can be translated to 226 amino acid sequences. The S_{32}-*RNase* is 838 bp long, and includes a 151 bp intron at positions 253 through 403 (Fig. 2B). Its 838 bp nucleotide sequence can be translated to 228 amino acid sequences. Comparisons of the amino acid sequences for these new *S-RNases* with those of 15 other apple *S-RNases* revealed five conserved domains (C1, C2, C3, C4, and C5) and one RHV, and showed that the intron region site is located within that region (Fig. 3). The two new *S*-alleles contain two histidine and eight cysteine conserved residues, which are thought to have structural and functional roles in ribonucleases. The first histidine residue is located within the C2 conserved region whereas the second histidine residue is located between the C3 conserved region and the fourth cysteine residue.

Amino acid identity among these 17 *S-RNases* ranges from 58% to 94% (Table 3). Although the amino acid sequence for S_{16a}-*RNase* is 100% identical to S_{16b}-*RNase*, their nucleotide sequences differ in three bases. The amino acid sequence of S_{31}-*RNase* has high homology with S_1- (94%), S_{20}- (94%), and S_{24}-*RNase* (93%), but homology of the amino acid sequences between S_{32}-*RNase* and the 16 *S-RNases* is lower, ranging from 58% (S_{24}) to 76% (S_{25}).

Three primers (ASR5'F1, ASR5'F2, and ASR3'R1) were synthesized from the 5'- and 3'-flanking regions to amplify the ORF regions of the two new *S-RNases*. We successfully obtained the complete *S-RNase* products by PCR using the primer pair ASR5'F1 and ASR3'R1 (data not shown). Although PCR could also be used to identify new *S-RNases*, it is uncertain whether this method can amplify the ORF regions of all *S-RNases* because, here, we examined the nucleotide sequences of only nine *S-RNases*.

S-allele-specific PCR Analysis

When the *S*-allele-specific primer pair was used for each apple cultivar, only one allele-specific fragment was amplified

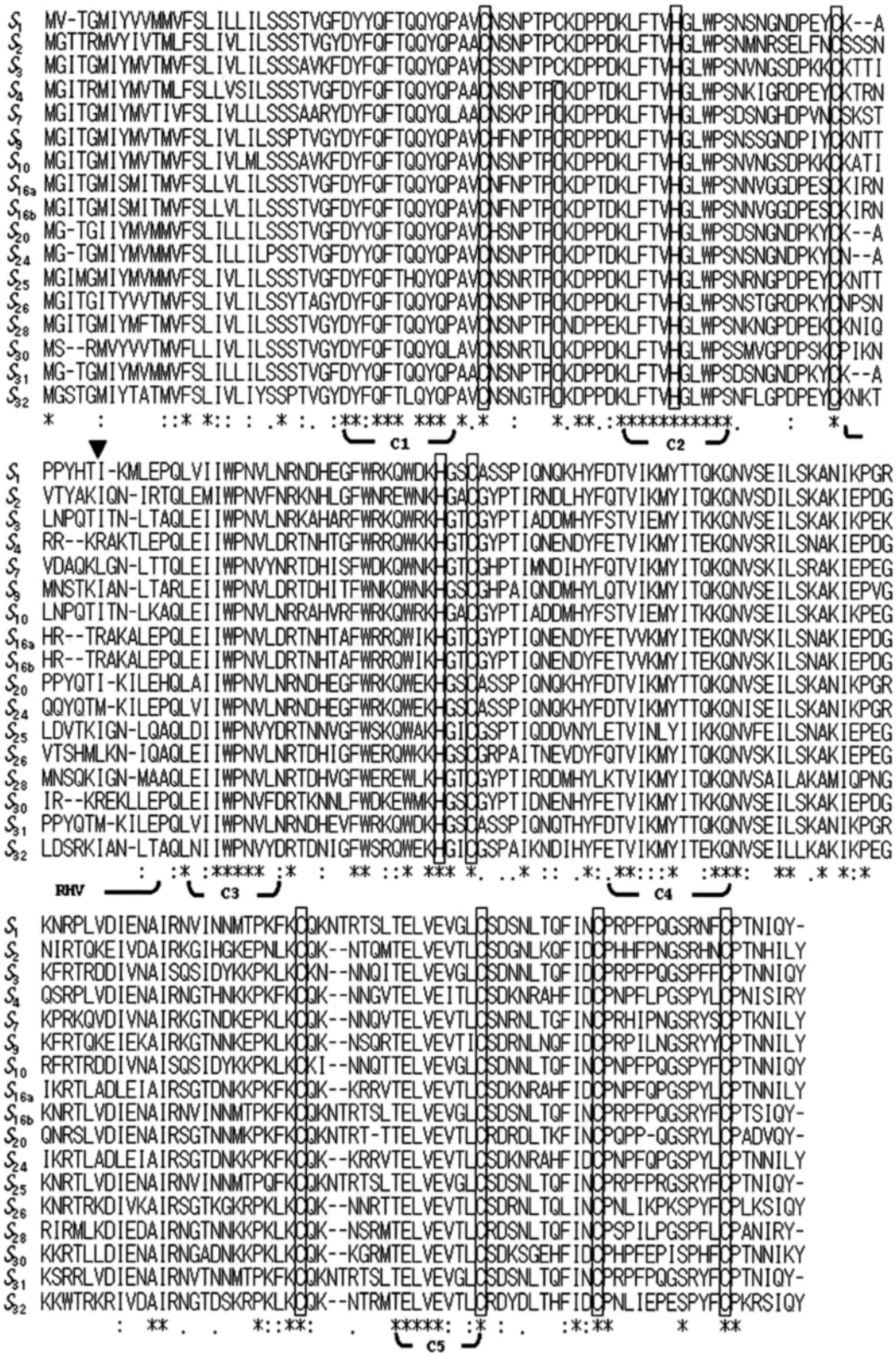

Figure 3. Alignment of amino acid sequences for 2 new *S-RNases* and 15 *S-RNases* in apple. Five conserved regions (C1 to C5) and one hypervariable region (RHV) are shown below *S-RNase* sequences. Conserved eight cysteine residues and two histidine residues essential for RNase activity are boxed. Intron region is indicated by arrowhead.

(Fig. 4). For example, the S_1-*RNase*-specific fragment (254 bp) was obtained from 'Winesap' (S_1S_{28}) and 'Stark Earliblaze' (S_1S_{28}), the S_2-*RNase*-specific fragment (449 bp) from 'Charden' ($S_2S_3S_4$) and 'York Imperial' (S_2S_{31}), the S_3- and S_{20}-*RNase*-specific fragments from 'Charden' ($S_2S_3S_4$; 375 bp) and 'Burgundy' ($S_{20}S_{32}$; 920 bp), the S_{28}-*RNase* specific

Table 3. Analysis of amino acid sequence homology (%) among the 17 *S-RNases* of apple.

	S_1	S_2	S_3	S_4	S_7	S_9	S_{10}	S_{16a}	S_{20}	S_{16b}	S_{24}	S_{25}	S_{26}	S_{28}	S_{30}	S_{31}	S_{32}
S_1		59	66	63	60	64	66	62	93	62	92	61	66	65	61	94	59
S_2			66	64	70	70	66	62	58	62	58	62	68	62	64	58	63
S_3				65	69	71	94	65	66	65	64	69	65	65	64	65	66
S_4					64	66	65	84	61	84	62	66	69	63	71	63	64
S_7						74	69	63	61	63	59	67	69	64	60	62	65
S_9							69	68	65	68	64	70	73	68	64	65	71
S_{10}								65	67	65	65	71	66	66	63	66	66
S_{16a}									60	100*	61	65	67	63	73	61	65
S_{20}										60	93	61	65	65	59	94	60
S_{16b}											61	65	67	63	73	62	65
S_{24}												60	64	64	59	93	58
S_{25}													66	65	62	60	76
S_{26}														68	65	65	69
S_{28}															58	65	64
S_{30}																60	66
S_{31}																	59

*Amino acid sequence is same but nucleotide sequence is different in three bases.

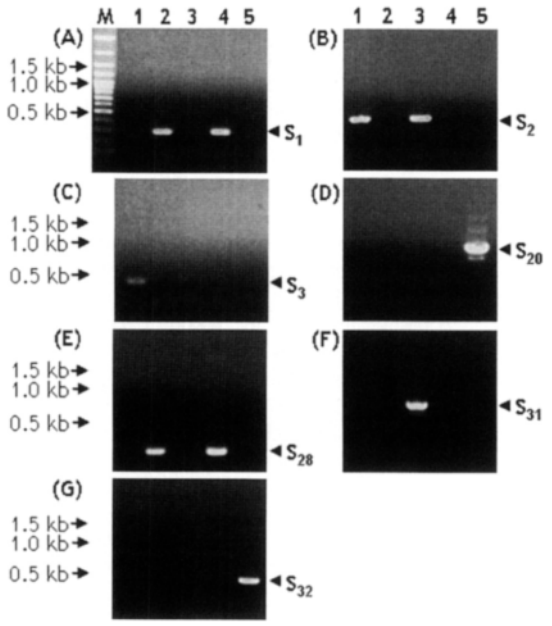

Figure 4. *S*-allele-specific PCR analysis for apple cultivars: 1, 'Charden' ($S_2S_3S_4$); 2, 'Winesap' (S_1S_{28}); 3, 'York Imperial' (S_2S_{31}); 4, 'Stark Earliblaze' (S_1S_{28}); 5, 'Burgundy' ($S_{20}S_{32}$). *S-RNase* fragments amplified by PCR with *S*-allele-specific primers S_1- (**A**), S_2- (**B**), S_3- (**C**), S_{20}- (**D**), S_{28}- (**E**), S_{31}- (**F**), and S_{32}-allele (**G**).

fragment (227 bp) from 'Winesap' (S_1S_{28}) and 'Stark Earliblaze' (S_1S_{28}), and the S_{31}- and S_{32}-*RNase*-specific fragments from 'York Imperial' (S_2S_{31}; 757 bp) and 'Burgundy' ($S_{20}S_{32}$; 451 bp). Conducting *S*-allele-specific PCR, we then confirmed the *S*-genotypes for our five cultivars (Table 4).

ACKNOWLEDGMENTS

We sincerely thank Dr. Akihiko Goto and Dr. Kazuyuki Abe from the Department of Apple Research National Institute of Fruit Tree Science (NIFTS), Morioka, Japan, for supplying the apple plant materials and valuable comments. This study was supported by the Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry, Republic of Korea (Grant No. 707001-02-1-CG000).

Received December 31, 2007; accepted February 2, 2008.

LITERATURE CITED

Boškoviæ R, Tobutt KR (1999) Correlation of stylar ribonuclease isoenzymes with incompatibility alleles in apple. Euphytica

Table 4. *S*-genotypes of apple cultivars as determined by *S*-allele-specific PCR analysis and nucleotide sequencing.

Acc. No.	Cultivar	PCR analysis*							*S*-genotype
		S_1	S_2	S_3	S_{20}	S_{28}	S_{31}	S_{32}	
1	'Charden'	–	+	+	–	–	–	–	$S_2S_3S_4$
2	'Winesap'	+	–	–	–	+	–	–	S_1S_{28}
3	'York Imperial'	–	+	–	–	–	+	–	S_2S_{31}
4	'Stark Earliblaze'	+	–	–	–	+	–	–	S_1S_{28}
5	'Burgundy'	–	–	–	+	–	–	+	$S_{20}S_{32}$

*+, amplified with *S*-allele specific primers; –, not amplified with *S*-allele-specific primers.

107: 29-43
Broothaerts W (2003) New findings in apple S-genotype analysis resolve previous confusion and request the re-numbering of some S-alleles. Theor Appl Genet 106: 703-714
Broothaerts W, Janssens GA, Proost P, Broekaert WF (1995) cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. Plant Mol Biol 27: 499-511
Bus VG, Spiers AG, Brewster DT, Hofstee ME (1996) Preliminary screening of apple germplasm for resistance to silverleaf infection. J NZ Crop Hort Sci 24: 1-6
Galli Z, Halasz G, Kiss E, Heszky L, Dobranszki J (2005) Molecular identification of commercial apple cultivars with microsatellite markers. HortScience 40(7): 1974-1977
Janssens GA, Goderis IJ, Broekaert WF, Broothaerts W (1995) A molecular method for *S*-allele identification in apple based on allele-specific PCR. Theor Appl Genet 91: 691-698
Janssens GA, van Haute AM, Keulemans J, Broothaerts W, Broekaert WF (1996) PCR analysis of self-incompatibility alleles in apple applied to leaves, seed embryos and in vitro shoots. Acta Hort 484: 403-407
Kim HT, Hirata Y, Lee IH, Cho KS, Kim YK, Kang SS, Kim MS, Son DS, Koh GC, Nou IS (2004) PCR-RFLP screening of intergeneric hybrid between pear (*Pyrus* sp.) and apple (*Malus* sp.). Kor J Breed 36(5): 330-337
Kim HT, Hattori G, Hirata Y, Kim DI, Hwang JH, Shin YU, Nou IS (2006) Determination of self-incompatibility genotypes of Korean apple cultivars based on S-RNase PCR. J Plant Biol 49(6): 448-454
Kitahara K, Matsumoto S (2002a) Sequence of the S_{10} cDNA from McIntosh apple and a PCR-digestion identification method. HortScience 37: 187-190
Kitahara K, Matsumoto S (2002b) Cloning of the S_{25} cDNA from McIntosh apple and an S_{25}-allele identification method. J Hortic Sci Biotechnol 77: 724-728
Kitahara K, Soejima J, Komatsu H, Fukui H, Matsumoto S (2000) Complete sequences of the *S*-genes 'Sd-' and 'Sh-RNase' cDNA in apple. HortScience 35: 712-715
Kobel F, Steinegger P, Anliker J (1939) Weitere Untersuchungen uber die Befruchtungsverhaltnisse der Apfel-und Birnsorten. Landw Jb Schweiz 53: 160-191
Komori S, Soejima J, Abe K, Kato H, Kotoda N, Kudo K (1999) Analyses of *S*-allele genotypes of 'McIntosh', 'Kitakami', 'Worcester Pearmain', etc. J Jpn Soc Hort Sci 68(2): 94
Komori S, Soejima J, Abe K, Kotoda N, Kato H (2000) Analysis of S-allele genotypes and genetic diversity in the apple. Acta Hort 538: 83-86
Li T-Z, Katoh N, Kasai A, Okuno T (2004) S-RNase from *Malus domestica*, cultivar Ontario. GenBank submission no. AY744080
Matityahu A, Stern RA, Schneider D, Goldway M (2005) Molecular identification of a new apple S-RNase-S29-cloned from 'Anna', a low-chilling-requirement cultivar. HortScience 40(3): 850-851
Matsumoto S, Furusawa Y (2005) Genomic DNA sequence of $S_{16c(=16)}$-RNase in apple: Re-numbering of $S_{16(=27a)}$- and $S_{22(=27b)}$-allele to S_{16a} and S_{16b}. Sci Rep Rac Educ Gifu Univ (Nat. Sci.) 29: 7-12
Matsumoto S, Kitahara K (2000) Discovery of a new self-incompatibility allele in apple. HortScience 35: 1329-1332
Matsumoto S, Kitahara K, Komori S, Soejima J (1999) A new *S*-allele in apple, '*Sg*', and its similarity to the '*Sf*' allele from Fuji. HortScience 34: 708-710
Matsumoto S, Suzuki M, Kitahara K, Soejima J (2000) Possible involvement of a new S-gene 'St-RNase' (Accession No. AB035928) in the wild apple possessing high similarity to the 'S_3-' and 'S_5-RNase' in the Japanese pear. Plant Physiol 122: 620
Matsumoto S, Kitahara K, Komatsu H, Soejima J (2001a) A functional *S*-allele, '*Sg*', in the wild apple possessing a single amino acid, S-RNase 'Sg-RNase', different from 'Sg-RNase' in *Malus* × *domestica* cultivars. J Hortic Sci Biotechnol 76: 163-166
Matsumoto S, Hayashi S, Kitahara K, Soejima J (2001b) Genomic DNA sequence encoding *Malus* × *domestica* Borkh. "Akane", "Delicious" and *Malus transitoria* S-RNases. DNA Seq 12: 381-383
Matsumoto S, Furusawa Y, Kitahara K, Komori S, Soejima J (2003) 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. Plant Biotechnol 20(4): 323-329
de Nettancourt D (1977) Incompatibility in angiosperms, *In* R Frankel et al., eds, Monographs on Theoretical and Applied Genetics. Springer-Verlag, New York, pp 28-57
de Nettancourt D de (2001) Incompatibility and Incongruity in Wild and Cultivated Plants. Springer-Verlag
Okuno T (2000) S-RNase from *Malus domestica*, cultivar Starking Delicious. GenBank submission no. AB017636
Sakurai K, Brown SK, Weeden NF (1997) Determining the self-incompatibility alleles of Japanese apple cultivars. HortScience 32: 1258-1259
Sakurai K, Brown SK, Weeden NF (2000) Self-incompatibility alleles of apple cultivars and advanced selections. HortScience 35: 116-119
Sassa H, Mase N, Hirano H, Ikehashi H (1994) Identification of self-incompatibility related glycoproteins in styles of apple (*Malus* × *domestica*). Theor Appl Genet 89: 201-205
Sassa H, Nishio T, Kowyama Y, Hirano T, Koba T, Ikehashi H (1996) Self-incompatibility (S) alleles of the Rosaceae encode members of a distinct class of the T2/S ribonuclease superfamily. Mol Gen Genet 250: 547-557
Schneider D, Stern RA, Eisikowitch D, Goldway M (2001) Analysis of *S*-alleles by PCR for determination of compatibility in the Red Delicious apple orchard. J Hortic Sci Biotech 76: 596-600
Steiner PW, van der Zwet T, Biggs AR (2006) Fire Blight, *Erwinia amylovora*. Available online at http://www.caf.wvu.edu/kearneysville/disease_descriptions/omblight.html (verified 3 Aug 2006).
van Nerum I, Geerts M, van Haute A, Keulemans J, Broothaerts W (2001) Re-examination of the self-incompatibility genotype of apple cultivars containing putative 'new' S alleles. Theor Appl Genet 103: 584-591
Verdoodt L, van Haute A, Goderis IJ, de Witte K, Keulemans J, Broothaerts W (1998) Use of the multi-allelic self-incompatibility gene in apple to assess homozygosity in shoots obtained through haploid induction. Theor Appl Genet 96: 294-300