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Determination of Self-Incompatibility Genotypes of Korean Apple Cultivars Based on S-RNase PCR

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To prevent self-fertilization, apple has a gametophytic self-incompatibility mechanism, part of a widespread intraspecific system, that is controlled by a multi-allelic locus. This attribute has been exploited in breeding programs for new cultivars. Likewise, many apple orchards depend on artificial pollination. Therefore, molecular analysis and early identification of the self-incompatibility (S) genotype could greatly improve breeding schemes and pollen donors selection. Here, we PCR-amplified the S-RNase PCR fragments from a total of 14 cultivars and parents, using new primers (ASPF3+ASPR3) common to 23 S-alleles in apple. The S-genotypes were determined for the following: 'Hongro' (S_1S_3), 'Gamhong' (S_1S_9), 'Saenara' (S_1S_3), 'Chukwang' (S_3S_9), 'Hwahong' (S_3S_9), 'Seokwang' (S_3S_5), 'Hwarang' (S_1S_9), 'Sunhong' (S_3S_9), 'S.E.B.' (S_1S_{19}), 'S.G.D.' (S_2S_3), and 'Mollie's Delicious' (S_3S_7). We also confirmed the characteristics of the S-genotypes for eight Korean apple cultivars by PCR-Southern blot analysis, using seven S-RNases as probes.

Keywords: apple, gametophytic self-incompatibility, PCR, S-genotype, S-RNase

Many flowering plants have self-incompatibility mechanisms to prevent self-fertilization and promote out-crosses. Apple (*Malus* × *domestica*), which belongs to the family Rosaceae, exhibits stylar monofactorial gametophytic self-incompatibility (GSI). This GSI mechanism is controlled by a single locus (S-locus) with multiple alleles. The S-locus products in the pistil are cytotoxic proteins with ribonuclease (S-RNase) activity. During an incompatible interaction, the S-RNases act on elongating pollen tubes, entering their cytoplasm and degrading the pollen RNA (Broothaerts et al., 1995; Kim et al., 1999; de Nettancourt, 2001). The pistil S-gene encodes a family of ribonucleases (McClure et al., 1989); a pollen S-gene candidate encodes an F-box protein in almond (Ushijima et al., 2003) and in sweet cherry (Yamane et al., 2003). The presence of this mechanism suggests that successful fruit set in species exhibiting this kind of self-incompatibility depends upon the availability of compatible pollen. Consequently, knowledge of the S-haplotype and the assignment of cultivars to different incompatibility groups are essential to the design of productive commercial apple orchards and to the development of effective breeding programs. Analyses 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}), with the S-genotypes of 14 diploid and 12 triploid varieties having 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_l, S_z) and reporting their correspondence to four of the Kobel's S-alleles.

The S-RNases of apple have been isolated and characterized by Sassa et al. (1994) and Broothaerts et al. (1995). Furthermore, biochemical and molecular methods have been developed to identify the S-allele and classify the incompatibility groups of different cultivars in rosaceous fruit tree species (Janssens et al., 1995; Bošković and Tobutt, 1999; Ishimizu et al., 1999; Tamura et al., 2000; Kim et al., 2004a, b). Sassa et al. (1994, 1996) have now discriminated the S_a- to S_f-RNases by their gene products, revealing characteristic migration patterns in the alkaline regions of isoelectric focusing (IEF) or 2D-PAGE gels. Likewise, the S-genotypes of 56 apple cultivars have been determined by separation of stylar proteins via IEF, non-equilibrium pH gel electrofocusing (NEPHGE), and staining of RNase (Bošković and Tobutt, 1999). From that research, the genotypes of 10 cultivars have been reported for the first time, and 14 new S-alleles, S_{12} to S_{25}, have been proposed in European apple (Bošković and Tobutt, 1999).

S-genotypes have been identified through various methods (Janssens et al., 1995) that have been extended and modified several times (Verdoodt et al., 1998; van Nerum et al., 2001). Molecular approaches also have been used to determine the S-genotype (Sakurai et al., 1997, 2000; Matsumoto and Kitahara, 2000; Kitahara and Matsumoto, 2002a, b) and to confirm the identity of cultivars by multiplex PCR (Broothaerts et al., 2001). Furthermore, 10 apple S-RNases (S_c-, S_d-, S_e-, S_f-, S_g-, $S_{g'}$-, S_h-, S_i-, S_t-, and S_z-RNase) have been cloned and sequenced in Japan (Sassa et al., 1996; Matsumoto et al., 1999a, 2000, 2001a, b; Matsumoto and Kitahara, 2000; Kitahara et al., 2000; Kitahara and Masumoto, 2002a, b), as have 11 S-RNases (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) in Europe (Broothaerts et al., 1995; Janssens et al., 1995; Verdoodt et al., 1998; Schneider et al., 2001; van Nerum et al., 2001). However, no molecular investigations have yet been reported for the S-genotypes of Korean cultivars.

The objective of this study was to identify the self-incom-

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patible genotypes of eight Korean apple cultivars and their six parents by PCR, using primers (ASPF3+ASPR3) common to 23 S-alleles, plus sequence analysis. We also developed an S-RNase analysis method that involved S-allele-specific PCR primers designed from the intron, variable region, and the molecular typing system of S-haplotypes. This protocol was based on Southern blots with seven S-allele-specific probes.

MATERIALS AND METHODS

Plant Materials

Eight Korean apple cultivars bred at the National Horticulture Research Institute in Korea and their six parents were used here (Table 1). Their leaves were collected and stored at -80°C.

DNA Isolation and PCR

Genomic DNA was isolated with the DNeasy Plant Mini Kit (QIAGEN, Germany), and used as template for PCR amplification. We designed common primers for PCR analysis based on the conserved sequences of 23 apple S-RNases (Table 2) (Matsumoto et al., 1999a, 2001, 2003; Broothaets, 2003). The basic reaction mixture contained 2.5 units of *Taq* DNA polymerase (TaKaRa, Japan), 2.5 μL of 10× buffer [Component: 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$], 2.5 mM each of dNTP, 200 nM each of primers, and 50 ng of template DNA. PCR reactions, in an ASTEC PCR Thermal Cycler (PC-320; Astec, Japan), comprised an initial denaturing at 94°C for 3 min; then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min; with a final extension at 72°C for 5 min.

Sequencing Analysis of S-RNases

Amplified PCR products were purified with a Geneclean Kit (BIO 101, USA) and subcloned using the pGEM-T Easy vector with TA cloning kits (Promega, USA). The nucleotide sequences of several clones were determined with an ABI PRISM 377 DNA sequencer (Applied Biosystems, USA) and a DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences, USA). DNA sequences were aligned manually via GENETYX-WIN 5.0 (GENETYX, Japan), and then analyzed using the BLAST and CLUSTALW programs to examine their homologies with other nucleotide sequences.

Table 2. Nucleotide sequences of primers used in this study.

Primer Name	Forward/ Reverse	Primer sequences
ASPF1	Forward	5′-GATCCTMKGAMAARTTG-3′
ASPF2	Forward	5′-TTTACBGTTCAYGGDTTGTGG-3′
ASPF3	Forward	5′-CAATTTACGCAGCARTATCAG-3′
ASPFN	Forward	5′-AARTTGTTTACBGTTCA-3′
ASPR1	Reverse	5′-CGTTBGGCCAAATHATTDCCA-3′
ASPR2	Reverse	5′-GTACATTYYGAYTACTGT-3′
ASPR3	Reverse	5′-CAAAGASHGACCTCAACYAATTC-3′
ASPRN	Reverse	5′-TGTACATTYYGAYTACWGT-3′

S-Allele-Specific PCR Analysis

DNA sequences of allele-specific primer pairs for the S_3- and S_5-RNases were designed from the variable regions (Table 3). The PCR reaction was performed as described above, except that we used annealing temperatures of 57°C for S_3-RNase and 58°C for S_5-RNase.

Southern Blot Analysis of PCR Products

Southern blot analysis was performed according to the DIG application protocol (Roche Diagnostics, Germany) and as described by Tateishi et al. (2002) and Kim et al. (2006). After electrophoresis on an 1% agarose gel, the PCR prod-

Table 1. Apple cultivars collected from the National Horticulture Research Institute in Korea.

Acc. No.	Cultivar	Parents	Expected S-genotype
1	Hongro	Spur Early Blaze (S?S?)[z] × Spur Golden Delicious (S?S?)	S?S?
2	Gamhong	Spur Early Blaze (S?S?) × Spur Golden Delicious (S?S?)	S?S?
3	Saenara	Spur Early Blaze (S?S?) × Spur Golden Delicious (S?S?)	S?S?
4	Chukwang	Fuji (S_1S_9) × Mollie's Delicious (S?S?)	S_1S?, S_9S?
5	Hwahong	Fuji (S_1S_9) × Sekaiichi (S_3S_9)	S_1S_3, S_3S_9
6	Seokwang	Mollie's Delicious (S?S?) × Gala (S_2S_5)	S_2S?, S_5S?
7	Hwarang	Fuji (S_1S_9) (Mutant)	S_1S_9
8	Sunhong	Hongro (S?S?) × Chukwang (S?S?)	S?S?

[z] The S-genotype is unclear.

Table 3. Nucleotide sequences of S-allele-specific primers for S_3- and S_5-RNases.

Type of S gene	Name	Forward /Reverse	Primer sequences	Annealing temperature (°C)	Estimated size of PCR product (bp)
S_3-RNase	AS3MPF	Forward	5′-GTACCCATTAATTTTCAATTC-3′	57	1445
	APR3	Reverse	5′-CAAAGASHGACCTCAACYAATTC-3′		
S_5-RNase	AS5MPF	Forward	5′-GAGTCAGTTCCATAATTTC-3′	58	1380
	APR3	Reverse	5′-CAAAGASHGACCTCAACYAATTC-3′		

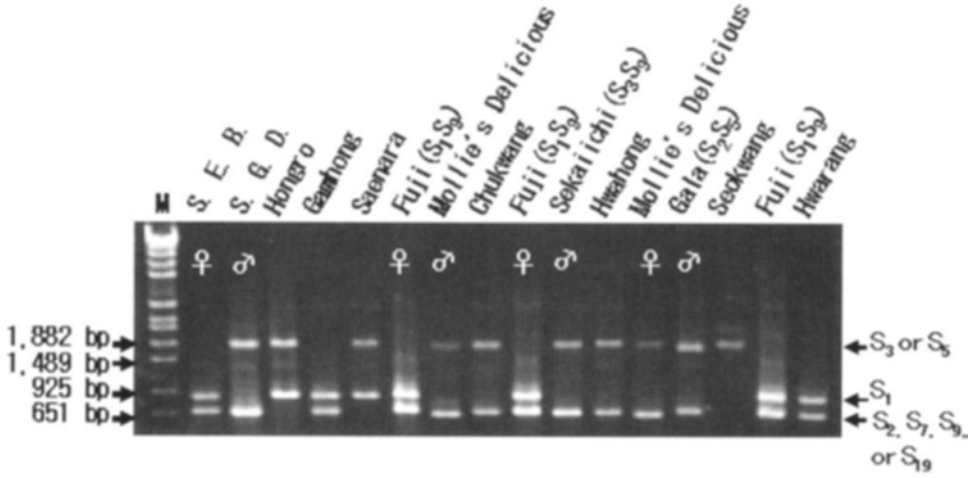

Figure 1. Electrophoretic separation of S-RNase fragments obtained from apple cultivars by PCR with common primers (ASPF3 + ASPR3). M, λ-*Eco*T14I/*Bgl*II digests. 'Hongro' (S_1S_3), 'Gamhong' (S_1S_9), and 'Saenara' (S_1S_3) were derived from cross between 'S.E.B.' (S_1S_{19}) and 'S.G.D.' (S_2S_3). 'Chukwang' (S_3S_9) was derived from cross between 'Fuji' (S_1S_9) and 'Mollie's Delicious' (S_3S_7), 'Hwahong' (S_3S_9) from cross between 'Fuji' (S_1S_9) and 'Sekaiichi' (S_3S_9), 'Seokwang' (S_3S_5) from cross between 'Mollie's Delicious' (S_3S_7) and 'Gala' (S_2S_5), and 'Hwarang' (S_1S_9) from bud mutant of 'Fuji' (S_1S_9) cultivar.

ucts were transferred onto a Hybond™–N⁺ nylon membrane (Amersham, UK) and probed with specific fragments of the S_1-, S_2-, S_3-, S_7-, S_9-, and S_{19}-RNases. The probe was labeled by the PCR-labeling method with DIG-dUTP (Roche Diagnostics, Germany), using S-RNase-specific primers (ASPF3 +ASPR3). Membranes were hybridized for 16 h at either 50 or 60°C under low- or high-stringency conditions, with DIG-labeled probes in 5x SSC containing 1% (w/v) blocking reagent, 0.1% (w/v) sodium N-lauroryl sarcosinate, and 0.02% (w/v) SDS. Afterward, the membranes were washed twice in 2x SSC, 0.1% SDS (w/v) for 5 min at room temperature, followed by two washing steps in 0.1x SSC, 0.1% SDS (w/v) for 15 min either at 50 or 60°C, under low- or high-stringency conditions. Hybridization products were detected by NBT/BCIP, according to the DIG kit protocol.

RESULTS AND DISCUSSION

Determination of Self-Incompatibility Genotypes for Korean Apple Cultivars

Until now, efficient identification of a self-incompatibility genotype in apple has required that PCR amplification be performed mostly with S-allele-specific primers because that species has many S-alleles with various nucleotide sequences (Matsumoto et al., 1999, 2000, 2001a, 2001b, 2003; Kitahara et al., 2000; Matsumoto and Kitahara, 2000; Kitahara and Matsumoto, 2002a, b; Broothaerts, 2003). Although such identifications are possible by this method, those of new S-RNase types are difficult. Thus, the primer combinations of four forward primers (ASPF1, ASPF2, ASPF3, and ASPFN) and four reverse primers (ASPR1, ASPR2, ASPR3, and ASPRN) were designed based on the conserved sequences of 23 S-RNases in apple (Table 2). The most useful combination was ASPF3 and ASPR3.

Using this primer pair, two S-RNase fragments were amplified from each of the 14 apple cultivars, but only one band, i.e., from 'Seokwang', was revealed (Fig. 1). Sizes of the amplified S-RNase fragments were segregated into three groups: 1600 bp in S_3 and S_5, 850 bp in S_1, and 630 bp in S_2, S_7, S_9, and S_{19}. The known S-genotypes of two apple cultivars -- 'Hwahong' (S_3S_9) and 'Hwarang' (S_1S_9) – also were verified by this experiment, but those of the other cultivars could not be because the S-genotypes of their parents have yet to be determined. Furthermore, two S-RNase fragments amplified by PCR were of the same size and showed only one band (i.e., S_3- and S_5-RNase). In all, the amplified S-RNase fragments of 14 cultivars were cloned and sequenced, with seven S-alleles being identified and the nucleotide sequences of their exon and intron regions being determined (Table 4; Fig. 2). The S_3-('Saenara') and S_5-RNase ('Seokwang') introns -- 1290 bp and 1156 bp, respectively – were larger than the other S-RNase introns. For example, the 843 bp S_1-RNase ('Hwarang') had an intron of 343 bp, while the 633 bp S_{19}-RNase ('Super Early Blaze') had a 169 bp intron.

Table 4. Expected sizes of PCR fragments and their introns (b).

S-allele	PCR fragment	Intron	Cultivar
S_1	843	343	Hwarang
S_2	638	147	Spur Golden Delicious
S_3	1781	1290	Saenara
S_5	1650	1156	Seokwang
S_7	609	118	Mollie's Delicious
S_9	635	144	Chukwang
S_{19}	633	169	Spur Early Blaze

The exon regions of the seven S-RNases revealed high homology, ranging from 78% (S_1 and S_2, S_2 and S_5) to 85% (S_7 and S_9) (Fig. 2). In contrast, low homology (28%) was seen with the intron regions of S_1 and S_7 compared with a high homology (89.2%) for the S_2- and S_9-RNase introns. Therefore, based on these PCR and sequence analyses data, we determined the self-incompatibility genotypes of eight

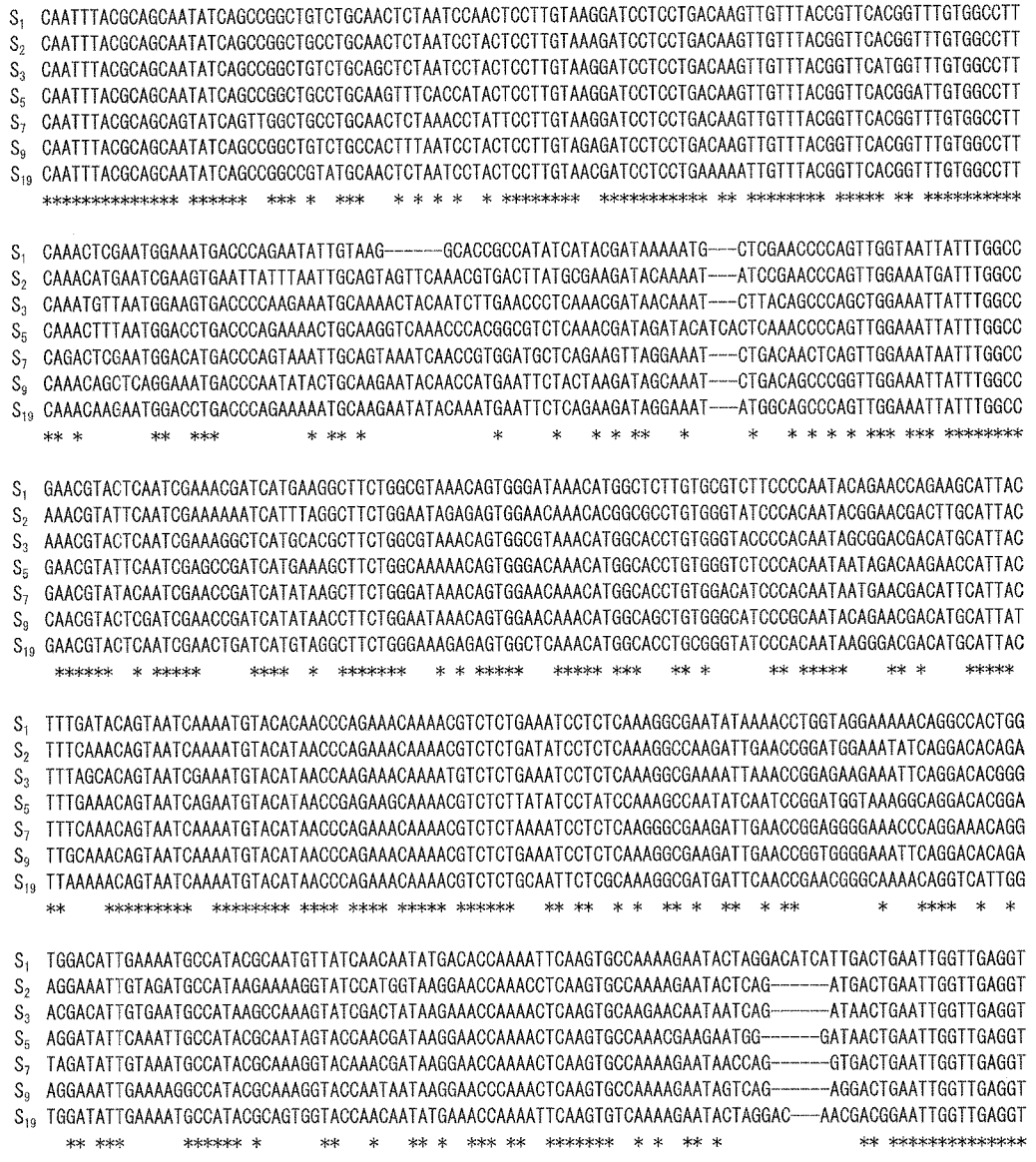

Figure 2. Alignment of nucleotide sequences in exon regions for S_1-, S_2-, S_3-, S_5-, S_7-, S_9-, and S_{19}-RNase fragments amplified from 7 apple cultivars (Table 4) by PCR, using ASPF3 + ASPR3 primers. Nucleotide sequences conserved in all RNases are indicated with asterisks. Gaps are marked by dashes.

Table 5. Determination of S-genotypes by PCR and sequence analyses.

Cultivar	Parents	S-genotype
Hongro	Spur Early Blaze × Spur Golden Delicious	S_1S_3
Gamhong	Spur Early Blaze × Spur Golden Delicious	S_1S_9
Saenara	Spur Early Blaze × Spur Golden Delicious	S_1S_3
Chukwang	Fuji (S_1S_9) × Mollie's Delicious	S_3S_9
Hwahong	Fuji (S_1S_9) × Sekaiichi (S_3S_9)	S_3S_9
Seokwang	Mollie's Delicious × Gala (S_2S_5)	S_3S_5
Hwarang	Fuji (S_1S_9) (Mutant)	S_1S_9
Sunhong	Hongro × Chukwang	S_3S_9
Spur Early Blaze (S. E. B.)		S_1S_{19}
Spur Golden Delicious (S. G. D.)		S_2S_3
Mollie's Delicious		S_3S_7

Korean-bred apple cultivars and their six parents to be the following (Table 5): 'Hongro' (S_1S_3), 'Gamhong' (S_1S_9), 'Saenara' (S_1S_3), 'Chukwang' (S_3S_9), 'Hwahong' (S_3S_9), 'Seokwang' (S_3S_5), 'Hwarang' (S_1S_9), 'Sunhong' (S_3S_9), 'S.E.B.'

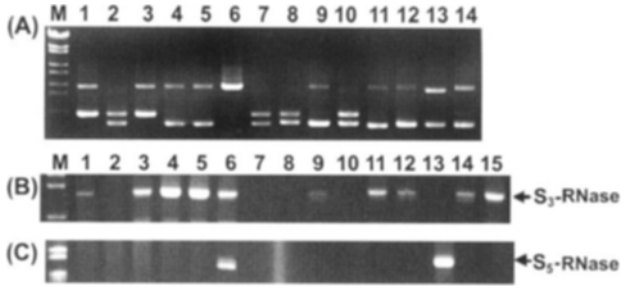

Figure 3. (**A**) S-RNase fragments amplified by PCR with common primers (ASPF3 + ASPR3). M, 1-kb ladder; 1, 'Hongro' (S_1S_3); 2, 'Gamhong' (S_1S_9); 3, 'Saenara' (S_1S_3); 4, 'Chukwang' (S_3S_9); 5, 'Hwahong' (S_3S_9); 6, 'Seokwang' (S_3S_5); 7, 'Hwarang' (S_1S_9); 8, 'S.E.B.' (S_1S_{19}); 9, 'S.G.D.' (S_2S_3); 10, 'Fuji' (S_1S_9); 11, 'Mollie's Delicious' (S_3S_7); 12, 'Sekaiichi' (S_3S_9); 13, 'Gala' (S_2S_5); 14, 'G.D.' (S_2S_3); and 15, 'Sunhong' (S_3S_9). Electrophoretic separation of fragments for S_3-RNases (**B**) and S_5-RNases (**C**) amplified by PCR with S-allele-specific primers.

(S_1S_{19}), 'S.G.D.' (S_2S_3), and 'Mollie's Delicious' (S_3S_7).

The S-genotype of 'Gamhong' was determined as 'S_1S_9', even though this cultivar originated from the cross between 'S.E.B.' (S_1S_{19}) and 'S.G.D' (S_2S_3). We then repeated our PCR analysis of S-RNases using newly collected leaf samples and re-confirmed that result. It is probable that the pollen parent of 'Gamhong' is 'Fuji' (S_1S_9) because 'Fuji' contains S_9-RNase and was often used in 1981 as a parent for cross breeding in Korea.

Matsumoto et al. (1999b) have identified the S-genotypes of 13 apple cultivars and 10 progenies of 'Megumi' by S-allele-specific PCR-restriction fragment length polymorphism (RFLP) analysis. They have reported that the S-genotypes of 'Kinsei' (S_2S_9) and 'Kizashi' (S_2S_3) are inconsistent with the S-genotypes expected from their parents ('Golden Delicious' (S_2S_3) × 'Ralls Janet' (S_1S_2), and 'Gala' (S_2S_5) × 'Fuji' (S_1S_9), respectively), indicating that they have other pollen parents with the S_9- or S_3-allele. Matsumoto et al. (1999b) have also suggested that, although the pollen parents of 'Akagi', 'Tsugaru', and 'Youkou' are still unknown, those with the S_7-, S_7-, and S_9-allele may have been used in their respective production.

Confirming S-RNase Types by S-Allele-Specific PCR and Southern Blot Analysis

In our PCR-RFLP analysis, the S_3- and S_5-RNase fragments were of the same size, and the fragments of 'Seokwang' (S_3S_5) showed only one band (Fig. 3A, Lane 6). Therefore, new S_3- and S_5-allele-specific primers were designed to confirm the presence of those S-RNases (Table 3). The S_3-RNase-specific fragments from six Korean apple cultivars -- 'Hongro' (S_1S_3), 'Saenara' (S_1S_3), 'Chukwang' (S_3S_9), 'Hwahong' (S_3S_9), 'Seokwang' (S_3S_5), and 'Sunhong' (S_3S_9) (Fig. 3B) -- and the S_5-RNase-specific fragments from 'Seokwang' (S_3S_5) and 'Gala' (S_2S_5) were amplified here (Fig. 3C). For 'Seokwang' (Lane 6), amplified with the common primers, the S-RNase fragments showed one band, but the S_3- and S_5-RNase fragments were amplified independently by using S-allele-specific primers.

Broothaerts (2003) has designed 15 S-allele-specific primer

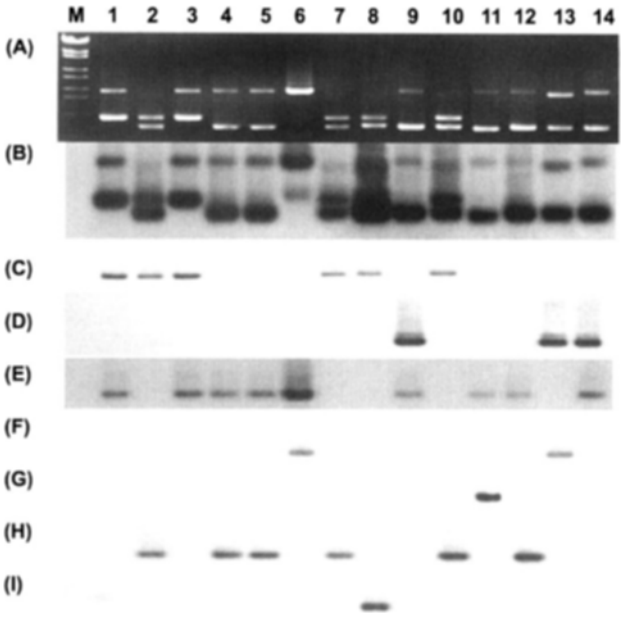

Figure 4. PCR Southern blot analysis of S-RNases in 14 apple cultivars. (**A**), PCR S-RNases were amplified by PCR with common primers. (**B**), Pre-hybridization and hybridization with S_1-RNase probe at 50°C. Hybridization at 57°C with S_1-RNase probe (**C**), S_2-RNase probe (**D**), S_3-RNase probe (**E**), S_5-RNase probe (**F**), S_7-RNase probe (**G**), S_9-RNase probe (**H**), and S_{19}-RNase probe (**I**). M, λ*Bst*PI; 1, 'Hongro' (S_1S_3); 2, 'Gamhong' (S_1S_9); 3, 'Saenara' (S_1S_3); 4, 'Chukwang' (S_3S_9); 5, 'Hwahong' (S_3S_9); 6, 'Seokwang' (S_3S_5); 7, 'Hwarang' (S_1S_9); 8, 'S.E.B.' (S_1S_{19}); 9, 'S.G.D.' (S_2S_3); 10, 'Fuji' (S_1S_9); 11, 'Mollie's Delicious' (S_3S_7); 12, 'Sekaiichi' (S_3S_9); 13, 'Gala' (S_2S_5); and 14, 'G.D.' (S_2S_3).

pairs based on an alignment of cDNA nucleotide sequences of the S-allele. Homology of nucleotide sequences among those alleles varies considerably, with the S_1 and S_2 sequences showing lower homology (about 70%), S_3 and S_{10} being >90%, and over 95% between S_{20} and S_{24}. The synthesis of S-allele-specific primers within such high-homology regions is difficult, and a single S-allele cannot be selectively amplified. Thus, we re-designed the S_3- and S_5-allele-specific forward primers based on the hypervariable intron region.

We also performed PCR Southern blot analysis to examine whether the amplified PCR fragments were S-RNase-specific (Fig. 4). Under high-stringency conditions at 57°C, cultivars with the S_1-, S_2-, S_3-, S_5-, S_7-, S_9-, and S_{19}-haplotypes yielded hybridization signals with the S_1-, S_2-, S_3-, S_5-, S_7-, S_9-, and S_{19}-RNases probes, respectively (Fig. 4C-I). In contrast, under low-stringency conditions at 50°C, all 14 examined cultivars produced S-haplotype-specific bands corresponding to seven S-haplotypes (Fig. 4B). This result was the same as those obtained by PCR and sequence analyses. Because the S-genotypes of our 14 cultivars were also confirmed by PCR-Southern blot analysis, we are now able to type the S-haplotype with the S-RNase probe, not only in Korean apple cultivars, but in others as well.

In conclusion, we have determined the self-incompatibility genotypes of eight Korean apple cultivars, using newly developed molecular markers (common primers and S-allele-specific primers), based on PCR and sequencing

analyses. Furthermore, we have confirmed the characteristics of S-genotypes for 14 cultivars by PCR-Southern blot analysis, with S_1-, S_2-, S_3-, S_5-, S_7-, S_9-, and S_{19}-RNases serving as probes. Therefore, we propose that these molecular techniques can be applied to the selection of pollinator trees and to the identification of self-incompatibility genotypes of the progeny gained through new breeding programs.

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