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Determination of partial genomic sequences and development of a CAPS system of the *S-RNase* gene for the identification of 22 *S* haplotypes of apple (*Malus* × *domestica* Borkh.)

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Abstract Information about self-incompatibility (*S*) genotypes of apple cultivars is important for the selection of pollen donors for fruit production and breeding. Although *S* genotyping systems using *S* haplotype-specific PCR of *S-RNase*, the pistil *S* gene, are useful, they are sometimes associated with false-positive/negative problems and are unable to identify new *S* haplotypes. The CAPS (cleaved amplified polymorphic sequences) system is expected to overcome these problems, however, the genomic sequences needed to establish this system are not available for many *S-RNases*. Here, we determined partial genomic sequences of eight *S-RNase*s, and used the information to design new primer and to select 17 restriction enzymes for the discrimination of 22 *S-RNases* by CAPS. Using the system, the *S* genotypes of three cultivars were determined. The genomic sequence-based CAPS system would be useful for *S* genotyping and analyzing new *S* haplotypes of apple.

Keywords Apple · CAPS · *S* genotype · Self-incompatibility · S-RNase

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Abbreviations

GSI	Gametophytic self-incompatibility
PCR	Polymerase chain reaction
RFLP	Restriction fragment length polymorphism
CAPS	Cleaved amplified polymorphic sequences

Introduction

Apple (*Malus* × *domestica* Borkh.), belonging to the family Rosaceae, exhibits gametophytic self-incompatibility (GSI) which prevents inbreeding and promotes out crossing. The GSI of Rosaceae is controlled by a single *S* locus, which has multiple haplotypes and encodes a ribonuclease, called S-RNase, for pistil-part specificity (de Nettancourt 1977, 2001). Pollen tube growth is inhibited and fertilization does not take place when the pollen *S* haplotype matches one of those of the pistil. For this reason, apple trees are planted with other compatible cultivars including crab apples in orchards, in order to make sure the fruit sets. Identification of the *S* genotypes is thus important for the selection of appropriate pollen donors in fruit production, as well as in breeding programs.

Analyzes of pollen tube growth in the style, as well as fruit and seed formation, have revealed 11 different *S* haplotypes (S^1 to S^{11}) (Kobel et al. 1939). Komori et al. (1999, 2000) have investigated incompatibility relationships among Japanese apple cultivars by pollination test, assigned 10 *S* haplotypes, S^a to S^i and S^z, and reported their correspondence to four of the *S* haplotypes described by Kobel et al. (1939).

The S^a- to S^f-RNases of apple were identified by protein analyzes, isoelectric focusing (IEF) or two-dimensional polyacrylamide gel electrophoresis (Sassa et al. 1994, 1996). *S* haplotype typing of fifty-six cultivars of apple has been carried out by separation of stylar protein extracts using IEF or non-equilibrium pH gradient electrophoresis followed by staining for ribonuclease activity (Bošković and Tobutt 1999). Although these protein-based analyzes are advantageous over pollination-based genotyping as they are not affected by field conditions, they require floral organs, limiting their applicability.

Cloning of the *S-RNase* genes enabled the development of PCR-based analyzes of the sequence polymorphisms of the *S-RNase*s for *S* genotyping. Several genotyping methods which use *S* haplotype-specific primer pairs have been reported to date (Janssens et al. 1995; Verdoodt et al. 1998; Matsumoto et al. 1999a, b, 2003a, b, 2006; Kitahara et al. 2000; Broothaerts 2003; Ershadi and Talaii 2007). The primer pairs specific to respective *S-RNase*s are usually designed based on alignments of cDNA sequences of the *S-RNase*s of apple (Broothaerts 2003). However, specific primer-based methods are sometimes associated with false-negative results. Low quality and/or quantity DNA samples would lead to mistyping; absence of the haplotype. False positive problems have also been reported for specific primer-based methods. The primers developed for the specific amplification of the S^{24} haplotype by Verdoodt et al. (1998) also amplified the S^{20} haplotype of 'Braeburn' and 'Worcester Pearmain' (Ershadi and Talaii 2007). Furthermore, primers designed to amplify the $S^{16a=27a}$ haplotype in 'Baskatong' amplified the S^4, $S^{16b=19}$ and S^{23} haplotypes in 'Champagne Reinette', 'Alkmene' and 'Delbard Jubilee', respectively (Ershadi and Talaii 2007). Matsumoto et al. (2000) reported that the S^{21} haplotype seemed to correspond to the $S^{30=t}$ haplotype because a PCR product very close to 259 bp was obtained from '*Malus transitoria*' ($S^{20}S^{30=t}$) and 'Ribston Pippin' ($S^1S^9S^{21}$) using the $S^{30=t}$ haplotype-specific primers. Broothaerts (2003) renumbered the S^{28} haplotype as $S^{6b=19}$ based on the amplification of a S^{19}-'specific' PCR product from Bohnapfel ($S^9S^{16}S^{19}$). However, the deduced amino acid sequence of the $S^{6b=19}$ haplotype was different from that of the S^{28} haplotype (Matsumoto et al. 2003a).

Another PCR-based approach, CAPS (cleaved amplified polymorphic sequence; Konieczny and Ausubel 1993)/PCR-RFLP, is expected to solve the false-negative/positive problems associated with haplotype-specific primer-based methods. Low-quality/quantity DNA samples can be readily detected, as the CAPS system simultaneously amplifies all the haplotypes followed by the detection of a specific sequence by restriction digestion. It is also more tolerant of false-positives as the detection of a specific target is dependent on restriction digestion instead of PCR amplification which is usually more vulnerable to experimental conditions than restriction digestion. One difficulty with the CAPS system is to design a primer pair that can simultaneously and equally amplify all the highly diverged target sequences such as the *S-RNase*s. In Japanese pear

(*Pyrus pyrifolia*), a relative of apple, the identification of new *S* haplotypes has been required to update primer pairs for CAPS-based *S* genotyping (Kim et al. 2007). Another problem when establishing a CAPS system is the selection of restriction enzymes for the detection of specific sequences. Although more than 20 *S* haplotypes have been reported in apple to date, available genomic sequences of the *S-RNase*s are limited, making it difficult to select *S*-specific restriction enzymes.

In this study, we first tested the effectiveness of different primer pairs for CAPS, and designed a new pair, ASPF3 and ASPR3S. We then determined the nucleotide sequences of eight *S-RNase* fragments amplified with ASPF3 and ASPR3S, and selected 17 restriction enzymes based on the sequences to identify haplotype-specific fragments for the discrimination of 25 *S-RNases*. The newly developed CAPS system effectively identified all the available 22 *S* haplotypes of 17 apple cultivars with known *S* genotypes. Using the system, the *S* genotypes of three Japanese apple cultivars ('HAC6', 'Takane' and 'Maoi') were determined.

Materials and methods

Plant materials and isolation of genomic DNA

Young leaves of 16 apple cultivars with known *S* genotypes and three uncharacterized cultivars, 'Maoi' ('Mantet' (S^7S^{25}) × 'HAC6'), 'HAC6' ('Golden Delicious' (S^2S^3) × 'Empire' ($S^{10}S^{28}$)), 'Takane' (Natural cross seedling of 'Red Gold' (S^2S^9), Triploid), were collected at the Department of Apple Research, National Institute of Fruit Tree Science (NIFTS), Morioka, Japan. The leaves were frozen in liquid nitrogen immediately after harvest, and stored at −80°C prior to use. Genomic DNA was isolated from leaves based on the method described by Sassa (2007) and used for PCR amplification.

PCR analysis

PCR amplification was conducted using the four *S-RNase*-specific primer combination, FTQQYQ and anti-$^{I}/_{T}$IWPNV (Takasaki et al. 2004), FTQQYQ and anti-$^{I}/_{M}$IWPNV (Matsumoto and Kitahara 2000), ASPF3 and ASPR3 (Kim et al. 2006), and ASPF3 and ASPR3S (this study; 5′ CAAAGASHGACCTC AACYAATTS 3′). PCR conditions were as described previously (Kim et al. 2006).

Cloning and sequencing of *S-RNases*

By using the ASPF3 and ASPR3S primers, the amplified PCR products were cloned into the pT7Blue-T vector (Novagen, Madison, USA). DNA sequences of the inserts of several clones were determined by using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems) and the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Sequence data were analyzed with BioEdit ver 7.0 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and ClustalW (Thompson et al. 1994).

CAPS analysis

The *S-RNase* fragments amplified with ASPF3 and ASPR3S were digested with the haplotype-specific restriction enzymes *Mva*I, *Eco*RV, *Bcl*I, *Hin*dIII, *Bfm*I, *Sph*I, *Asp*A2I, *Bst*SNI, *Nco*I, *Afl*II, *Bbv*CI, *Bsm*I, *Bpl*, *Pci*I, *Bfu*AI, *Bpm*I or *Pml*I + *Bst*SNI. *Bsm*I and *Bfu*AI were incubated for 3 h at 65 and 50°C, respectively, and the other restriction enzymes were incubated for 3 h at 37°C. Expected fragment sizes are shown in Table 1. Digested fragments were separated on 2% agarose gels in TAE buffer and visualized by staining with ethidium bromide.

Results and discussion

S-RNase amplification analysis

We compared the three common primer pairs for amplifying the 22 *S-RNase* fragments from 17 apple cultivars; FTQQYQ and anti-$^{I}/_{T}$IWPNV (Takasaki et al. 2004), FTQQYQ and anti-$^{I}/_{M}$IWPNV (Matsumoto and Kitahara 2000), and ASPF3 and ASPR3 (Kim et al. 2006). For FTQQYQ and anti-$^{I}/_{T}$IWPNV, the S^2-, S^7-, S^{25}- and S^{32}-*RNase* fragments of expected sizes, 347, 318, 2,560 and 351 bp, respectively, were not amplified, and S^3-, S^9- and S^{11}-*RNase* fragments of 1,491, 344 and 372 bp, respectively, were amplified weakly (Fig. 1a). The S^7- and S^{25}-*RNase* fragments of expected sizes, 318 and 2,560 bp, respectively, were not amplified by

Table 1 Selection of *S* haplotype-specific restriction enzymes and estimated restriction fragment sizes (bp) of the 25 *S-RNases*

S haplotype	PCR fragment	*Mva* I	*Eco* RV	*Bcl* I	*Hind* III	*Bfm* I	*Sph* I	*Asp* A2I	*Bst* SNI	*Nco* I	*Afl* II	*Bbv* CI	*Bsm* I	*Bpl* I	*Pci* I	*Bfu*AI*	*Bpm* I	*Pml* I+*Bst* SNI
$S^1 = S^1$	844	*712/132*	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
$S^2 = S^a$	647	–	*495/152*	–	–	–	–	–	–	576/71	–	–	–	–	–	–	–	–
$S^3 = S^b$	1790	–	–	*1230/560*	–	1760/30	899/891	–	–	–	–	–	1704/86	–	–	–	–	–
S^4	637	–	–	*470/167*	–	–	–	–	–	–	–	–	–	–	–	–	–	–
S^5	1659	–	–	–	*1384/275*	–	–	–	–	–	–	–	–	–	–	–	–	–
$S^{6b} = S^{17} = S^{19}$	670	–	–	–	–	*533/137*	–	–	–	–	–	–	–	–	–	–	–	–
$S^7 = S^d$	618	505/82/31	–	–	*343/275*	–	–	–	–	–	–	–	–	–	–	–	–	–
$S^9 = S^c$	644	–	–	–	–	–	*408/236*	–	–	–	–	–	–	–	–	–	–	–
$S^{10} = S^i$	2218	–	–	–	–	–	–	*1288/930*	–	1061/931/226	–	–	–	–	–	–	1333/885	–
$S^{11} = S^{13} = S^{14}$	672	–	–	–	–	–	–	–	*590/82*	–	–	–	–	–	–	–	–	329/261/82
$S^{16a} = S^{27a}$	3110	–	*2116/994*	–	–	–	–	–	–	2780/330	–	–	–	–	2690/420	–	–	–
$S^{16b} = S^{22} = S^{27b}$	2627	–	–	–	–	–	–	–	–	*2307/320*	–	–	–	–	1999/628	–	–	–
S^{16c**}	2218	–	–	–	–	–	–	–	–	*331/2384*	–	–	–	–	2014/204	–	–	–
$S^{20} = S^g$	818	–	–	–	–	–	–	–	–	–	*522/296*	–	–	–	–	–	–	–
S^{21}	674	–	–	–	–	–	*348/326*	–	–	–	–	–	–	–	–	–	–	–
$S^{23} = S^{10b}$	647	–	–	–	–	–	–	–	–	–	–	*495/152*	–	–	–	(500/147)	–	–
$S^{24} = S^h$	839	–	–	–	–	–	–	–	*751/88*	–	–	–	–	–	–	–	–	751/88
$S^{25} = S^x$	2860	*1272/818/508/262*	–	–	–	–	–	–	–	1860/1000	2008/52	–	–	–	1000/1860	–	2076/784	2629/231
S^{26}	659	–	–	–	–	–	–	–	–	–	–	–	*437/222*	–	–	–	–	–
$S^{28} = S^e$	672	–	–	385/287	–	–	–	–	–	–	–	–	–	*399/241/32*	–	(437/235)	–	–

Table 1 continued

S haplotype	PCR fragment	*Mva* I	*Eco* RV	*Bcl* I	*Hind* III	*Bfm* I	*Sph* I	*Asp* A2I	*Bst* SNI	*Nco* I	*Afl* II	*Bbv* CI	*Bsm* I	*Bpl* I	*Pci* I	*Bfu*AI*	*Bpm* I	*Pml* I+*Bst* SNI
S^{29**}	726	–	–	–	–	–	–	–	*644/82*	–	–	–	–	–	–	–	–	–
S^{30**}	677	–	–	–	–	–	–	–	–	–	–	–	–	–	*262/451*	–	–	–
S^{31}	777	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*432/333/12*	–	–
S^{32}	651	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*403/246/2*	–
S^{kb}	674	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	*411/263*

Italics: Unambiguously identifiable *S* haplotype-specific bands

* *Bfu*AI is not suitable for detecting S^{23} and S^{28} haplotypes, because at least two copies of the *Bfu*AI site are required for efficient cleavage

** These enzymes were not used in this study because of the unavailability of plant materials

The DDBJ/EMBL/GenBank accession numbers are S^{f} (D50837), S^{2} (U12199), S^{3} (U12200), S^{4} (AF327223), S^{5} (U19791), S^{17} (AB105062), S^{7} (U19792), S^{9} (U19793), S^{i} (AB052683), S^{13} (AB105060), S^{27a} (AF016919), S^{27b} (AF327222), S^{16c} (AB126322), S^{g} (AB096138), S^{21} (AB094494), S^{10b} (AF239809), S^{24} (AF016920), S^{x} (AB062100), S^{26} (AF016918), S^{28} (AF201748), S^{29} (AY039702), S^{30} (AB035928), S^{31} (DQ135990), S^{32} (DQ135991) and S^{kb} (EU443101)

FTQQYQ and anti-$^{I}/_{M}$IWPNV (Fig. 1b). Using the same primers, Matsumoto and Kitahara (2000) did observe the amplification of S^{7}-*RNase*, probably because of some differences in PCR conditions. Faint bands of S^{3}-, S^{9}- and S^{11}-*RNase* were observed at a size of 1,491, 344 and 372 bp, respectively.

ASPF3 and ASPR3 amplified 21 *S-RNase* fragments but not S^{kb}-*RNase* (DDBJ/GenBank/EMBL accession number EU443101; data not shown), probably because the ASPR3 (5′ CAAAGASHGACC TCAACYAATTC 3′) has a mismatch at the 3′ end with the corresponding region of the S^{kb}-*RNase* (5′ CA ATTGGTTGAGATCACTCTTTG 3′). Therefore, we modified ASPR3 to 'ASPR3S' (5′ CAAAGASHGA CCTCAACYAATTS 3′). The ASPF3 and ASPR3S primers showed amplification of all 22 *S-RNase* fragments with expected sizes from 17 apple cultivars (Fig. 1c). Based on the lengths of the amplified fragments, the 22 *S-RNases* were separated into four groups: group I, 618–674 bp including 12 *S-RNases* (S^{2}, S^{4}, S^{6b}, S^{7}, S^{9}, S^{11}, S^{21}, S^{23}, S^{26}, S^{28}, S^{32} and S^{kb}); group II, 777–844 bp including 4 *S-RNases* (S^{1}, S^{20}, S^{24} and S^{31}); group III, ca. 1,700 bp including 2 *S-RNases* (S^{3} and S^{5}); and group IV, 2,218–3,110 bp including 4 *S-RNases* (S^{10}, S^{16a}, S^{16b} and S^{25}) (for genomic sequences including introns, see below).

Determination of partial genomic sequences of eight *S-RNases* and selection of *S* haplotype-specific enzymes

For selection of the *S* haplotype-specific restriction enzymes for CAPS, sequence data for all the amplicons are needed. However, intron-containing genomic sequence data are not available for many apple *S-RNase*s. To select restriction enzymes based on the target sequence data, PCR fragments amplified by the ASPF3 and ASPR3S primers were cloned and sequenced for eight *S-RNases*. The sequences of the eight *S-RNases* (partial genomic DNA) were registered in DDBJ/GenBank/EMBL; S^{3}-*RNase* (AB428425), S^{4}-*RNase* (AB428426), S^{5}-*RNase* (AB428427), S^{10}-*RNase* (AB428428), S^{16a}-*RNase* (AB428429), S^{16b}-*RNase* (AB428430), S^{25}-*RNase* (AB428431) and S^{26}-*RNase* (AB428432). Genomic sequences have been reported for 17 *S-RNases* (Kim et al. (2008) (S^{31}, S^{32}), Matsumoto et al. (1999a) (S^{1}, S^{20}), Matsumoto et al. (1999b) (S^{2}, S^{9}), Matsumoto et al. (2001) (S^{7}, S^{24}), Matsumoto et al. (2003a) (S^{6b}, S^{11}, S^{21},

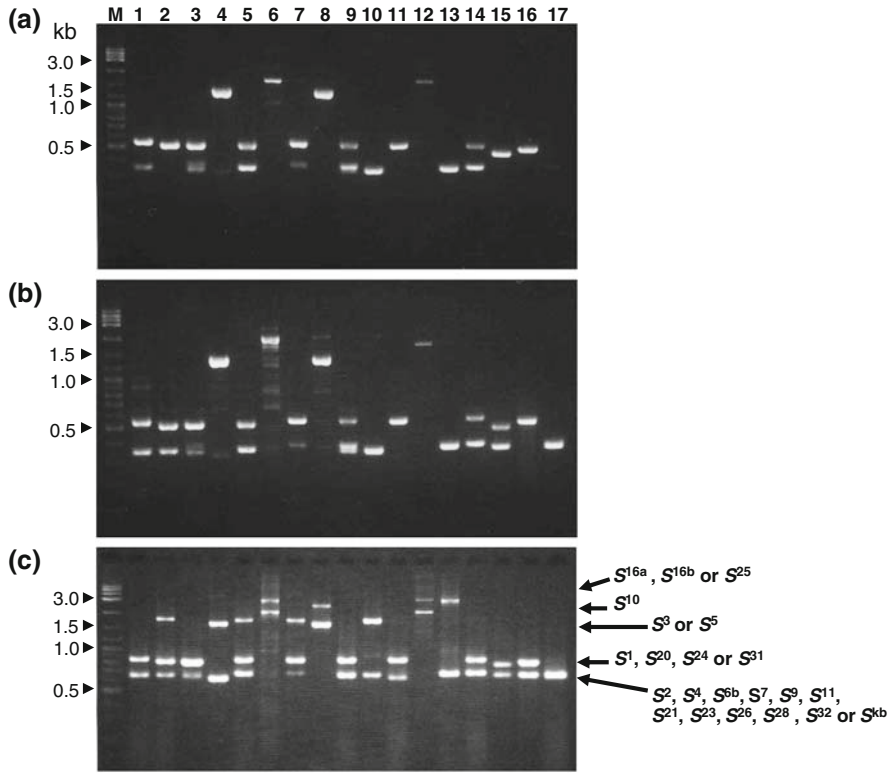

Fig. 1 Comparison of amplification of *S-RNase* fragments using different primer pairs. The *S-RNase* fragments of different *S* genotypes were amplified by PCR using primers **a** FTQQYQ and anti-(I/T)IWPNV, **b** FTQQYQ and anti-(I/M)IWPNV and **c** ASPF3 and ASPR3. The cultivars examined were Lane 1, Fuji (S^1S^9); 2, Mutsu ($S^2S^3S^{20}$); 3, Gravenstein ($S^4S^{11}S^{20}$); 4, Sansa (S^5S^7); 5, Blenheim Orange ($S^1S^3S^{6b}$); 6, Maypole ($S^{10}S^{16a}$); 7, Jacques Lebel ($S^1S^3S^{11}$); 8, Alkmene (S^5S^{16b}); 9, Ribston Pippin ($S^1S^9S^{21}$); 10, Granny Smith (S^3S^{23}); 11, Akane (S^7S^{24}); 12, McIntosh ($S^{10}S^{25}$); 13, Baskatong ($S^{16a}S^{26}$); 14, Winesap (S^1S^{28}); 15, York Imperial (S^2S^{31}); 16, Burgundy ($S^{20}S^{32}$) and 17, Maoi (S^2S^{kb}, this study)

S^{28}, S^{30}), Matsumoto and Furusawa (2005) (S^{16c}), Matityahu et al. (2005) (S^{29}), Schneider et al. (2001) (S^{23}) and EU443101 (S^{kb}).

Among the 22 *S-RNases*, S^7*-RNase* is the shortest (618 bp) while S^{16a}*-RNase* is the longest (3,110 bp). The lengths of the PCR fragments are as follows: S^7 (618 bp) < S^4 (637 bp) < S^9 (644 bp) < S^2 and S^{23} (647 bp) < S^{32} (651 bp) < S^{26} (659 bp) < S^{6b} (670 bp) < S^{11} and S^{28} (672 bp) < S^{21} = S^{kb} (674 bp) < S^{31} (777 bp) < S^{31} (777 bp) < S^{20} (818 bp) < S^{24} (839 bp) < S^1 (844 bp) < S^5 (1,659 bp) < S^3 (1,790 bp) < S^{10} (2,218 bp) < S^{16b} (2,627 bp) < S^{25} (2,860 bp) < S^{16a} (3,110 bp). Based on the sequence data of the 22 *S-RNases*, we selected 16 restriction enzymes, which are expected to generate haplotype-specific fragments for all 22 *S-RNases*; *Mva*I (S^1, S^7 and S^{25}), *Eco*RV (S^2 and S^{16a}), *Bcl*I (S^3, S^4 and S^{28}), *Hin*dIII (S^5 and S^7), *Bfm*I (S^3 and S^{6b}), *Sph*I (S^3, S^9 and S^{21}), *Asp*A2I (S^{10}), *Bst*SNI (S^{11} and S^{24}), *Nco*I (S^2, S^{10}, S^{16a}, and S^{16b}), *Afl*II (S^{20} and S^{25}), *Bbv*CI (S^{23}), *Bsm*I (S^3 and S^{26}), *Bpl*I (S^{28}), *Bfu*AI (S^{23}, S^{28} and S^{31}), *Bpm*I (S^{10}, S^{25} and S^{32}) and *Pml*I + *Bst*SNI (S^{11}, S^{24}, S^{25} and S^{kb}) (Table 1). Since genomic sequences including introns were not available for many apple *S-RNases*, *S*-specific restriction enzymes for those haplotypes had to be selected in a trial-and-error manner by referring to cDNA sequence data only. The intron-containing *S-RNase* genomic sequences determined in this study will be useful when a new *S*-specific enzyme is needed upon the identification of a new *S* haplotype.

In addition, specific restriction enzymes for the identification of S^{16c}, S^{29} and S^{30} haplotypes were selected based on reported sequences; *Nco*I (S^{16c}), *Bst*SNI (S^{29}) and *Pci*I (S^{30}), although plant materials were not available in this study. The 2,218 bp fragment of S^{16c}*-RNase* is expected to be digested into 331 and 2,384 bp fragments by *Nco*I. The 726 bp fragment of S^{29} haplotype and the 677 bp fragment of the S^{30} haplotype are expected to be split into 644 and 82 bp fragments by *Bst*SNI and 262 and 415 bp fragments by *Pci*I, respectively.

Pattern of digestion of *S-RNase* fragments by *S* haplotype-specific enzymes

The utility of the CAPS system was assessed experimentally by the amplification of 22 *S-RNase* fragments from 17 apple cultivars with the ASPF3 and ASPR3S primers followed by digestion with

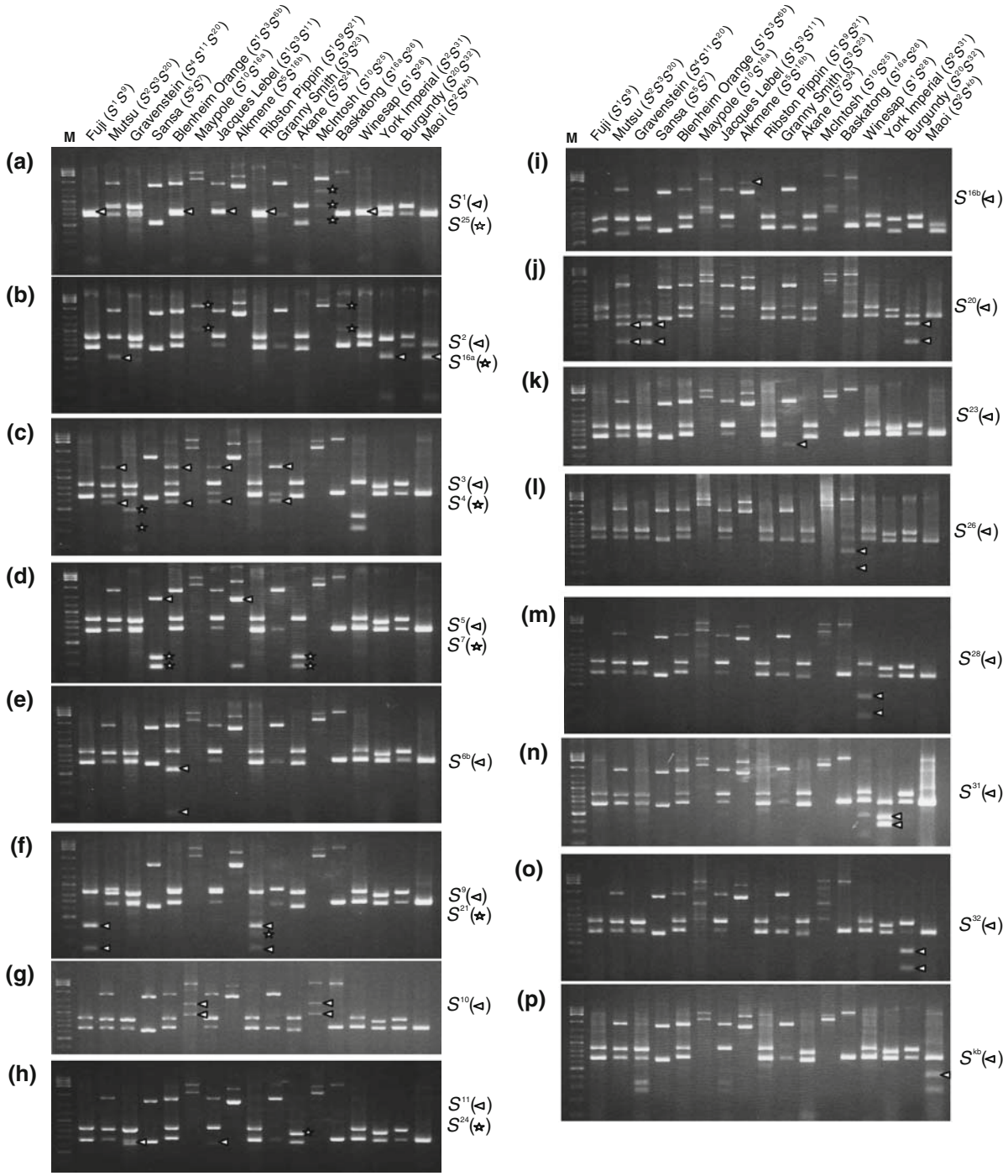

Fig. 2 CAPS analysis for the identification of the *S* genotypes of 17 apple cultivars. The *S-RNase* fragments were amplified by PCR using ASPF3 and ASPR3S primers and digested with *S* haplotype-specific restriction enzymes; **a** *Mva*I, **b** *Eco*RV, **c** *Bcl*I, **d** *Hin*dIII, **e** *Bfm*I, **f** *Sph*I, **g** *Asp*A2I, **h** *Bst*SNI, **i** *Nco*I, **j** *Afl*II, **k** *Bbv*CI, **l** *Bsm*I, **m** *Bpl*I, **n** *Bfu*AI, **o** *Bpm*I and **p** *Pml*I + *Bst*SNI. Arrow heads and stars show the digested fragments. The band sizes of the molecular marker are from top to bottom as follows: 10,000, 8,000, 6,000, 5,000, 4,000, 3,000, 2,000, 1,500, 1,200, 1,000, 900, 800, 700, 600, 500, 400, 300, 200, 100 bp

selected restriction enzymes (Table 1). The results of the CAPS analysis are shown in Fig. 2.

With *Mva*I digestion, S^1 haplotype-specific fragments of 'Fuji' (S^1S^9), 'Blenheim Orange' ($S^1S^3S^{6b}$), 'Jacques Lebel' ($S^1S^3S^{11}$), 'Ribston Pippin' ($S^1S^9S^{21}$) and 'Winesap' (S^1S^{28}), and S^{25} haplotype-specific fragment of 'McIntosh' ($S^{10}S^{25}$) were detected (Fig. 2a). S^2 haplotype-specific fragments of 'Mutsu' ($S^2S^3S^{20}$), 'York Imperial' (S^2S^{31}) and 'Maoi' (S^2S^{kb}), and S^{16a} haplotype-specific fragments of 'Maypole' ($S^{10}S^{16a}$) and 'Baskatong' ($S^{16a}S^{26}$) were detected by *Eco*RV digestion (Fig. 2b). With *Bcl*I digestion, the S^3 haplotype fragments of 'Mutsu' ($S^2S^3S^{20}$), 'Blenheim Orange' ($S^1S^3S^{6b}$), 'Jacques Lebel' ($S^1S^3S^{11}$) and 'Granny Smith' (S^3S^{23}), and S^4 haplotype fragment of 'Gravenstein' ($S^4S^{11}S^{20}$) were observed (Fig. 2c). The S^5 haplotype fragments of 'Alkmene' (S^5S^{16b}) and 'Sansa' (S^5S^7), and S^7 haplotype fragments of 'Akane' (S^7S^{24}) and 'Sansa' (S^5S^7) were generated by *Hin*dIII digestion (Fig. 2d). The S^{6b} haplotype fragment of 'Blenheim Orange' ($S^1S^3S^{6b}$) was released by *Bfm*I (Fig. 2e). The S^9 haplotype fragments of 'Fuji' (S^1S^9) and 'Ribston Pippin' ($S^1S^9S^{21}$), and S^{21} haplotype fragment of 'Ribston Pippin' ($S^1S^9S^{21}$) were detected using *Sph*I (Fig. 2f). The S^{10} haplotype fragments of 'Maypole' ($S^{10}S^{16a}$) and 'McIntosh' ($S^{10}S^{25}$) were released by *Asp*A2I (Fig. 2g). Using *Bst*SNI, the S^{11} haplotype fragment of 'Gravenstein' ($S^4S^{11}S^{20}$) and 'Jacques Lebel' ($S^1S^3S^{11}$), and the S^{24} haplotype fragment of 'Akane' (S^7S^{24}) were observed (Fig. 2h). The S^{16b} haplotype fragment of 'Alkmene' (S^5S^{16b}) was digested by *Nco*I (Fig. 2i). The S^{20} haplotype fragments of 'Mutsu' ($S^2S^3S^{20}$), 'Gravenstein' ($S^4S^{11}S^{20}$) and 'Burgundy' ($S^{20}S^{32}$) were released by *Afl*II (Fig. 2j). The S^{23} haplotype fragment of 'Granny Smith' (S^3S^{23}) was released by *Bbv*CI (Fig. 2k). The S^{26} haplotype fragment of 'Baskatong' ($S^{16a}S^{26}$) was released by *Bsm*I (Fig. 2l). The S^{28} haplotype fragment of 'Winesap' (S^1S^{28}) was detected using *Bpl*I (Fig. 2m). With *Bfu*AI digestion, the S^{31} haplotype fragment of 'York Imperial' (S^2S^{31}) was observed (Fig. 2n). The S^{32} haplotype fragment of 'Burgundy' ($S^{20}S^{32}$) was released by *Bpm*I (Fig. 2o). The S^{kb} haplotype fragment of 'Maoi' (S^2S^{kb}, genotype determined in this study. See below.) was detected using *Pml*I + *Bst*SNI (Fig. 2p). Collectively, these results show that the specific restriction fragments for 22 *S-RNase*s predicted from sequence data are detected by the CAPS analysis, demonstrating the utility of this system for the *S* genotyping of apple.

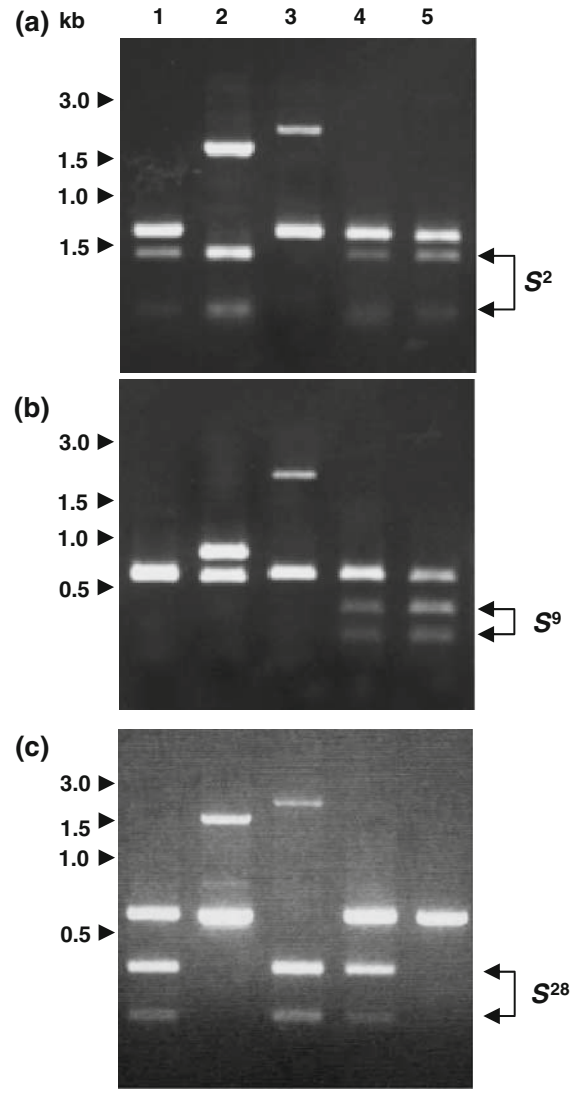

Fig. 3 Determination of the *S* genotypes of 'HAC6' and 'Takane'. The *S-RNase* fragments amplified by PCR using ASPF3 and ASPR3 were treated with the restriction enzymes **a** *Eco*RV (S^2-specific), **b** *Sph*I (S^9-specific) and **c** *Bpl*I (S^{28}-specific). Lane 1, HAC6 (S^2S^{28}); 2, Golden Delicious (S^2S^3); 3, Empire ($S^{10}S^{28}$); 4, Takane ($S^2S^9S^{28}$); 5, Red Gold (S^2S^9). The digested fragments are indicated by arrows

Identification of the *S* genotypes of three Japanese apple cultivars with the CAPS system

By using the CAPS system, *S* genotypes of the uncharacterized apple cultivars 'HAC6', 'Takane'

and 'Maoi' were analyzed. In the PCR of 'HAC6' DNA, an *S-RNase* fragment of ca. 650 bp was amplified. The long fragments of 1,790 bp (S^3) and 2,218 bp (S^{10}) of the parents ['Golden Delicious' (S^2S^3) and 'Empire' ($S^{10}S^{28}$)] were not observed. Thus, the *S* genotype of 'HAC6' was expected to be S^2S^{28} based on the PCR analysis. The PCR fragment of 'HAC6' was further digested with S^2 and S^{28} haplotype-specific restriction enzymes (Fig. 3). The 650 bp PCR fragment was cut with *Eco*RV into two fragments of 495 and 152 bp (S^2-specific) and cut with *Bpl*I into 399, 241 and 32 bp (S^{28}-specific), respectively. Therefore the *S* genotype of 'HAC6' was determined as S^2S^{28} by the CAPS analysis.

'Takane' is a triploid cultivar reported to be derived from a natural cross seedling of 'Red Gold' (S^2S^9) (Ueda et al. 1989). In 'Takane', the *S-RNase* fragment was detected as a ca. 660 bp band. The PCR fragment of ca. 660 bp was digested with the S^2, S^9 and S^{28} haplotype-specific restriction enzymes, *Eco*RV, *Sph*I and *Bpl*I, respectively (Fig. 3). Therefore the *S* genotype of 'Takane' is $S^2S^9S^{28}$.

'Maoi' was reported to be derived from a cross between 'Mantet' (S^7S^{25}) and 'HAC6' (S^2S^{28}, this study) (Yoshida et al. 2002). A PCR product of ca. 650 bp was amplified from 'Maoi' (Fig. 1c). CAPS analysis of the PCR product using the *S* haplotype-specific restriction enzymes revealed that the *S* genotype of 'Maoi' is S^2S^{kb} (Fig. 2), suggesting that 'Maoi' was not derived from a cross between 'Mantet' (S^7S^{25}) and 'HAC6' (S^2S^{28}). The S^{kb} haplotype of 'Maoi' was further confirmed by sequence analysis of the PCR fragment.

The usefulness of the CAPS system was demonstrated by the successful identification of *S* genotypes of the three apple cultivars. Although the pollen parent of the triploid cultivar 'Takane' is not known, the *S* genotype of 'Takane' ($S^2S^9S^{28}$) determined by this study implies that 'Takane' was derived from the fertilization of an unreduced egg of 'Red Gold' (S^2S^9) and pollen with the S^{28} haplotype, suggesting that the CAPS system is effective even for *S* genotyping of cultivars with unknown parentage.

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References

Bošković R, Tobutt KR (1999) Correlation of stylar ribonuclease isoenzymes with incompatibility alleles in apple. Euphytica 107:29–43. doi:10.1023/A:1003516902123

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

de Nettancourt D (1977) Incompatibility in Angiosperms. In: Frankel R et al (eds) Monographs on theoretical and applied genetics. Springer-Verlag, New York, pp 28–57

de Nettancourt D (2001) Incompatibility and incongruity in wild and cultivated plants. Springer-Verlag, New York

Ershadi A, Talaii AR (2007) Identification of S-alleles in 40 apple (*Malus* × *domestica* Borkh) cultivars by allele-specific PCR amplification. Acta Hortic 760:111–115

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. doi:10.1007/BF00223298

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 49:448–454

Kim HT, Kakui H, Koba T, Hirata Y, Sassa H (2007) Cloning of a new *S-RNase* and development of a PCR-RFLP system for determination of the *S*-genotypes of Japanese pear. Breed Sci 57:159–164. doi:10.1270/jsbbs.57.159

Kim H, Park J, Hirata Y, Nou I (2008) Molecular characterization of two new *S-RNases* ('S_{31}' and 'S_{32}') in apple (*Malus* × *domestica* Borkh.). J Plant Biol 51:202–208

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) Analyzes of *S*-allele genotypes of 'McIntosh', 'Kitakami', 'Worcester Pearmain', etc. J Jpn Soc Hortic Sci 68(suppl. 2):94 (article in Japanese)

Komori S, Soejima J, Abe K, Kotoda N, Kato H (2000) Analysis of *S*-allele genotypes and genetic diversity in the apple. Acta Hortic 538:83–86

Konieczny A, Ausubel FM (1993) A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J 4:403–410. doi: 10.1046/j.1365-313X.1993.04020403.x

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 40:850–851

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 (1999a) A new *S*-allele in apple, '*Sg*', and its similarity to the '*Sf*' allele from Fuji. HortScience 34:708–710

Matsumoto S, Komori S, Kitahara K, Imazu S, Soejima J (1999b) S-genotypes of 15 apple cultivars and self-compatibility of 'Megumi'. J Jpn Soc Hortic Sci 68:236–241

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, Hayashi S, Kitahara K, Soejima J (2001) Genomic DNA sequence encoding *Malus* × *domestica* Borkh. "Akane", "Delicious" and *Malus transitoria* S-RNases. DNA Seq 12:381–383. doi:10.3109/10425170109084462

Matsumoto S, Furusawa Y, Kitahara K, Komori S, Soejima J (2003a) 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 20:323–329

Matsumoto S, Furusawa Y, Komatsu H, Soejima J (2003b) *S*-allele genotypes of apple pollenizers, cultivars and lineages including those resistant to scab. J Hortic Sci Biotechnol 78:634–637

Matsumoto S, Kitahara K, Komatsu H, Abe K (2006) Cross-compatibility of apple cultivars possessing *S*-RNase alleles of similar sequence. J Hortic Sci Biotechnol 81:934–936

Sassa H (2007) A technique to isolate DNA from woody and herbaceous plants by using a silica-based plasmid extraction column. Anal Biochem 363:166–167. doi:10.1016/j.ab.2007.01.004

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. doi:10.1007/BF00225142

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 Biotechnol 76:596–600

Takasaki T, Okada K, Castillo C, Moriya Y, Sato T, Sawamura Y, Norika N, Norika S, Nakanishi T (2004) Sequence of the S_9-RNase cDNA and PCR-RFLP system for discriminating S_1-to S_9-allele in Japanese pear. Euphytica 135:157–167. doi:10.1023/B:EUPH.0000014907.50575.d0

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680. doi:10.1093/nar/22.22.4673

Ueda A, Koike H, Makita H, Kobayashi Y, Takemae S, Miyagawa K (1989) New apple cultivar 'Takane'. Nagano Fruit Tree Stn Rep 2:1–6 (article in Japanese)

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. doi:10.1007/s001220050739

Yoshida M, Muramatsu H, Minegishi T, Ogano R, Watanabe H, Kakizaki M, Tanaka S, Inagawa Y, Noda T (2002) A new apple variety ''Maoi''. Hokkaido Agr. Exp Stn Rep 82:41–48 (article in Japanese)