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ORIGINAL PAPER

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cDNA cloning of nine *S* alleles and establishment of a PCR-RFLP system for genotyping European pear cultivars

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Abstract Nine full-length cDNAs of *S* ribonucleases (*S*-RNases) were cloned from stylar RNA of European pear cultivars by RT-PCR and 3′ and 5′ RACE. Comparison of the nucleotide sequences between the nine *S*-RNases cloned and 13 putative *S* alleles previously amplified by genomic PCRs revealed that seven corresponded to *Sa*, *Sb*, *Sd*, *Se*, *Sh*, *Sk* and *Sl* alleles, and the other two were new *S* alleles (designated as *Sq* and *Sr* alleles). Genomic PCR with a set of 'FTQQYQ' and 'EP-anti-IIWPNV' primers was used to amplify nine *S* alleles; 1,414 bp (*Sl*), ca. 1.3 kb (*Sk* and *Sq*), 998 bp (*Se*), 440 bp (*Sb*) and ca. 350 bp (*Sa*, *Sd*, *Sh* and *Sr*). Among these, *S* alleles of similar size were discriminated by digestion with *Bae*I, *Bgl*II, *Bss*HII, *Hin*dIII, *Eco*O109I and *Sph*I. The PCR amplification of *S* allele following digestion with the restriction enzymes provided a PCR-RFLP system for rapid *S*-genotyping European pear cultivars harboring nine *S* alleles. The PCR-RFLP system assigned a total of 63 European pear cultivars to 25 genotypes. Among these, 14 genotypes were shared by two or more cultivars, which were cross-incompatible. These results suggested that the genes cloned represented the *S*-RNases from European pear, and that there were many cross-incompatible combinations among European pear varieties.

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Introduction

Self-incompatibility is a genetic mechanism to prevent self-fertilization, if both the pollen and pistil are fertile (reviewed in de Nettancourt 2001). European pear (*Pyrus communis* L.), Japanese pear (*Pyrus pyrifolia* Nakai) and apple (*Malus domestica* Borkh.) belong to the Maloideae subfamily, Rosaceae, and exhibit gametophytic self-incompatibility (GSI) controlled by a single *S*-locus with multi-alleles (Kikuchi 1929; Kobel et al. 1939; Lewis and Modlibowska 1942). These fruit trees are given a stable fruit set by cross-pollination with the pollen bearing an *S* allele different from either of the *S* alleles of the pistils, and their *S*-genotype assignment is important for the management of pollination in commercial orchards. Japanese pear and apple cultivars are classified into various cross-incompatible groups assigned with various pairs of *S* alleles by cross-incompatibility with pollen parents and between cultivars (Kobel et al. 1939; Terami et al. 1946). However, most European pear cultivars are cross-compatible (Crane and Thomas 1939; Crane and Lewis 1942; Griggs and Iwakiri 1954) and have not been assigned *S*-genotypes. Using a pollen tube growth test, Sanzol and Herrero (2002) investigated cross-incompatibility among 'Bartlett', 'Coscia' and three hybrids of 'Bartlett' × 'Coscia', and assigned them to five genotypes comprised of pairs of four *S* alleles. However, genotyping through pollination requires a great deal of labor and time because of the need to grow the cross progeny to flowering.

The *S* alleles of the Japanese pear and apple encode *S*-RNase as a stylar product (Sassa et al. 1994; Ishimizu et al. 1996a). *S*-RNase genes have been cloned from some cultivars assigned to various pairs of *S* alleles by pollination and pollen tube growth tests (Broothaerts et al. 1995; Norioka et al. 1996; Sassa et al. 1996; Ishimizu et al. 1998). The alignment of the deduced amino acid sequences of *S*-RNases defines five conserved regions (C1, C2, C3, RC4 and C5), and one hypervariable (HV) region involved in allelic specificity in self-incompatible reactions (Ishimizu et al. 1998;

Ushijima et al. 1998). Based on the nucleotide sequences of the *S*-RNases, PCR-based methods are established for rapid *S*-genotyping: an apple system composed of genomic PCR with *S* allele specific primers (Janssens et al. 1995); a Japanese pear system, PCR-restriction fragment length polymorphism (PCR-RFLP) systems composed of genomic PCR with consensus primers for *S* alleles following digestion with restriction endonucleases (Ishimizu et al. 1999; Takasaki et al. 2004).

Recently, genomic PCRs amplified 13 putative *S* alleles from 'Bartlett' and 'Coscia' and some European pear cultivars that were not genotyped by pollination and pollen tube growth tests. Using PCR with a set of primers designed from the C1 and C3 regions of apple *S*-RNases, Zuccherelli et al. (2002a; b) amplified six putative *S* alleles *Sa*, *Sb*, *Sc*, *Sd*, *Se* and *Sh*, and discriminated between them using digestion with four restriction enzymes. Among six cultivars assigned to pairs of *S* alleles, 'Abbé fétal' and 'Doyenné du Comice' assigned to the same genotype (*SaSb*) were cross-incompatible. Using PCR with a set of primers designed from C1 and C5 regions of Japanese pear *S*-RNases, Zisovich et al. (2004) amplified eight putative *S* alleles *Si*, *Sj* (=*Se*), *Sk*, *Sl*, *Sm*, *Sn*, *So* and *Sp* from eight cultivars and assigned their cultivars to pairs of *S* alleles. These putative *S* alleles had the structural features of *S*-RNase, but their stylar expression and linkage with *S* allele have not been confirmed yet.

Here, we cloned nine full-length cDNA sequences of *S*-RNases from stylar RNA of European pear cultivars using Reverse transcript-PCR (RT-PCR) and rapid amplification of cDNA ends (RACE). Based on cDNA and genomic sequences of *S*-RNases, we established a PCR-RFLP system for rapid *S*-genotyping of European pear cultivars and genotyped a total of 63 European pear cultivars. Cross-incompatibility was confirmed between the cultivars assigned to identical genotypes.

Materials and methods

Plant material

The plant material comprised a total of 64 diploid European pear cultivars planted at three orchards in Japan, at Fujisaki farm, the Teaching and Research Center for Bio-coexistence, Hirosaki University in Fujisaki, Aomori, at the Nagano Fruit Tree Experimental Station in Suzaka, Nagano and at the Department of Apple Research, National Institute of Fruit Tree Science (NIFTS) in Morioka, Iwate. Young leaves and styles at the white stage of flower bud development (Norioka et al. 1996) were collected, frozen in liquid nitrogen, and stored at −80°C until use.

RNA isolation

Total RNA was extracted from the styles and leaves of European pear cultivars using the RNeasy Plant mini Kit (QIAGEN). mRNAs were isolated from total stylar RNA using the Micro-FastTrack™ 2.0 mRNA Isolation Kit (Invitrogen).

RT-PCR cloning

The cDNA fragments of *S*-RNase were amplified from about 1 µg of the total stylar RNA by the Titan™ One Tube RT-PCR system (Roche Diagnostics) with a set of primers, 'FTQQYQ' (5′-TTTACGCAGCAATATCAG-3′) and 'anti-(I/T)IWPNV, (5′-AC(A/G)TTCGGCCAA ATA(A/G)TT-3′) designed from the C1 region and a conserved region downstream of the HV region of Japanese pear *S*-RNases, respectively (Takasaki et al. 2004). The RT-PCR was carried out in a 50 µl reaction for 10 cycles of 30 s at 94°C, 30 s at 55°C and 45 s at 68°C, followed by 20 cycles of 30 s at 94°C, 30 s at 55°C and 45 s at 68°C, adding a 5 s autoextension for each cycle, and a final extension of 7 min at 68°C.

3′ and 5′ RACE cloning

The 3′ end of cDNA was amplified from about 100 ng of stylar mRNA using the Titan™ One Tube RT-PCR system (Roche Diagnostics). The first PCR with a set of primers, 'FTQQYQ' and an adapter primer *Not*I-$(dT)_{18}$ (Amersham-Pharmacia) was carried out in a 50 µl reaction for 10 cycles of 30 s at 94°C, 30 s at 55°C and 45 s at 68°C, followed by 25 cycles of 30 s at 94°C, 30 s at 55°C and 45 s at 68°C, adding a 5 s autoextension for each cycle, and a final extension of 7 min at 68°C. For nested PCR, two forward primers, 'C2F1' (5′-TCTAATCCTA CTCCTTGT-3′) and 'C2F2' (5′-GATCCTCCT GACAAG-3′) were designed from the C2 region of RT-PCR products. One microliter of this first PCR reaction was used in a nested PCR with a set of primers; 'C2F1' and '*Not*I-dT' (5′-AACTGGAAGAATTCGCGGCCG-CAGGATT-3′) for *Sd*- and *Se*-RNases, and with another set of primers; 'C2F2' (5′-GATCCTCCT GACAAGT-3′) and '*Not*I-dT' for the other. Using the Expand High-Fidelity PCR system (Roche Diagnostics), the nested PCR was carried out in 30 µl reaction for 10 cycles of 15 s at 94°C, 30 s at 48°C and 2 min at 72°C, followed by 20 cycles of 15 s at 94°C, 30 s at 60°C, and 2 min at 72°C, and a final extension of 7 min at 70°C.

The 5′ end of the cDNA was amplified using 5′ RACE System 2.0 (Invitrogen). About 100 ng of stylar mRNA was subjected to first strand cDNA synthesis for 50 min at 42°C using primer 'EPSq-anti-IIWPNV' (5′-A CGTTTGGCCAAATAATT-3′) for *Sq*-RNase or 'anti-IIWPNV' for the other. After degradation of the cDNA/RNA hybrid with the RNase mix, the cDNA was purified using a S.N.A.P. column (Invitrogen). An oligo dC anchor sequence was then added to the 5′ end of the cDNA using Terminal Deoxynucleotide Transferase (TdT). The dC-tailed cDNA was amplified using the first PCR with two primer combinations of 'AAP' (Abridged Anchor Primer) and 'anti-IIWPNV', and of 'AAP' and 'EPSq-anti-IIWPNV' for 35 cycles of 1 min at 94°C,

1 min at 48°C and 2 min at 72°C. Five microliters of the first PCR reaction was used in a nested PCR with a forward primer 'AUAP' (Abridged Universal Amplification Primer) and nine reverse primers specific for each *S*-RNase: 'HVSa' (5′-GGGCTGTCAGATTTGCT-3′), 'HVSb' (5′-GGG GTTCGAGTATTTTC-3′), 'HVSd' (5′-GCTGCCATA TTTCCTATCT-3′), 'HVSe' (5′-GG TTTGAGTGATG GATCTA-3′), 'HVSh' (5′-GGGGT-TCGAGTTTTT TTGC-3′), 'HVSk' (5′-GGCTTTAAG ATCTGTTATC G-3′), 'HVSl' (5′-GGGGTTTGAGTG ATGTATCTA-3′), 'HVSq' (5′-CTTATCGTTCGAGG TTTC-3′) and 'HVSr' (5′-GCTTGGATATTTGTTA TCC-3′). Using the Expand High-Fidelity PCR system (Roche Diagnostics), the nested PCR was carried out in a 50 µl reaction for 30 cycles of 1 min at 94°C, 1 min at 48°C and 2 min at 72°C.

PCR-RFLP analysis

Genomic DNA was extracted from young leaves using a CTAB method (Castillo et al. 2001). PCR was conducted using the Expand High-Fidelity PCR system (Roche Diagnostics). About 50 ng of genomic DNA was mixed with 0.3 mM of each primer, 200 µM dNTP, 1× PCR-buffer, 1U Taq polymerase and distilled water to make a final volume of 30 µl. PCR amplification with a set of primers with 'FTQQYQ' and 'EP-anti-IIWPNV' (5′-AC(A/G)TT(C/T)GGCCAAATAATT-3′) was carried out for 10 cycles of 15 s at 94°C, 30 s at 48°C and 3 min at 72°C, followed by 20 cycles of 15 s at 94°C, 30 s at 48°C and 3.5 min at 72°C, with a final extension for 7 min at 70°C. Five microliters of PCR products were digested with six restriction endonucleases; *Bae*I, *Bgl*II, *Bss*HII, *Hin*dIII, *Eco*O109I and *Sph*I. *Bae*I and *Bss*HII were reacted for 4 h at 25°C and at 50°C, respectively, and the other endonucleases for 4 h at 37°C. The PCR products before or after the digestion were analyzed in 2% agarose gel and were visualized by staining with ethidium bromide.

Nucleotide and amino acid sequence analysis

The 5′ RACE and genomic PCR products were run on 2% agarose gel, extracted from the gel using a Gene clean II Kit (Bio 101) and directly sequenced. The RT-PCR and 3′ RACE products were cloned into pCR 2.1 plasmid vector using the TA Cloning Kit (Invitrogen) and sequenced. The nucleotide sequences were determined by the dideoxy-nucleotide chain termination method with the primers described above, using an ABI PRISM™ 310 DNA capillary sequencer. Sequence data were analyzed with Genetyx-Mac ver. 13 software (Genetyx). A putative signal peptide was predicted using SignalP ver. 2.0 (Nielsen et al. 1999).

Pollination tests

Pollination tests were conducted at the three orchards described above. One flower was selected from the second to fourth flowers in a flower cluster at 1 to 2 days before anthesis and then carefully emasculated. Self- and cross-pollinations with one flower per cluster were conducted. All treated flowers were covered with paper bags to avoid pollen contamination. Initial fruit sets were recorded 4 weeks after pollination (before June drop). Fruit set and the number of viable seeds were counted 10 to 11 weeks after pollination (after June drop). Large viable seeds were distinguished from aborted seeds. The number of viable seeds per fruit (the number of viable seeds/the number of fruit sets) and the number of viable seeds per flower (the number of viable seeds/the number of pollinated flowers) were calculated (Moriya et al. 2005).

Results

Cloning cDNA coding *S*-RNases

RT-PCR was carried out with a set of primers, 'FTQQYQ' and 'anti-(I/T)IWPNV, on total RNA isolated from styles and leaves of three cultivars genotyped previously by genomic PCR (Zuccherelli et al. 2002a); 'Bartlett' (*Se*), 'Conference' (*SdSh*) and 'Doyenné du Comice' (*SaSb*). In addition six cultivars of unknown genotype were studied; 'Aurora', 'Flemish Beauty', 'General Leclerc', 'La France', 'Le Lectier' and 'Winter Nelis'. cDNA fragments of ca. 200 bp were amplified from stylar RNA, but not from the leaves (data not shown). Comparison of nucleotide sequences was used to classify the cDNA fragments into nine classes, and two classes were assigned to each cultivar. Thirteen putative *S* alleles were amplified from European pear cultivars by genomic PCRs, and their nucleotide sequences deposited in the EMBL and Genebank databases (Zuccherelli et al. 2002a, b; Zisovich et al. 2004); *Sa* (accession no. AJ458181), *Sb* (accession no. AJ458182), *Sc* (accession no. AJ459774), *Sd* (accession no. AJ459775), *Se* = *Sj* (accession no. AJ457053 and AF457594, *Sj* sharing the same nucleotide sequence with *Se* was denoted as *Se* described below.), *Sh* (accession no. AJ459776), *Si* (accession no. AF518319), *Sk* (accession no. AY103408), *Sl* (accession no. AY103409), *Sm* (accession no. AY159323), *Sn* (accession no. AY195840), *So* (accession no. AY261994) and *Sp* (accession no. AY421968). The nucleotide sequences of cDNA fragments cloned were compared to those of the 13 putative *S* alleles reported. Out of nine classes of cDNA fragments, seven were found to represent the nucleotide sequences of putative *Sa*, *Sb*, *Sd*, *Se*, *Sh*, *Sk* and *Sl* alleles reported previously, but the other two were different from all 13 published *S* alleles and were designated as putative *Sq* and *Sr* alleles. Based on these putative *S* alleles, the nine cultivars analyzed were genotyped as follows: 'Aurora' (*SaSe*), 'Bartlett' (*SeSl*), 'Conference' (*SdSr*), 'Doyenné du Comice' (*SaSb*), 'Flemish Beauty' (*SdSe*), 'General Leclerc' (*SlSq*), 'La France' (*SeSr*), 'Le Lectier' (*SbSq*) and 'Winter Nelis' (*ShSk*).

To determine the full-length sequences of the nine putative *S* alleles, 3′ and 5′ RACE cloning was performed on stylar mRNAs of five European pear cultivars; 'Doyenne du Comice' (*SaSb*), 'Flemish Beauty' (*SdSe*), 'General Leclerc' (*SlSq*),'La France' (*SeSr*) and 'Winter Nelis' (*ShSk*). Overlapping nucleotide sequences of the 5′ and 3′ end cDNAs provided nine full-length cDNA sequences containing an open reading frame of 684–696 nucleotides encoding 226–232 amino acids. The amino acid sequences deduced from the nine cDNA sequences are shown in Fig. 1, and these have the primary structural organization of Japanese pear and apple *S*-RNases; a putative signal peptide comprising 25 or 27 amino acids, two histidine residues essential for *T2*/*S* type RNase activity (Kawata et al. 1989) and eight cysteine residues important for the tertiary structure (Ishimizu et al. 1996b). The alignment of the deduced amino acid sequences predicted five conserved regions (C1, C2, C3, RC4 and C5) and one HV region at similar positions in the Japanese pear and apple *S*-RNases (Ishimizu et al. 1998; Ushijima et al. 1998). These common structural features observed in the Maloideae *S*-RNases suggest that the nine cDNAs encode *Sa*-, *Sb*-, *Sd*-, *Se*-, *Sh*-, *Sk*-, *Sl*-, *Sq*- and *Sr*-RNases, respectively. Amino acid sequence identity among the *S*-RNases ranged from 61.8% (*Sa*- and *Sl*-RNase) to 89.5% (*Sl*- and *Sk*-RNase).

PCR-RFLP system

For PCR amplification of the nine *S* alleles from genomic DNA, a new reverse primer 'EP-anti-IIWPNV,' was designed. PCR with a set of 'FTQQYQ' and 'EP-anti-IIWPNV' primers resulted in two PCR products with different lengths from six European pear cultivars; 1,414 and 1,283 bp from 'General Leclerc' (*SlSq*), 1,274 and 345 bp from 'Winter Nelis' (*ShSk*), 998 and 369 bp from 'Flemish Beauty' (*SdSe*), 998 and 353 bp from 'La France' (*SeSr*) and 440 and 345 bp from 'Doyenne du Comice' (*SaSb*), respectively (Fig. 2a). These PCR products were sequenced and compared with the nucleotide sequences of the nine *S*-RNase cDNAs cloned, which revealed the presence of an intron ranging from 145 to 1,211 bp in a position of the HV region (Table 1; Fig. 1), and the *S*-RNase genomic product size corresponding to each *S* allele was determined as follows: *Sl* (1,414 bp), *Sq* (1,283 bp), *Sk* (1,274 bp), *Se* (998 bp), *Sb* (440 bp), *Sd* (369 bp), *Sr* (353 bp), *Sa* (345 bp) and *Sh* (345 bp).

Agarose gel electrophoresis after PCR was used to distinguish between *Sl*, *Se* and *Sb* alleles detected at 1,414, 998 and 440 bp, respectively, but could not differentiate between the other *S* alleles of similar sizes; *Sk* and *Sq* of ca. 1.3 kb or *Sa*, *Sd*, *Sh* and *Sr* alleles of ca. 350 bp (Fig. 2a). We searched for restriction endonucleases that could digest these *S* alleles specifically on the nucleotide sequences of 15 putative *S* alleles adding putative *Sc*, *Si*, *Sm*, *Sn*, *So* and *Sp* alleles, and selected six restriction endonucleases, *Bgl*II, *Hin*dIII, *Sph*I, *Eco*O109I, *Bss*HII and *Bae*I. The ca. 1.3 kb products could be distinguished by digestion with *Bgl*II and *Hin*dIII: *Bgl*II digested only the *Sk* allele, yielding 1,022 and 214 bp fragments (Fig. 2b, lane 2); *Hin*dIII digested only the *Sq* allele, yielding 960 and 323 bp fragments (Fig. 2c, lane 1). While ca. 350 bp products could be distinguished by digestion of with *Sph*I, *Eco*O109I, *Bss*HII and *Bae*I: *Sph*I only digested the *Sa* allele, yielding 233 and 112 bp fragments (Fig. 2d, lane 5); *Eco*O109I not only digested the *Sd* allele, yielding 260 and 109 bp fragments (Fig. 2e, lane 3) but also the *Sl* allele, yielding 1,149 and 265 bp fragments (Fig. 2e, lane 1); *Bss*HII only digested the *Sh* allele, yielding 210 and 135 bp fragments (Fig. 2f, lane 2); *Bae*I not only digested the *Sr* allele, yielding 195 and 125 bp fragments (Fig. 2g, lane 4), but also the *Sl* allele, yielding 1,282 bp fragment (Fig. 2g, lane 1). Taken together, the *Sk* and *Sq* alleles of ca. 1.3 kb were distinguished by digestion with *Bgl*II and *Hin*dIII, and the *Sa*, *Sd*, *Sh* and *Sr* alleles of ca. 350 bp were distinguished by digestion of with *Sph*I, *Eco*O109I *Bss*HII and *Bae*I.

Consequently, genomic PCR with a set of 'FTQQYQ' and 'EP-anti-IIWPNV' primers following digestion with six restriction endonucleases (*Bae*I, *Bgl*II, *Bss*HII, *Hin*dIII, *Eco*O109I and *Sph*I) provided a PCR-RFLP system for genotyping European pear cultivars harboring nine *S*-alleles *Sa*, *Sb*, *Sd*, *Se*, *Sh*, *Sk*, *Sl*, *Sq* and *Sr* (Table 1).

Genotyping cultivars

The PCR-RFLP system was employed to genotype the European pear cultivars described in Table 2. Genomic PCR with 'FTQQYQ' and 'EP-anti-IIWPNV' primers resulted in amplifying products of 1,414 bp (*Sl*), ca. 1.3 kb, 998 bp (*Se*), 440 bp (*Sb*) and ca. 350 bp. The products of ca. 1.3 kb and ca. 350 bp were distinguished by digestion with the six restriction endonucleases. Two different products were amplified from all cultivars except for 'Passe Crassane,' from which only the *Sr* allele was amplified. A total of 63 cultivars were classified into 25 genotypes assigned to pairs of the *Sa*, *Sb*, *Sd*, *Se, Sh*, *Sk*, *Sl*, *Sq* and *Sr* alleles (Table 2). Out of the 25 genotypes, 14 were shared among two and more cultivars. 'Red Clapp's' (a red fruit pigmentation of 'Clapp's Favourite', *SdSe*) and 'Max red Bartlett' (a red fruit pigmentation of 'Bartlett', *SeSl*), were assigned to the same genotype as their parents. Six cultivars were genotyped as follows: 'Abbé fétal' (*SaSb*), 'Doyenné du Comice' (*SaSb*), 'Docteur jules Guyot' (*SaSe*), 'Cascade' (*SbSe*) and 'Red Clapp's' (*SdSe*), and these genotypes were consistent with the previous genotyping (Zuccherelli et al. 2002a; Zisovich et al. 2004). However, 'Conference' (*SdSr*) and 'Passe Crassane' (*Sr*) did not agree with the genotypes reported previously by Zuccherelli et al. (2002a).

Pollination tests

The functionality of the nine *S* alleles *Sa*, *Sb*, *Sd*, *Se, Sh*, *Sk*, *Sl*, *Sq* and *Sr*, were revealed by cross-incompatibility between the cultivars assigned to identical genotypes.

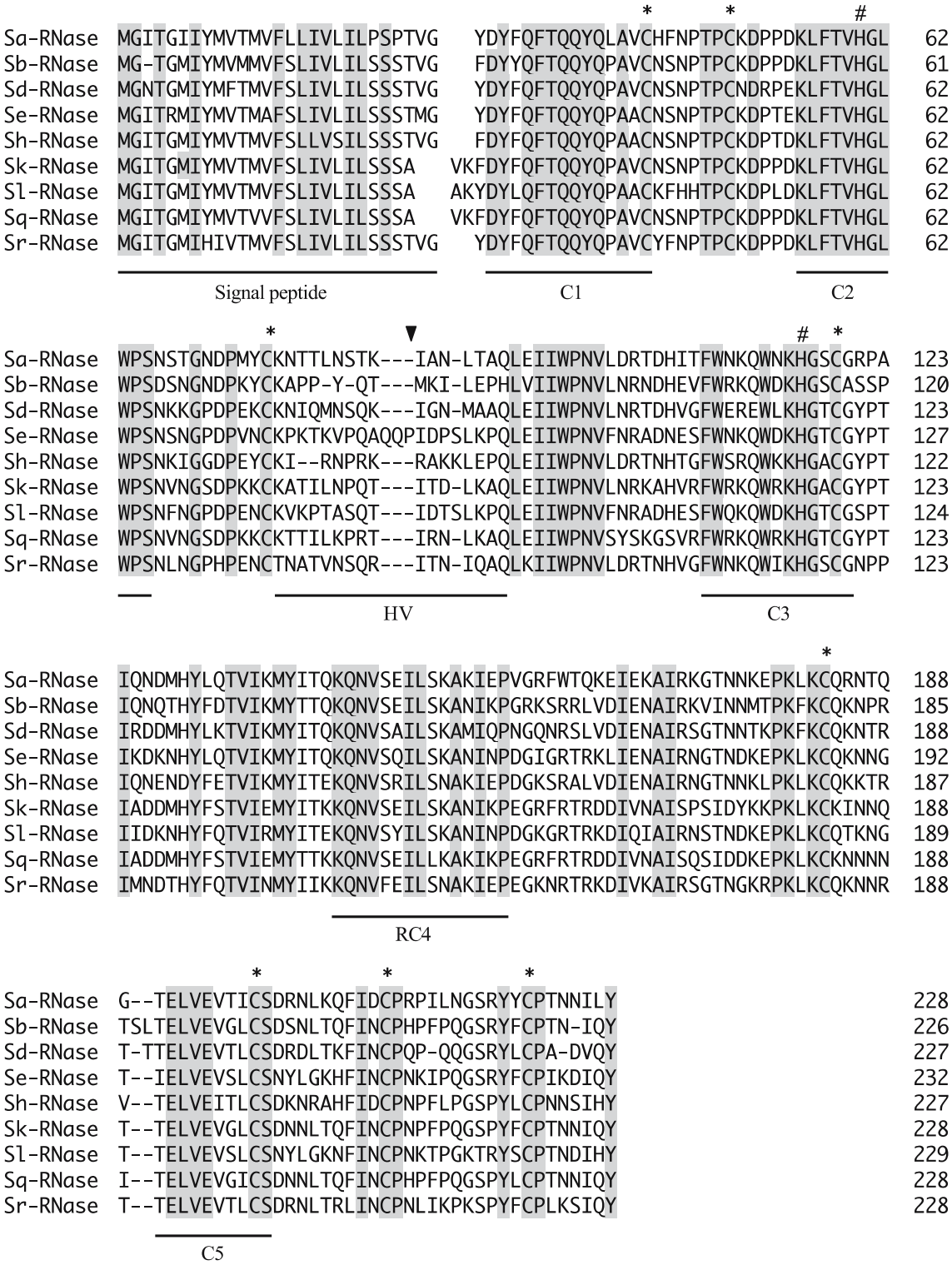

Fig. 1 Alignment of the deduced amino acid sequences of nine *S*-RNases of European pear. The amino acid residues conserved among nine *S*-RNases are *shaded*. Eight cysteine residues conserved and two histidine residues essential for the RNase activity are marked with *asterisks* and *hashes* over the amino acid sequence, respectively. The putative signal, a hypervariable (HV) region and five conserved regions (C1, C2, C3, RC4 and C5) are *underlined*. The *arrowhead* indicates the position of the intron. Accession numbers of nucleotide sequences for nine *S*-RNases are as follows: AB236430 (*Sa*), AB236429 (*Sb*), AB236427 (*Sd*), AB236428 (*Se*), AB236431 (*Sh*), AB236432 (*Sk*), AB236425 (*Sl*), AB236424 (*Sq*) and AB236426 (*Sr*)

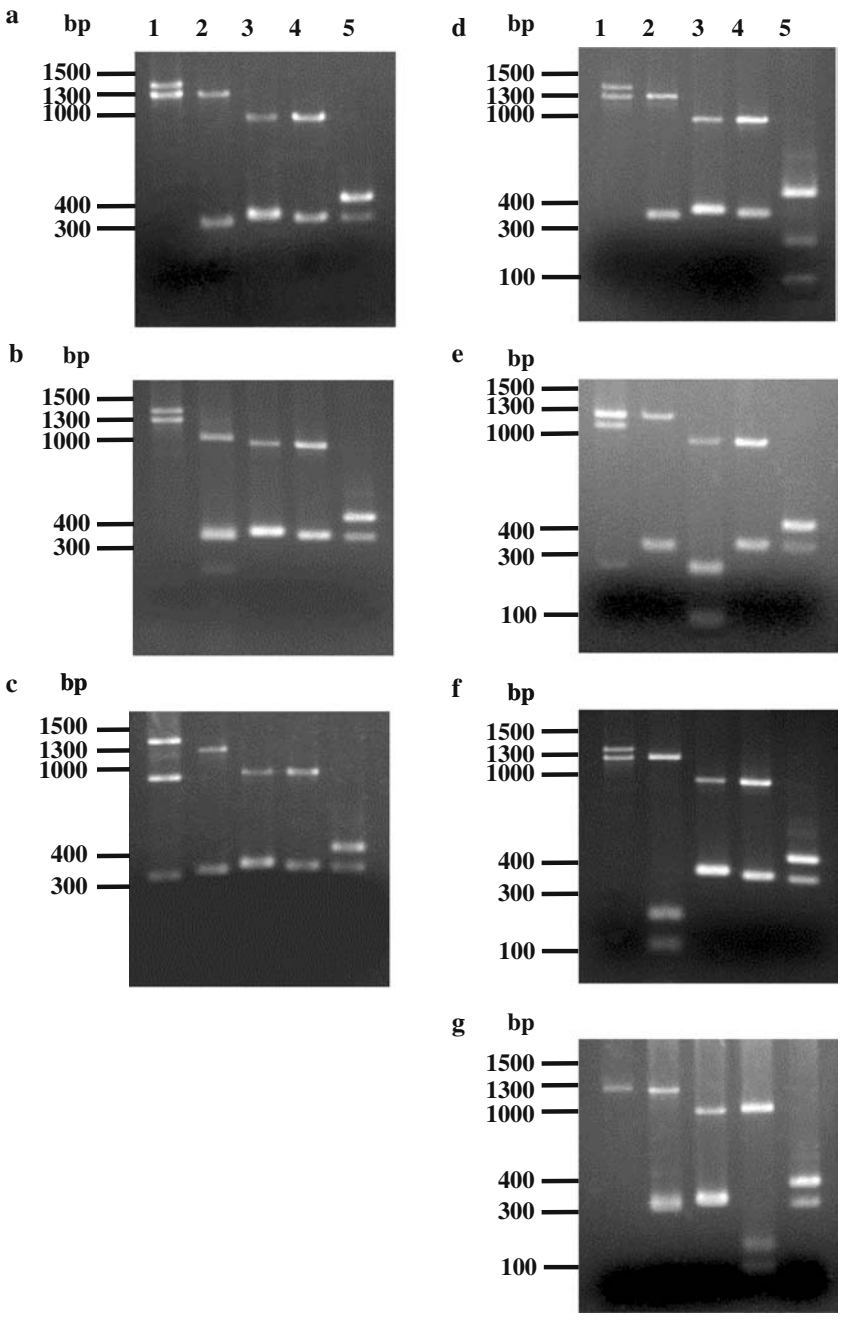

Fig. 2 PCR products from genomic DNA of five European pear cultivars using a set of primers, 'FTQQYQ' and 'EP-anti-IIWPNV' (**a**), following digestion with six restriction endonucleases; *Bgl*II (**b**), *Hin*dIII (**c**), *Sph*I (**d**), *Eco*O109I (**e**), *Bss*HII (**f**) and *Bae*I (**g**). *lane 1* General Leclerc (*SlSq*), *lane 2* Winter Nelis (*ShSk*), *lane 3* Flemish Beauty (*SdSe*), *lane 4* La France (*SeSr*), *lane 5* Doyenne du Comice (*SaSb*)

Self-pollinations were performed on all 64 cultivars described in Table 2, and cross-pollinations on the cultivars belonging to the 13 genotypes that were shared among two or more cultivars (Table 3). Incompatibility was distinguished from compatibility using the number of viable seeds per flower as a criterion. All 61 cultivars assigned were self-incompatible, except for 'Ayers', 'Magness' and 'Orient' which were male sterile (data not shown). Crosses between cultivars assigned to the different genotypes proved to be compatible, showing high fruit set % and 3.3 or more seeds per flower. Whereas, crosses between the identical genotypes resulted in a variable fruit set % depending on the parthenocarpic potential of the cultivars used as the seed parent, but produced 0.5 or fewer seeds per flower. The pollen from cultivars used as pollen parents provided many viable seeds for pistils assigned to their different genotypes (data not shown). The result of these pollination tests proved cross-incompatibility between cultivars assigned to identical genotypes, and demonstrated the functionality of all nine *S* alleles in styles and pollen.

Discussion

'La France' represented 64% of the total production (33,800 ton) in Japan in 2003, followed by 'Bartlett' 7%,

Table 1 Size (bp) of PCR products with an intron length and fragments yielded by digestion with six restriction endonucleases

S allele	PCR product	Intron	Restriction endonucleases					
			*Bgl*II	*Hin*dIII	*Sph*I	*Eco* O109I	*Bss*HII	*Bae*I
Sl	1,414	1,211	–	–	–	1149, 265	–	1282, 99, 33
Sk	1,274	1,083	1022, 214, 38	–	–	–	–	–
Sq	1,284	1,074	–	960, 323	–	–	–	–
Se	998	786	–	–	–	–	–	–
Sb	440	246	–	–	–	–	–	–
Sa	345	145	–	–	233, 112	–	–	–
Sd	369	169	–	–	–	260, 109	–	–
Sh	345	145	–	–	–	–	210, 135	–
Sr	353	153	–	–	–	–	–	195, 125, 33

– PCR products undigested with the restriction endonuclease

Table 2 *S*-genotypes of European pear cultivars assigned by PCR-RFLP analysis

PCR *S*-genotype	Cultivars	PCR *S*-genotype	Cultivars
SaSb	Doyenné du Comice	*SeSh*	El Dorado
	Abbé fetal		Winter Cole
SaSe	Aurora	*SeSk*	Fondante Thirriot
	Docteur jules Guyot		Packam's Triumph
	Harrow Delight	*SeSl*	Ayers
	Magness		Bartlett
	Tyson		Délices d'Hardenpont
SaSl	Koonce		Harvest Queen
	Harrow Sweet		Max red Bartlett
	Pierre Tourasse		Orient
SaSq	Beurré Clairgeau		Pera d'Agua
SaSr	Angelys		Seckel
SbSe	California		Seigneur d'Espéren
	Cascade	*SeSr*	Ballad
	Hartman		Doyenné d'hiver
	Highland		Idaho
	Howell		La France
SbSk	Alexandrine Douillard	*SeSq*	Besi de Saint-Waast
SbSl	Beurré Jean Van Geert		Bon-Chretien d'Hiver
	Canal Red		Covert
	Honey Sweet		Pierre Cornelle
	Joséphine de Malines	*ShSl*	Michaelmas Nelis
SbSq	Le Lectier	*ShSk*	Winter Nelis
SbSr	Condo	*SlSq*	General Leclerc
	Urbaniste		Ovid
SdSe	Bautomne	*SlSr*	Bristol Cross
	Clapp's Favorite		Emile d'Heyst
	Flemish Beauty		Kieffer
	Red Clapp's		Koshisayaka
SdSl	Doyenné Gris	*SkSl*	Santa Maria
SdSq	Devoe	*SkSr*	Ankara
SdSr	Conference	*Sr*	Passe Crassane

'Le Lectier' 7%, 'Silver Bell' (an open pollinated seedling of 'La France') 4%, 'General Leclerc' 4% and 'Aurora' 3% and 'Margueritte Marillat' 3%. 'Alexandrine Douillard', 'Bautomne', 'California', 'Conference', 'Devoe', 'Docteur jules Guyot', 'Doyenné du Comice', 'El Dorado', 'Flemish Beauty', 'Highland', 'Red Clapp's', 'Passe Crassane', 'Seigneur d'Espéren' and 'Winter Nelis' were also cultivated as minor cultivars. Two new cultivars, 'Ballad' and 'Koshisayaka' were selected from seedlings of 'Bartlett' × 'La France' (Abe et al. 1996; Matsumoto and Otake 1998). However, few *S*-genotypes of these cultivars have been assigned to pairs of *S* alleles by pollination tests.

To assign *S*-genotype of these cultivars, we started to clone *S*-RNase cDNAs from stylar RNA of nine European pear cultivars grown in Japan, and obtained the full-length cDNAs of nine *S*-RNases; *Sa*-, *Sb*-, *Sd*-, *Se*-, *Sh*-, *Sk*-, *Sl*-, *Sq*- and *Sr*-RNases. These *S*-RNases genes were expressed in styles but not in leaves, and their deduced amino acid sequences possessed the common structural features of the Maloideae *S*-RNases (Ishimizu et al. 1998). The correlation of the nine *S*-RNases with *S*-genotypes was evidenced by the cross-incompatibility among the cultivars assigned to the same genotype by the PCR-RFLP system. These results strongly suggested that the genes cloned represented the *S*-RNases from European pear.

Table 3 Fruit set and number of seeds formed by cross-pollinations among the cultivars assigned to the same or different genotype by PCR-RFLP analysis

Seed parent	*S*- genotype	Pollen parent	*S*- genotype	No. of flowers pollinated	Initial fruit set (%)	Fruit set (%)	No. of seeds/fruit	No. of seeds/flower
Doyenné du Comice	*SaSb*	Abbé fétal	*SaSb*	10	10.0	10.0	0	0
		La France	*SeSr*	10	80.0	80.0	6.8	5.4
Aurora	*SaSe*	Docteur jules Guyot	*SaSe*	30	3.3	3.3	0	0
		La France	*SeSr*	30	96.7	90.0	7.3	6.5
Highland	*SbSe*	California	*SbSe*	30	30.0	3.3	0	0
		Cascade	*SbSe*	29	31.0	0	0	0
		La France	*SeSr*	30	96.7	76.7	5.3	4.0
Beurré Jean Van Geert	*SbSl*	Joséphine de Malines	*SbSl*	30	20.0	20.0	0	0
		Howell	*SbSe*	29	82.8	79.3	7.9	6.2
Condo	*SbSr*	Urbaniste	*SbSr*	30	6.7	0	0	0
		Hartman	*SbSe*	30	90.0	60.0	8.4	5.1
Red Clapp's	*SdSe*	Flemish Beauty	*SdSe*	30	53.3	50.0	0.9	0.5
		La France	*SeSr*	29	96.6	96.6	8.8	8.5
Winter Cole	*SeSh*	El Dorado	*SeSh*	20	70.0	55.0	0	0
		Packam's Triumph	*SeSk*	11	100.0	100.0	3.6	3.6
Fondante Thirriot	*SeSk*	Packam's Triumph	*SeSk*	30	6.7	6.7	1.0	0.1
		La France	*SeSr*	30	93.3	93.3	9.2	8.5
Seigneur d'Espéren	*SeSl*	Bartlett	*SeSl*	30	23.3	16.7	0.4	0.1
		La France	*SeSr*	30	96.3	86.7	3.9	3.3
Ayers	*SeSl*	Bartlett	*SeSl*	30	20.0	6.7	1.0	0.1
		Pera d'Agua	*SeSl*	30	0.0	0	0	0
		Besi de Saint Waast	*SeSq*	30	86.7	80.0	9.0	7.2
Pierre Cornelle	*SeSq*	Covert	*SeSq*	30	76.7	26.7	0	0
		La France	*SeSr*	30	93.3	93.3	6.4	6.0
La France	*SeSr*	Ballad	*SeSr*	14	100.0	100.0	0	0
		Passe Crassane	*Sr*	60	98.3	98.3	7.9	7.9
General Leclerc	*SlSq*	Ovid	*SlSq*	29	93.1	89.7	0	0
		Le Lectier	*SbSq*	30	100.0	96.7	7.4	7.2
Bristol Cross	*SlSr*	Emile d'Heyst	*SlSr*	30	3.3	3.3	0	0
		Seigneur d'Espéren	*SeSl*	30	90.0	90.0	9.2	8.3

PCR-based methods are useful for rapid *S*-genotyping because they require no flowering material (Janssens et al. 1995; Ishimizu et al. 1999; Takasaki et al. 2004). Our genomic PCR with a set of 'FTQQYQ' and 'EP-anti-IIWPNV' primers was successful in amplifying the nine *S* alleles from European pear cultivars. *Sl*, *Se* and *Sb* alleles amplified as products of 1,414, 998 and 440 bp, respectively, could be distinguished from the other *S* alleles by agarose gel electrophoresis. However, *S* alleles with an intron of similar length, *Sk* and *Sq* alleles of ca. 1.3 kb or *Sa*, *Sd*, *Sh* and *Sr* alleles of ca. 350 bp, could not be distinguished, but were discriminated by digestion with six restriction endonucleases; *Bgl*II (*Sk* specific), *Hin*dIII (*Sq* specific), *Sph*I (*Sa* specific), *Eco*O109I (*Sd* specific), *Bss*HII (*Sh* specific) and *Bae*I (*Sr* specific). The set of 'FTQQYQ' and 'EP-anti-IIWPNV' primers could amplify putative *Sc*, *Si*, *Sm*, *Sn* and *Sp* alleles of ca. 350 bp, and *So* allele of 1,291 bp, which were not digested with the six restriction endonuclease because their recognition sequences were absent on the nucleotide sequences of putative *Sc*, *Si*, *Sm*, *Sn*, *So* and *Sp* alleles (Zuccherelli et al. 2002a; Zisovich et al. 2004). *Eco*O109I and *Bae*I digested not only *Sd* and *Sr* alleles but also *Sl* allele (Fig. 2e, g, lane 1). The digestion of *Sl* allele with *Eco*O109I and *Bae*I is not a problem for allele discrimination because *Sl* allele was amplified as products of 1,414 bp. Consequently, we established a PCR-RFLP system for genotyping European pear cultivars harboring *Sa*, *Sb*, *Sd*, *Se*, *Sh*, *Sk*, *Sl*, *Sq* and *Sr* alleles using the PCR with the 'FTQQYQ' and 'EP-anti-IIWPNV' primers following digestion with the six restriction endonucleases.

Using the PCR-RFLP system, we assigned a total of 63 cultivars to the 25 genotypes comprised of pairs of different *S*-alleles (Table 2). Among the cultivars analyzed, some were bred by selections from controlled hybridization (Crawford 1996; Sanzol and Herrero 2002). Eleven hybrid cultivars and their parents were assigned to pairs of *S*-alleles as follows: 'Angelys' (*SaSr*) [a hybrid of 'Doyenné d'hiver' (*SeSr*) × 'Doyenné du Comice (*SaSb*)], 'Ballad' (*SeSr*) and 'Koshisayaka' (*SlSr*) [hybrids of 'Bartlett' (*SeSl*) × 'La France' (*SeSr*)], 'Bristol Cross' (*SlSr*) [a hybrid of 'Bartlett' × 'Conference' (*SdSr*)], 'Bautomne' (*SeSd*) [a hybrid of 'Conference' × 'Doyenné d'hiver'], 'Condo' (*SbSr*) [a hybrid of 'Conference' × 'Doyenné du Comice'], 'Highland' (*SbSe*) [a hybrid of 'Bartlett' × 'Doyenné du Comice'], 'Hartman' (*SbSe*) [a hybrid of 'Doyenné du Comice' × 'Max red Bartlett' (*SeSl*)], 'Cascade' (*SbSe*) and 'California' (*SbSe*) [hybrids of 'Max red Bartlett' × 'Doyenné du Comice'] and 'Santa Maria' (*SlSk*) [a hybrid of 'Bartlett' × 'Coscia' (*SbSk*, Zisovich et al. 2004)]. Each hybrid cultivar inherited an *S* allele from the seed and pollen parents, respectively, indicating the behavior expected of *S* alleles. The cross-incompatibility and the inheritance

of *S* alleles from the parents correlated with our assignment of *S*-RNase sequences to *S* alleles and proved the reliability of the PCR-RFLP system.

S-genotypes of 'Passe Crassane' and 'Conference' did not agree with those previously assigned by Zuccherelli et al. (2002a). A single *Sr* allele was amplified from 'Passe Crassane', which Zuccherelli et al. (2002a) assigned to *Sa* allele resulting from genomic PCR following digestion with four restriction enzymes, *Bam*HI, *Eco*RI, *Hae*III and *Taq*I. Digestion of both *Sa* and *Sr* alleles with *Bam*HI, *Eco*RI and *Hae*III yielded the same restriction fragment length polymorphism, probably causing miss-assignment of the *Sa* allele. Cross-compatibility between 'La France' (*SeSr*) and 'Passe Crassane' which suggested that 'Passe Crassane' had a new *S* allele that could not have been amplified by PCR with a set of 'FTQQYQ' and 'EP-anti-IIWPNV' primers. 'Conference' was assigned to *SdSr*, whose genotype differed from *SdSh* proposed by Zuccherelli et al. (2002a). We could not explain the two genotypes of 'Conference', but the *S*-genotypes of 'Bristol Cross' (*SlSr*) and 'Condo' (*SbSr*) supported that the existence of *Sr* allele in 'Conference'.

Early pollination studies report that most European pear cultivars are cross-compatible (Crane and Thomas 1939; Crane and Lewis 1942; Griggs and Iwakiri 1954). However, 14 out of 25 genotypes were shared by two or more cultivars. In particular, nine cultivars have an identical genotype of *SeSl*. These results indicated that there are many cross-incompatible combinations among European pear cultivars. Genotyping other cultivars by using the PCR-RFLP system would increase the number of cultivars assigned to the same genotypes. Only a few fruits were set by most crosses between the cultivars assigned to identical genotypes, but many seedless fruits were produced by crosses when 'La France', 'General Leclerc' and 'Winter Cole' were used as seed parents. The large number of seedless fruits was due to the expression of parthenocarpy (Table 3). Without cross-pollination, 'La France' and 'General Leclerc' set seedless fruits as large as the cross-pollinated fruits, but their seedless fruits were inferior to the cross-pollinated with regards to soluble solids content (Moriya et al. 2005). Therefore, the *S*-genotype assignment is essential for a stable fruit set and good-quality fruit production in European pear cultivars. Our PCR-RFLP system will be valuable for genotyping cultivars and seedlings harboring nine *S*-alleles *Sa*, *Sb*, *Sd*, *Se*, *Sh*, *Sk*, *Sl*, *Sq* and *Sr*.

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