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A molecular method for *S*-allele identification in apple based on allele-specific PCR

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Abstract cDNA sequences corresponding to two self-incompatibility alleles (*S*-alleles) of the apple cv 'Golden Delicious' have previously been described, and now we report the identification of three additional *S*-allele cDNAs of apple, one of which was isolated from a pistil cDNA library of cv 'Idared' and two of which were obtained by reverse transcription-PCR (RT-PCR) on pistil RNA of cv 'Queen's Cox'. A comparison of the deduced amino acid sequences of these five *S*-allele cDNAs revealed an average homology of 69%. Based on the nucleotide sequences of these *S*-allele cDNAs, we developed a molecular technique for the diagnostic identification of the five different *S*-alleles in apple cultivars. The method used consists of allele-specific PCR amplification of genomic DNA followed by digestion of the amplification product with an allele-specific restriction endonuclease. Analysis of a number of apple cultivars with known *S*-phenotype consistently showed coincidence of phenotypic and direct molecular data of the *S*-allele constitution of the cultivars. It is concluded that the *S*-allele identification approach reported here provides a rapid and useful method to determine the *S*-genotype of apple cultivars.

Key words *Malus* × *domestica* · Apple · Self-incompatibility · *S*-alleles · PCR

Introduction

Self-incompatibility (SI) is a feature of many flowering plants that prevents inbreeding and promotes outcrossing. In the simplest case, SI is determined by a single locus, the *S*-locus, with multiple alleles (de Nettancourt 1977). Apple (*Malus* × *domestica* Borkh.) exhibits gametophytic self-incompatibility in which the SI phenotype of the pollen is determined by its own haploid genotype. Thus, fertilization is blocked when the *S*-allele carried by the pollen matches one of the two *S*-alleles expressed in the pistil.

Gametophytic SI has been studied mainly in solanaceous species, such as *Nicotiana alata*, *Lycopersicon* spp., *Petunia* spp. and *Solanum* spp., and only a few studies have been devoted to species from other plant families (for a recent review see Clarke and Newbigin 1993). The *S*-allele-associated proteins (S-proteins) are highly basic (pI = 8–10.5) glycoproteins of approximately 30 kDa that are abundant within the pistil. They have been shown to possess ribonuclease activity and are therefore called S-RNases (McClure et al. 1989; Singh et al. 1991; Broothaerts et al. 1991; Kaufmann et al. 1991). Until recently, there has been no direct proof that the S-RNases were responsible for SI. Lee et al. (1994), however, showed that inhibition of the synthesis of S3-proteins in *Petunia inflata* plants of the *S2S3* genotype by introduction of an antisense *S3*-gene resulted in a failure of the transgenic plants to reject *S3* pollen. Moreover, expression of a transgene-encoded S3-protein in *P. inflata* plants of *S1S2* genotype conferred on the transgenic plants the ability to reject *S3* pollen. Similarly, Murfett et al. (1994) introduced an extra *S*-allele into *Nicotiana* plants and showed that the presence of the transgene-encoded S-RNase in the pistil was sufficient for allele-specific recognition and rejection of *Nicotiana* pollen. These experiments represent direct *in vivo* evidence that S-RNases control the SI behaviour of the pistil.

Most rosaceous fruit crops, including apple, are known to be gametophytically self-incompatible (de Nettancourt 1977). Because of this SI, part of the total number of fruit trees in orchards are "pollinator" trees who serve as pollen-donor to the main cultivar. Pollinators must be carefully chosen to guarantee optimal fruit set on a given main cultivar. Cross-fertility between

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cultivars is determined by carrying out manual pollinations followed by an assessment of fruit-setting efficiency. In these controlled pollinations, saturating quantities of pollen are used per stigma, thus making it difficult to demonstrate a clearcut distinction between compatible (both *S*-alleles are different) and semi-compatible (one *S*-allele in common) reactions. These analyses are also hampered by the fact that pollen tube growth in the style is strongly affected by environmental and physiological factors (de Nettancourt 1977). An unambiguous molecular method for *S*-allele identification would, therefore, be of particular interest to fruit tree breeders.

In our previous study on SI in apple (Broothaerts et al. 1995), two pistil-specific cDNAs corresponding to the *S2*- and *S3*-allele of apple were characterized. The gene product of the *S2*-allele was isolated from pistils of cv 'Golden Delicious' and shown to be an RNase. In the investigation described here three additional *S*-alleles of apple were identified. Based on the comparison of the nucleotide sequences of the five identified *S*-allele cDNAs, we developed an *S*-allele typing method using allele-specific polymerase chain reaction (PCR) amplification and restriction enzyme digestion. Different apple varieties were subjected to this PCR analysis, which allowed rapid and easy determination of the presence of any of the five different *S*-alleles in a given apple variety.

Materials and methods

Plant material

The plant material was comprised of 17 different cultivars of *Malus* × *domestica* Borkh., which were provided either by the Centre for Fruit Culture at our university (cultivars in Table 3B) or by the National Fruit Trials, Brogdale Farm, Kent, U.K. (cultivars in Table 3A). Styles (dissected from flower buds at the "balloon" stage of development) and leaves were immediately upon removal from the plant frozen in liquid nitrogen and then stored at −80 °C until use.

Isolation of genomic DNA

Genomic DNA was isolated from apple leaves according to the method of Aldrich and Cullis (1993), without further Chelex 100 or gel purification. DNA concentration was determined spectrophotometrically, and possible DNA degradation was checked by agarose gel electrophoresis.

cDNA library construction and screening

A cDNA library from pistils of cv 'Idared' was constructed essentially as previously described (Broothaerts et al. 1995), starting from 1.5 µg of poly(A)$^+$ RNA. The cDNA library consisted of 8×10^5 plaque-forming units, and 9×10^3 plaques were screened by plaque hybridization using the same mixture of genomic DNA probes as used previously (Broothaerts et al. 1995). The cDNA inserts from selected plaques were subcloned into the vector pEMBL18$^+$. DNA sequences were determined by dideoxy chain termination (Sanger et al. 1977) on an ALF DNA Sequencer (Pharmacia) using the AutoRead Sequencing Kit (Pharmacia).

Reverse transcription-PCR (RT-PCR) and cloning

Total RNA was isolated from five mature styles of cv 'Queen's Cox' by the method of Logemann et al. (1987) and resuspended in a final volume of 40 µl. Reverse transcription was performed at 42 °C for 1 h, in a total reaction volume of 20 µl containing 1 µl of RNA solution, 1 × *Taq* polymerase buffer (Appligene), 1 µ*M* oligo (dT) primer (Pharmacia), 0.01 units of RNase inhibitor (RNaseGuard, Pharmacia), 250 µ*M* dNTPs, 1 m*M* DTT and 1 unit of Mu-MMLV reverse transcriptase (Pharmacia).

Ten microliters of the RT reaction was used in a subsequent PCR under the standard conditions described in Broothaerts et al. (1995). The PCR amplification was carried out in a total reaction volume of 25 µl using the primers OWB 134 and oligo(dT), and was comprised of 30 cycles (denaturation 1 min 94 °C, annealing 1 min 50 °C, extension 1 min 72 °C). Sequences of the primers used are shown in Table 1. PCR products were cloned in the vector pEMBL 18$^+$, and DNA sequences were determined.

Allele-specific PCR amplification

Allele-specific PCR amplification was carried out with 50 ng of genomic DNA and two allele-specific primers (Table 2) in a total reaction volume of 35 µl under the standard conditions described in Broothaerts et al. (1995). The reaction was run for 30 cycles comprising denaturation at 94 °C for 1 min, annealing at an optimal temperature for each primer combination (Table 2) for 1 min and extension at 72 °C for 1 min. Sequences of the primers used are shown in Table 1. After PCR, the appropriate restriction buffer and an allele-specific restriction enzyme (Table 2) were added directly to one-half of the PCR reaction volume. Digestion was done for 1 h at 37 °C. PCR products and digests were analysed by agarose gel electrophoresis.

Table 1 Oligonucleotides used in PCR amplification reactions

Name	Sense/ antisense	Nucleotide sequence (5′ → 3′)	Position
OWB139	Sense	atgaattctgcaaggtcaaacccacg	Nucleotides 73–91 in *S5*
OWB138	Antisense	atgaattcatatggataatggtcaaccg	Nucleotides 630–649 in *S5*
OWB126	Sense	gccttcagactcgaatggaca	Nucleotides 44–64 in *S7*
OWB127	Antisense	tggcatttacaatatctacc	Nucleotides 344–363 in *S7*
OWB154	Sense	cagccggctgtctgccactt	Nucleotides 130–149 in *S9*
OWB 155	Antisense	cggttcgatcgagtacgttg	Nucleotides 309–328 in *S9*
OWB128	Sense	atgacggttcttatccatcc	Nucleotides 34–53 in D[b]
OWB75	Antisense	tgagccattcccgctggggc	Nucleotides 249–268 in D[b]
OWB122, 123, 134, 145[a]			

[a] Characteristics of OWB122 and OWB123 (*S2*), and OWB134 and OWB145 (*S3*) were published by Broothaerts et al. (1995)

[b] Sequence data for the genomic PCR fragment D (GenBank accession number U19794 (X1)) are from Broothaerts et al. (1995)

Table 2 Conditions for allele-specific PCR and restriction enzyme digestion

S-allele	Primers	Annealing temp (°C)	Band size (bp) of PCR product	Restriction enzyme	Band sizes (bp) after digestion
S2[a]	OWB122 OWB123	64	449	*Eco*RV	349 + 100
S3[a]	OWB134 OWB145	56	375	*Pst*I	226 + 149
S5	OWB139 OWB138	58	1700[b]	*Eco*RV	1580 + 120
S7	OWB126 OWB127	62	440[c]	*Acc*I	228 + 212
S9	OWB154 OWB155	62	343[c]	*Eco*RI	212 + 131
D	OWB128 OWB75	59	236	*Eco*RV	143 + 93

[a] Characteristics of the allele-specific PCR and restriction digestion concerning the *S2*- and *S3*-allele have already been published by Broothaerts et al. (1995)

[b] The size of the PCR product obtained using primer pair OWB139/OWB138 (*S5*-allele) was estimated on the basis of gel electrophoresis (precise length of the intron of the *S5*-allele is unknown; see results)

[c] The sizes of the PCR products obtained using primer pair OWB126/OWB127 (*S7*-allele) and primer pair OWB154/OWB155 (*S9*-allele) were calculated on the basis of the nucleotide sequences of the *S7*- and *S9*-allele, respectively, in combinatioin with sequence data of their introns as derived from the genomic fragments B and C, respectively (see results)

Results

Identification of three novel *S*-allele cDNAs in apple

We recently characterized cDNA clones for two *S*-alleles (*S2* and *S3*) isolated from apple cv 'Golden Delicious'. Both cDNA clones were obtained by hybridizing a pistil cDNA library with short genomic sequences of apple amplified by PCR. These PCR fragments were derived from DNA of several apple cultivars, including 'Golden Delicious', 'Idared' and 'Queen's Cox' using amplimers corresponding to conserved regions in the *S*-genes of Solanaceae (Broothaerts et al. 1995).

The same strategy was used in this study to identify cDNA clones representing the *S*-alleles of apple cv 'Idared'. This variety is known to be cross-fertile with 'Golden Delicious' and should, therefore, carry at least one *S*-allele that does not occur in 'Golden Delicious'. The screening of a pistil cDNA library of 'Idared' yielded six positive clones. After the subcloning and sequence analysis of these clones, two different cDNA sequences were identified. One cDNA sequence is identical to the previously identified *S3*-cDNA in 'Golden Delicious', indicating that both varieties contain one common *S*-allele. The other cDNA sequence, hereafter named *S7*-cDNA (see below), is 83% and 81% homologous to the corresponding part in the *S2*- and *S3*-cDNAs, respectively. The isolated *S7*-cDNA is, however, not a full-length cDNA. At the 5'-end the untranslated region and part of the coding sequence are missing, whereas a poly(A) tail is absent from the 3'-untranslated region. The sequence of the *S7*-cDNA is deposited under GenBank accession number U19792 (S7Mdo).

In order to identify additional *S*-alleles in apple, we applied RT-PCR on pistil RNA from cv 'Queen's Cox', which is known to be cross-fertile with 'Golden Delicious' and 'Idared'. Total RNA from 'Queen's Cox' pistils was reverse-transcribed into cDNA using oligo(dT) as a starting primer. The single-stranded cDNA was then used as a template for PCR amplification in the presence of one *S*-gene-specific primer (OWB 134) and oligo(dT). Primer OWB 134 was designed on the basis of the conserved sequence near the translation initiation codon of the *S2*- and *S3*-cDNA sequences (Table 1; see Broothaerts et al. 1995). After cloning and sequencing of the RT-PCR products, two novel cDNAs showing similarity to the other *S*-allele cDNAs of apple were obtained. One cDNA sequence, hereafter named *S9*-cDNA (see below), is 996 basepairs (bp) in length (including a poly(A) tail of 17 nucleotides) and contains an open reading frame of 228 amino acids, featuring a 27-amino acid putative amino-terminal signal peptide. The other cDNA sequence, hereafter named *S5*-cDNA (see below), is not full-length (nucleotides upstream of the internal *Bam*HI restriction site were not cloned) and encodes 179 amino acids of the mature protein. GenBank accession numbers of the *S9*- and *S5*-cDNA are U19793 (S9Mdo) and U19791 (S5Mdo), respectively.

We found that the cDNA sequences *S7* and *S9* correspond perfectly with two of the genomic DNA fragments (named fragments B and C, respectively) that were used as a probe to screen the cDNA library of 'Golden Delicious' (Broothaerts et al. 1995) and 'Idared' (this study). An alignment of cDNA and corresponding genomic sequences revealed that the *S7*-allele and the *S9*-allele contain a single small intron of 120 bp and 144 bp, respectively.

An alignment of the derived acid sequences of the mature protein domains of the *S2*-, *S3*-, *S5*-, *S7*- and *S9*-alleles is presented in Fig. 1. Visual inspection of the alignment reveals 96 perfectly conserved residues and an additional 21 sites that accomodate only conservative replacements. The longest stretch of perfectly conserved residues is near the amino-terminus of the mature protein and includes 15 residues.

Development of a method for *S*-allele-specific PCR amplification

In order to be able to assess the correspondence of the cloned *S*-allele sequences with the *S*-alleles assigned on a phenotypic basis by Kobel et al. (1939), we developed a diagnostic method for *S*-allele identification. Nucleotide sequence alignment of the *S2*-, *S3*-, *S5*-, *S7*- and *S9*-allele was carried out in order to design two specific primers for each *S*-allele, which would correspond to variable regions in the sequence. Table 1 shows the sequence of

```
      1                  20                  40
      "********** * *   * **"***************"    "   *
S2    YDYFQFTQQYQPAACNSNPTPCKDPPDKLFTVHGLWPSNMNRSELFNCSSS
S3    FDYFQFTQQYQPAVCSSNPTPCKDPPDKLFTVHGLWPSNVNGSDPKKCKTT
S5                            DPPDKLFTVHGLWPSNFNGPDPENCKVK
S7    YDYFQFTQQYQLAACNSKPIPCKDPPDKLFTVHGLWPSDSNGHDPVNCSKS
S9    YDYFQFTQQYQPAVCHFNPTPCRDPPDKLFTVHGLWPSNSSGNDPIYCKNT

              60                  80                 100
            "   " " **"***** "*  *  ** ""* ***"** *"* "
S2    NVTYAKIQ-NIRTQLEMIWPNVFNRKNHLGFWNREWNKHGACGYPTIRNDL
S3    ILNPQTI-TNLTAQLEIIWPNVLNRKAHARFWRKQWRKHGTCGYPTIADDM
S5    PTASQTIDTSLKPQLEIIWPNVFNRADHESFWQKQWDKHGTCGSPTIIDKN
S7    TVDAQKLG-NLTTQLEIIWPNVYNRTDHISFWDKQWNKHGTCGHPTIMNDI
S9    TMNSTKI-ANLTARLEIIWPNVLDRTDHITFWNKQWNKHGSCGHPAIQNDM

                        120                140
      **  *** **** ***** ***"* * *    *   "*  **  "   * *
S2    HYFQTVIKMYITQKQNVSDILSKAKIEPDGNIRTQKEIVDAIRKGIHGKEP
S3    HYFSTVIEMYITKKQNVSEILSKAKIKPEKKFRTRDDIVNAISQSIDYKKP
S5    HYFETVIRMYITEKQNVSYILSKANINPDGKGRTRKDIQIAIRNSTNDKEP
S7    HYFQTVIKMYITQKQNVSKILSRAKIEPEGKPRKQVDIVNAIRKGTNDKEP
S9    HYLQTVIKMYITQKQNVSEILSKAKIEPVGKFRTQKEIEKAIRKGTNNKEP

            160                 180                 200
       ***      ******""**"      **"**      "   ***  * *
S2    NLKCQKNTQMTELVEVTLCSDGNLKQFIDCPHHFPNGSRHNCPTNHILY
S3    KLKCKNNNQITELVEVGLCSDNNLTQFIDCPRPFPQGSPFFCPTNNIQY
S5    KLKCQTKNGITELVEVSLCSNYFGKNFINCPNKTPGKTRYSCPTNDIHY
S7    KLKCQKNNQVTELVEVTLCSNYFGKNFINCPRHIPNGSRYSCPTKNILY
S9    KLKCQKNSQRTELVEVTICSDRNLNQFIDCPRPILNGSRYYCPTNNILY
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Fig. 1 Alignment of the derived amino acid sequences of the mature protein domains of the *S2*-, *S3*-, *S5*-, *S7*- and *S9*-alleles of apple. Sequence data of the *S2*- and *S3*- allele (GenBank accession numbers are U12199 and U12200, respectively) are from Broothaerts et al. (1995). The first 22 amino acid residues of *S7* are derived from the nucleotide sequence of the genomic PCR fragment B (Broothaerts et al. 1995; see discussion). Gaps introduced to allow optimal alignment are marked with *dashes*; sites that are perfectly conserved between the sequences (as far as available) are indicated by *asterisks*; sites that have only conservative replacements are marked with " [amino acid groups defined in Dayhoff et al. (1979): C, STAPG, MILV, HRK, NDEQ, FYW]

the different primers. Each pair of allele-specific primers was used for PCR amplification on total genomic DNA of different apple cultivars. In order to increase the specificity of the method, subsequent digestion of the PCR product with an allele-specific restriction endonuclease was performed. Table 2 shows for each *S*-allele the primer pair used for PCR amplification and the specific restriction endonuclease. The PCR products and the digestion products could be visualized upon agarose electrophoresis gels.

Allele-specific amplification reactions were independently optimized for each primer pair by performing PCR reactions at different annealing temperatures. As a template in these optimization reactions, we used genomic DNA from the cultivars from which the corresponding sequence was obtained (positive control) and DNA from cultivars that had yielded different *S*-allele sequences (negative control). Allele-specific PCR amplification products for the *S2*-, *S3*-, *S7*-, and *S9*-alleles, respectively, had a size that conformed to the size predicted on the basis of the cDNA and intron sequences. For the *S5*-allele, an allele-specific amplification product of approximately 1700 bp was obtained, 577 bp of which were cDNA-derived. Further restriction analysis of this 1700 bp fragment (not shown) revealed that the *S5*-allele contained a single intron of approximately 1100 bp. A similar-sized intron was previously found to be present in the *S3*-allele (Broothaerts et al. 1995).

In our previous study we had identified a genomic fragment, called fragment D, which showed homology to the *S*-gene but which was unlikely to be linked to the *S*-locus because of its non-polymorphic nature (Broothaerts et al. 1995). We optimized the same selective PCR method for this genomic fragment (Tables 1, 2). As it was present in all apple cultivars, amplification of the genomic fragment D was used as a control for DNA integrity.

Figure 2 shows an example of the selective amplification technique applied to four apple varieties, i.e. 'Danziger Kantapfel', 'Transparent von Croncels', 'Queen's Cox' and 'Jonagold', the latter being a triploid variety. Using our *S*-allele typing method, we found the following *S*-allele sequences to be present in the four cultivars tested: 'Danziger Kantapfel', *S2* and *S7*; 'Transparent von Croncels', *S2* and *S3*; 'Queen's Cox' *S5* and *S9*; 'Jonagold', *S2*, *S3* and *S9*. These data clearly indicate that *S2*, *S3*, *S5*, *S7* and *S9* are polymorphic, in contrast to genomic fragment D, which is present in all cultivars. By using the *S5*-specific amplimers, we also obtained some minor amplification products, but these could be shown to have an non-polymorphic nature and were not further considered.

Analysis of different apple varieties by *S*-allele-specific PCR amplification and restriction enzyme digestion

To ensure that the cloned cDNA sequences are involved in SI, the *S*-allele typing approach was applied to a number of cultivars described in Kobel et al. (1939), and our data were compared with the phenotypic behaviour of the cultivars assayed. Kobel and coworkers determined the *S*-phenotype of the cultivars by hand-pollinating the pistils and then measuring *in situ* pollen tube growth by fluorescence microscopy. Table 3A shows the data from the PCR-based *S*-allele identification method for all five *S*-alleles compared with the *S*-classification system of Kobel. In addition to the cloned cDNAs from 'Golden Delicious', which had previously been associated with the *S2* and *S3*-allele (Broothaerts et al. 1995), each of the novel *S*-gene-similar cDNA sequences described in this paper could be related to a particular *S*-allele. The novel cDNA, which was isolated from 'Idared', corresponded to the *S7*-allele since only 'Danziger Kantapfel', which is the only cultivar in the classification system of Kobel that rejects *S7*- bearing pollen, appeared to contain the *S7*-cDNA. On the basis of a similar analysis, the two cDNAs from 'Queen's Cox' seemed to correspond to the *S5*- and *S9*- allele, respecti-

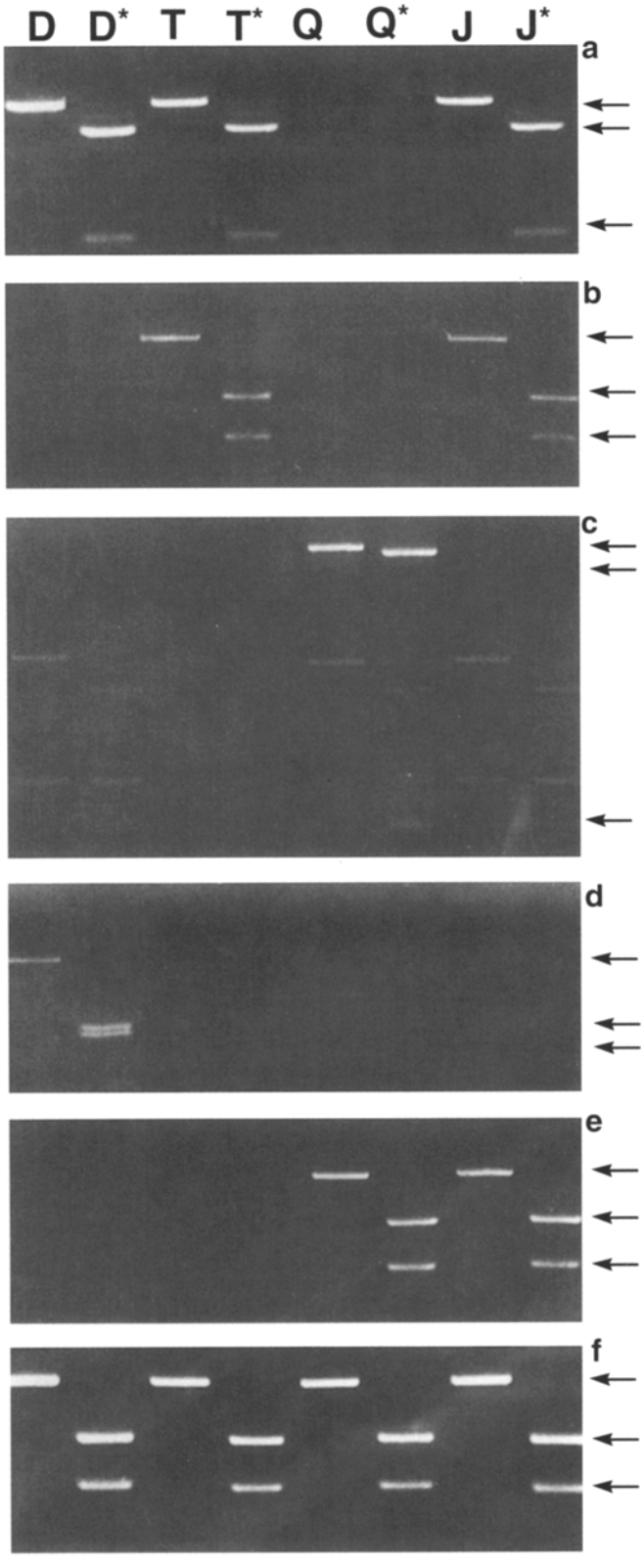

Fig. 2 *S*-allele-specific PCR analysis. Four apple varieties, i.e. 'Danziger Kantapfel' (*D*), 'Transparent von Croncels' (*T*), 'Queen's Cox' (*Q*) and 'Jonagold' (*J*), were analysed for the presence of the *S2*-, *S3*-, *S5*-, *S7*- and *S9*-allele (**a**, **b**, **c**, **d**, and **e**, respectively) and for the genomic fragment D (**f**). Tracks *D*, *T*, *Q* and *J* show the amplified PCR product; tracks *D**, *T**, *Q** and *J** show the PCR product after digestion with a specific restriction endonuclease (see Table 2). The position of the PCR products and the restriction fragments is indicated by an *arrow* (the corresponding band sizes are shown in Table 2)

vely. Indeed, *S5*-cDNA was present in cvs 'White Transparent' (*S1S5*) and 'Schöne von Boskoop' (*S2S3S5*), whereas the *S9*-cDNA was only present in cv 'Wellington' (*S8S9*). This means that the S-genotypes of cvs 'Idared' and 'Queen's Cox' are *S3S7* and *S5S9*, respectively. To avoid confusion, we have consistently used Kobel's *S*-allele numbers for the cloned sequences throughout this paper.

To further test the validity of the described method for *S*-allele identification, we analysed the varieties investigated for segregation of the iso-enzyme marker gene *GOT-1* by Manganaris and Alston (1987). These authors found that the deviant *GOT-1* segregation ratios that were observed in the progeny of crosses between certain varieties could be explained by assuming a close linkage between the *GOT-1* gene and the *S*-locus. On the basis of these *GOT-1* analyses, four different *S*-alleles were discriminated, and the *S*-genotype of a few cultivars was predicted (see Table 3B). In the paper mentioned, the *S*-alleles were named '*S1*' to '*S4*', a numbering that does not correspond with the numbering of *S*-alleles in the original paper of Kobel et al. (1939). To avoid confusion, the *S*-alleles deduced by Manganaris and Alston (1987) will be put between quotation marks (e.g. '*S1*'). Analysis of the varieties studied by Manganaris and Alston (1987) by our allele-specific PCR amplification method allowed the alleles termed '*S1*', '*S2*', '*S3*' and '*S4*' in their paper to be related to those assigned by Kobel et al. (1939). Since the *S7*-allele was only present in 'Idared' and 'Jonathan', the *S7*-allele could be related to the allele '*S4*'. The other *S*-allele of 'Idared', being the *S3*-allele, would therefore be identical to the allele '*S3*', which is consistent with the derived genotypes of 'Fiesta', 'Kent' and 'Granny Smith'. The linkage of the *S*-alleles of 'Idared' with the *S3*- and *S7*-alleles of 'Kobel', respectively, has also been confirmed by *GOT-1* analysis (F. Alston, personal communication). We found the *S3*-allele also to be present in 'Elstar', which implies that the *S5*-allele of 'Elstar' corresponds to allele 'S2'. The *S5*-allele was present in 'Queen's Cox', 'Fiesta' and 'Gala', which is also consistent with the data derived from *GOT*-1 analysis. The *S9*-allele of 'Queen's Cox' could be related to the allele termed '*S1*' by Manganaris and Alston (1987), which is also consistent with the derived *S*-genotype of 'Kent'.

Discussion

In this study we have identified cDNA sequences encoding different S-RNases of apple. The screening of a pistil cDNA library of cv 'Idared' yielded two cDNAs that show similarity to the *S*-cDNAs previously identified in

Table 3 *S*-allele typing of apple cultivars

Cultivar	*S*-phenotype[a]	PCR analysis *S2*	*S3*	*S5*	*S7*	*S9*	*D*	*S*-genotype[b]
A								
Roter Sauergrauech	*S1S3*	−	+	−	−	−	+	*S3*
White Transparent	*S1S5*	−	−	+	−	−	+	*S5*
Ontario	*S1S8*	−	−	−	−	−	+	
Transparent von Croncels	*S2S3*	+	+	−	−	−	+	*S2S3*
Champagner Reinette	*S2S4*	+	−	−	−	−	+	*S2*
Danziger Kantapfel	*S2S7*	+	−	−	+	−	+	*S2S7*
Wellington	*S8S9*	−	−	−	−	+	+	*S9*
Schöne von Boskoop (3n)	*S2S3S5*	+	+	+	−	−	+	*S2S3S5*
B								
Queen's Cox	*S5S9* (*'S2' 'S1'*)	−	−	+	−	+	+	*S5S9*
Idared	*S3S7* (*'S3' 'S4'*)	−	+	−	+	−	+	*S3S7*
Fiesta	*S3S5* (*'S3' 'S2'*)	−	+	+	−	−	+	*S3S5*
Kent	*S3S9* (*'S3'*, not *'S1'*)	−	+	−	−	+	+	*S3S9*
Jonathan	*S7*, not *S3* (*'S4'*, not *'S3'*)	−	−	−	+	+	+	*S7S9*
Granny Smith	*S3*, not *S9* (*'S3'*, not *'S1'*)	−	+	−	−	−	+	*S3*
Elstar	*S5* (*'S2'*)	−	+	+	−	−	+	*S3S5*
Gala	*S5* (*'S2'*)	+	−	+	−	−	+	*S2S5*
Golden Delicious	not *S5* (not *'S2'*)	+	+	−	−	−	+	*S2S3*

[a] **A**: assayed by pollination tests (Kobel et al. 1939); **B**: deduced from results of *GOT-1* linkage analysis (Mangaris and Alston 1987), according to the numbering of Kobel; *S*-phenotypes following the numbering of Manganaris and Alston (1987) are shown between brackets

[b] Determined by allele-specific PCR amplification and restriction enzyme digestion (this study)

apple and to the *S*-genes present in solanaceous species. Two other *S*-allele cDNAs were cloned after RT-PCR on pistil RNA of cv 'Queen's Cox'. One cDNA from cv 'Idared' was identical to the *S3*-cDNA that was previously isolated from a cDNA library of cv 'Golden Delicious' (Broothaerts et al. 1995). By allele-specific PCR analysis of different cultivars with known *S*-phenotype, which had been previously assigned on the basis of in situ pollen tube growth (Kobel et al. 1939), it was further shown that the other cloned cDNA from 'Idared' and the cDNAs from 'Queen's Cox' correspond with the *S7*-, *S5*- and *S9*-allele, respectively.

The cDNA sequences described herein, together with the *S*-allele cDNAs of 'Golden Delicious' described in our previous paper on SI in apple, are the first cDNA sequences reported for *S*-alleles in a plant belonging to the Rosaceae family. In styles of Japanese pear (*Pyrus serotina* Redh.), which is also a member of the Rosaceae, *S*-gene-related basic RNases were identified by isoelectric focusing with subsequent staining for RNase activity (Sassa et al. 1992). In a more recent study, the NH_2-terminal amino acid sequences of three *S*-allele-associated proteins in Japanese pear were published (Sassa et al. 1993). These partial sequences are very similar to their counterpart in the derived amino acid sequences of the cloned *S*-cDNAs. In addition, Sassa et al. (1994) identified SI-related glycoproteins in styles of apple cultivars using two-dimensional electrophoresis (2D-PAGE). One of the two S-proteins from 'Golden Delicious' identified by this means had a mo-lecular mass of 29 000 and could, therefore, be the 29-kDa ribonuclease that had been purified earlier from pistils of 'Golden Delicious' (Broothaerts et al. 1995).

The pairwise amino acid sequence similarities among the mature proteins S2, S3, S7 and S9 (alignment of the S5 amino acid sequence was omitted in these calculations as the S5 sequence is not full-length) range from 65% (between S2 and S3) to 74% (between S7 and S9). Most of the observed sequence homology occurs in defined regions throughout the whole protein sequence (see Fig. 1). This clustering of conserved residues leaves some of the intervening sequence regions quite variable, while others are more or less conserved. The high degree of sequence diversity between the allelic variants of the S-protein seems to be a characteristic feature of the *S*-gene (Ioerger et al. 1990).

The longest stretch of identity between the five S-proteins of apple (residues 21 to 39 in Fig. 1) corresponds with one of the conserved regions (C2) identified by comparison of 12 solanaceous S-proteins (Ioerger et al. 1991). Region C3 (residues 89 to 94 in Fig. 1) is also fairly well-conserved in apple *S*-allele sequences. The conserved regions C2 and C3 have previously been found to be highly similar to the active site of two fungal ribonu-cleases, namely RNase Rh and RNase T2 (McClure et al. 1989). The residues conserved in both the *S*-allele sequences of apple and the ribonucleases Rh and T2 include two histidines (His-33 and His-90 in Fig. 1) essential for RNA catalysis (Kawata et al. 1990) and four cysteines (Cys-48, Cys-93, Cys-157 and Cys-195 in Fig. 1) involved in disulfide bridges (Kawata et al. 1988).

To ensure that the cloned *S*-allele cDNAs are involved in SI, we attempted to relate them to the *S*-alleles assigned on a phenotypic basis by Kobel et al. (1939). Therefore, we developed a simple molecular technique for the identification of the *S*-allele sequences, which is based on allele-specific PCR amplification of genomic DNA followed by allele-specific restriction analysis. Allelic diversity between the *S*-allele sequences of apple was sufficiently high so as to be able to select a pair of specific PCR primers and a specific restriction endonuclease for each *S*-allele. PCR analysis was applied to a number of apple varieties with known *S*-phenotype,

based on cross-incompatibility between cultivars (Kobel et al. 1939), and yielded consistent results. In addition, our PCR data were also consistent with available data on the *S*-genotypes derived by *GOT-1* analysis, an iso-enzyme marker gene that is closely linked to the *S*-locus in apple (Manganaris and Alston 1987). Hence, these data, together with the striking homology to the well-studied *S*-genes from solanaceous plants, indicate that the identified *S*-cDNAs are derived from the *S*-locus alleles in apple.

On the basis of the 2D-PAGE profiles of apple *S*-glycoproteins (named S^a, S^b, S^c, S^d, S^e and S^f), Sassa et al. (1994) proposed putative *S*-genotypes of some apple cultivars. Comparison of the proposed *S*-genotypes of cvs 'Golden Delicious', 'Jonagold' and 'Jonathan' with the *S*-genotypes of these apple cultivars deduced by our *S*-allele-specific PCR analysis revealed that the S^c- and S^e-glycoprotein could be related to the *S9*- and *S7*- allele (described in this paper), respectively. The S^a-glycoprotein (as well as the S^b-glycoprotein) corresponds to either the *S2*- or the *S3*-allele.

Since the determination of the *S*-genotype by test pollinations or by progeny analysis for *GOT-1* segregation often is a complex task, it is desirable to replace these procedures by more direct methods allowing the immediate identification of the *S*-allele pattern in each individual plant considered. The *S*-allele selective identification method described here is simple and requires only small amounts of DNA, preferably obtained from young leaves, as starting material.

A similar molecular approach for the identification of *S*-allele in *Brassica oleracea* has been reported by Brace et al. (1994). The technique used by these authors is based on the PCR amplification of genomic DNA using a single pair of conserved primers, followed by digestion with six different restriction endonucleases. Since primers corresponding to conserved regions in *Brassica* *S*-alleles are used (in contrast to our method, which uses primers to variable regions), this method can be applied for the identification of *S*-alleles where appropriate sequence data are not available. However, a disadvantage of their method is that in heterozygotes there may be a preferential amplification of one of the two *S*-alleles, which will then hamper the interpretation of the results.

In our previous study on SI in apple, short genomic sequences from DNA of several apple cultivars were amplified by PCR using amplimers corresponding to conserved regions in the *S*-genes of Solanaceae. One of the genomic fragments was shown to be generally present in all apple cultivars tested (fragment D; Broothaerts et al. 1995). In our *S*-allele typing analysis, we routinely use specific amplification and specific restriction enzyme digestion of the genomic fragment D as a control for DNA integrity. Since fragment D appears to be non-polymorphic, we presume that fragment D is not involved in SI and is, therefore, derived from a gene that is not an *S*-allele. An *S*-gene-homologous sequence (and corresponding RNase), named X2, that is not associated with SI, has also been identified in *P. inflata,* a species exhibiting SI (Lee et al. 1992). RNase X2 shows significant homology to the S-RNases, is pistil-specific and is an extracellular enzyme as are the true S-RNases. It has been suggested that RNase X2 may help defend the pistil against pathogen attack. Other S-like-RNases that show similarity to the S- and fungal T2-type RNases have been isolated from several self-compatible plants (reviewed by Green et al. 1994). These S-like-RNases may participate in starvation rescue and senescence, as well as in general RNA turnover. Further work is needed to characterize the gene product of the genomic fragment D and its expression pattern.

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Note added in proof

During the preparation of this manuscript, a paper was published (Battle I, Alston FH, Evans KM (1995) The use of the isoenzymic marker gene *GOT-1* in the recognition of incompatibility *S*-alleles in apple. Theor Appl Genet 90:303–306) that further confirmed the linkage of the *GOT-1* gene to the *S*-alleles *S3*, *S5*, *S7* and *S9* of Kobel et al. (1939). These data are consistent with the results reported here.