Theor Appl Genet (2003) 106:703–714
DOI 10.1007/s00122-002-1120-0

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New findings in apple S-genotype analysis resolve previous confusion and request the re-numbering of some S-alleles

Received: 14 May 2002 / Accepted: 19 June 2002 / Published online: 7 November 2002
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Abstract Apple trees display gametophytic self-incompatibility which is controlled by a series of polymorphic S-alleles. To resolve the discrepancies in S-allele assignment that appeared in the literature, we have re-examined the identity of S-alleles known from domestic apple cultivars. Upon an alignment of S-allele nucleotide sequences, we designed allele-specific primer pairs to selectively amplify a single S-allele per reaction. Alternatively, highly similar S-alleles that were co-amplified with the same primer pair were discriminated through their distinct restriction digestion pattern. This is an extension of our previously developed allele-specific PCR amplification approach to reveal the S-genotypes in apple cultivars. Amplification parameters were optimised for the unique detection of the 15 apple S-alleles of which the nucleotide sequences are known. Both the old cultivars with a known S-genotype and a number of more common cultivars were assayed with this method. In most cases, our data coincided with those obtained through phenotypic and S-RNase analysis. However, three S-alleles were shown to relate to RNases that were previously proposed as being encoded by distinct S-alleles. For another S-allele the corresponding gene product has not been discriminated. Consequently, we propose the re-numbering of these four S-alleles. Furthermore, two alleles that were previously identified as S_{27a} and S_{27b} now received a distinct number, despite their identical S-specificity. To ease widespread future analysis of S-genotypes, we identified common cultivars that may function as a witness for bearing a particular S-allele. We discuss the assignment of new S-alleles which should help to avoid further confusion.

Keywords Allele-specific PCR · *Malus × domestica* · S-allele · S-RNase · Self-incompatibility · Genotyping

Communicated by H.F. Linskens

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Introduction

All apple varieties, without exception, exhibit a self-incompatibility mechanism, preventing fertilisation following self-pollination (reviewed in de Nettancourt 2001). It has been an area of much research during recent years to understand by which mechanism the pistil recognises (in)compatible pollen. In the gametophytic SI system that is operating in apple and many other species, one gene residing at the S-locus is known, i.e. the S-gene, which encodes a family of ribonucleases in the pistil. These S-RNases specifically interact with a component in the male partner, encoded by an as yet unknown gene residing at the same S-locus, and presumably acting as an inhibitor of all non-corresponding S-RNases (Golz et al. 2001). The recognition between the allelic products of the S-locus genes determines whether or not further pollen-tube growth is arrested in the style.

The simple genetics of the gametophytic SI system implies that the cross-pollination behaviour of a variety may be predicted from its S-allele constitution. This is obviously important for the design of fruit tree orchards (Schneider et al. 2001), but also in selecting suitable breeding strategies, avoiding sterile crossings. Cross-compatibility between varieties is difficult to assess phenotypically, as environmental and physiological factors have an impact on the outcome of pollination tests. Additionally, varieties differ significantly in the severity of the self-incompatibility response, which is never a complete barrier in apple, and fruit may also develop parthenocarpically. Nevertheless, careful pollination studies, involving microscopic evaluation of pollen-tube growth through the pistil, have allowed Kobel and co-

workers (1939) to discriminate 11 different S-alleles in apple (S_1 to S_{11}). His original work resolved the S-genotypes of 14 diploid and 12 triploid varieties. However, the S-genotype of five additional 3n varieties that were examined was only partially deduced, the remaining allele(s) being specified as Sx or Sy. A more recent investigation of the incompatibility relationships among mainly Japanese cultivars was carried out by Komori et al. (2000), who assigned a letter symbol to ten S-alleles (Sa to Si and Sz) and reported their correspondence to four of Kobel's S-alleles. Some of the latter S-alleles (Sa to Sf) were previously also discriminated by their gene products, which revealed characteristic migration patterns in the alkaline region of IEF or 2D-PAGE gels (Sassa et al. 1994, 1996). To complicate the area further, S^e defined by Sassa is different from Se defined by Komori (see Komori et al. 2000). Using IEF and NEPHGE followed by RNase activity staining, Bošković and Tobutt (1999) identified the gene product for S_1 to S_{11}. Interestingly, some of Kobel's results were rejected by the latter investigation, and the occurrence of 14 additional S-alleles (numbered S_{12} to S_{25}) was proposed. Most of the new S-alleles only occurred in a single cultivar and were discriminated on the basis of often small differences in mobility on IEF or NEPHGE gels. By nucleotide sequencing, Van Nerum et al. (2001) indeed showed that S_{22} (attributed to the apple cultivar Alkmene), S_{23} (Delbard Jubilé) and S_{25} (Merlijn) were identical and corresponded to the previously identified S_{27b}-allele (Verdoodt et al. 1998). As a further complication, the presumed S_{25}-allele in Telamon, however, was shown to correspond to the sequenced S_{10}-allele. The same study also showed that the "Japanese" Sg (cloned by Matsumoto et al. 1999) corresponded to S_{20} of Bošković and Tobutt (1999). Kobel's S_1 to S_{11}, except S_6, S_8 and S_{11}, have been cloned and were sequenced by various groups (S_2, S_3: Broothaerts et al. 1995; S_5, S_7, S_9: Janssens et al. 1995; S_1, S_9: Sassa et al. 1996; S_4, S_{10}: Van Nerum et al. 2001; S_{10}: Kitahara and Matsumoto 2002). The sequences of additional S-alleles with a number >11 have been reported by Verdoodt et al. (1998; S_{24}, S_{26}, S_{27a}), Matsumoto et al. (1999) and Matsumoto and Kitahara (2000; Sg, respectively Se), Van Nerum et al. (2001; S_{27b}) and Schneider et al. (2001; S_{28} and "S_{10}", but different from the real S_{10}, see below). The sequence of Se is identical to S_{28} cloned by Schneider et al. (2001) and was also named S_{30} and S-RNase I in the GenBank (AF201748 and AB017636 respectively).

In this paper, we tried to resolve many of the discrepancies and double annotations introduced in S-allele assignments during recent years. We attempted to link the known sequences of S-allele coding regions with the phenotypes observed by Kobel and with the S-RNase bands detected by Bošković and Tobutt (1999). The basics of the method employed are the use of allele-specific primers to generate sequence-characterised amplified regions (SCARs) from genomic DNA templates by PCR, and the identification of the amplification products on an electrophoresis gel. Occasionally, PCR products were digested with an allele-specific restriction enzyme to discriminate two or more co-amplified S-allele products. Extending our previous work on apple S-genotyping, we re-designed the primers used to amplify single S-alleles if necessary to increase the amplification specificity. In one case, an additional digestion step was introduced to identify S-alleles that were previously not discriminated. Furthermore, we designed new primers for those alleles that were not previously included in this analysis (i.e. "S_{10b}" and S_{28}). As a result, 15 S-alleles can be identified by their unique amplification/digestion profiles on the gel. Several new S-alleles discovered through sequencing were shown to correspond to S-RNases that were previously identified through RNase separation (Bošković and Tobutt 1999). Consequently, we propose a re-numbering of these S-alleles in order to match the nucleotide sequences with the corresponding S-RNase gene products. A few other S-numbers that had been introduced solely on the basis of RNase zymogram analysis were rejected, following sequence analysis that showed them to be identical.

Materials and methods

Plant material

Leaves were harvested during the growing season from field-grown apple trees (cultivar collection of the Fruitteeltcentrum at Rillaar) and either processed immediately, or frozen in liquid nitrogen, and either stored at –80 °C or lyophilized for later use. Leaves or budwood from less common varieties (those mentioned in Fig. 2) were obtained from the National Fruit Collections at Brogdale, UK, except for Brünnerling (Dresden, Germany), Oberrieder Glanzreinette, Oetwiler, and Kaiserapfel (Wädenswil, Switzerland); Adam's Pearmain was obtained from different collections (Balsgard, Sweden; Brogdale, UK; Gembloux, Belgium; Horticulture Research International, UK). I greatly acknowledge the help of E. Pauwels, I. Dewit, K. Tobutt, P. Martin, M. Lateur, H. Nybom, M. Fischer, M. Kellerhals and L. Royen in collecting this material.

Nucleotide alignment

Nucleotide sequences of S-alleles were retrieved from GenBank and aligned using the Clustal algorithm in the MegAlign software of DNASTAR.

S-allele-specific PCR

Genomic DNA was isolated from dried apple leaves, pulverized in a ribolyser apparatus (Savant-120), or from frozen tissues, using the Genomic DNA Purification Kit (MBI Fermentas). Allele-specific PCR amplification was done according to the conditions described in Table 1, using Perkin Elmer GeneAmp 2400 thermal cyclers, programmed as follows (referred to as the standard PCR programme): 3 min at 94 °C, 30 cycles of 15 s at 94 °C, 15 s at 60 °C, and 30 s at 72 °C, and finally 2 min at 72 °C, followed by cooling to 4 °C. Standard PCR conditions (in 20 µl total volume) included 1 × PCR buffer (Promega), 1.75 mM of $MgCl_2$, 200 µM dNTPs, 1 µM of each primer, and 0.6 U of *Taq* DNA polymerase (Promega). Roughly 100 ng of genomic DNA template were used per reaction. We tried to standardize the PCR conditions as much as possible, e.g. using an annealing temperature of 60 °C, and an extension time of 30 s. Under these conditions, the amplification

appeared sufficiently selective for the S-allele assayed, although some primer pairs could work at higher annealing temperatures. Only in a few cases, the standard conditions had to be adapted for optimal amplification (see Table 1). For discrimination of S_4/S_{27a}/S_{27b}, part of the amplification product was digested by the allele-specific restriction endonuclease *Taq*I (1 h, 65 °C), and digestion products were run on 2.5% agarose gels along the undigested PCR products. Similarly, the amplification product obtained using S_{20}-primers was digested with *Nar*I (overnight, 37 °C) to discriminate S_{20} from the group S_{14}/S_{17}/S_{21}. Remarks further indicated that the size of the (undigested) PCR product obtained using the S_{20}-primers reflects the presence of S_6 (850 bp) or $S_{20/14/17/21}$ (920 bp). Primer design was done manually taking into account the general principles for primer design (GC content and distribution, avoiding stretches of G/C or A/T nucleotides). All primers have been carefully tested against many different DNA samples and were replaced by others in case of non-specific amplification. Note that some of the primers described previously (Janssens et al. 1995; Verdoodt et al. 1998; Van Nerum et al. 2001) have been slightly modified. The sequences of the allele-specific primers employed are shown in Table 1.

Results

Design of allele-specific primer pairs for 15 S-alleles

In order to select primers for allele-specific amplification, the nucleotide sequences of all 15 S-alleles that had been cloned from different apple varieties were aligned (Fig. 1). Pairwise similarities varied considerably among alleles. At the lower end, S_1 and S_2 sequences were only 70% identical in the region shown, and the same divergence was found between S_2 and both S_{20} and S_{24}. On the other hand, S_3 and S_{10} were over 96% identical, and S_{20} and S_{24} over 95%. The S_{27a} and S_{27b} sequence share 99% identical residues, but as none of the substitutions are translated into the protein sequence nor have an effect on the SI phenotype, these sequences were originally considered different forms of the same S_{27}-allele (Van Nerum et al. 2001). However, because they de facto differ at the nucleotide level, these sequences must be considered as alleles and should, therefore, receive a different number (see below). Comparing all the sequences, several conserved regions can be identified (see asterisks above alignment in Fig. 1). The largest highly conserved region is located near the N-terminus of the unprocessed protein, consisting of 133 perfectly conserved residues on a stretch of 200 (from base 1 to 200). Some alleles were also extremely similar outside the conserved regions, which hampered the search for unique stretches of sequence that were sufficiently discriminating from the other S-alleles. Primer pairs were designed for the most-variable regions, ensuring that the amplified products in between the primer annealing sites were between 200 and 1,000 bp, allowing their separation by conventional agarose gel electrophoresis. In the case of S_3 and S_{10}, both of which have an intron of >1 kb, the primers had to be chosen in the second exon, which further complicated the discrimination of these highly similar sequences. The allele-specific primers employed in this work are marked in Fig. 1. We tried to select allele-specific sequences for both the forward and reverse primer to safeguard the specificity of the PCR against putative unknown S-alleles. In a few cases (see below), the original primers were found to co-amplify several S-alleles, and new primers had to be designed. For four alleles (S_1, S_3, S_5 and S_{10}), we purposely tested more than one primer pair in order to ensure that the amplified fragment corre-

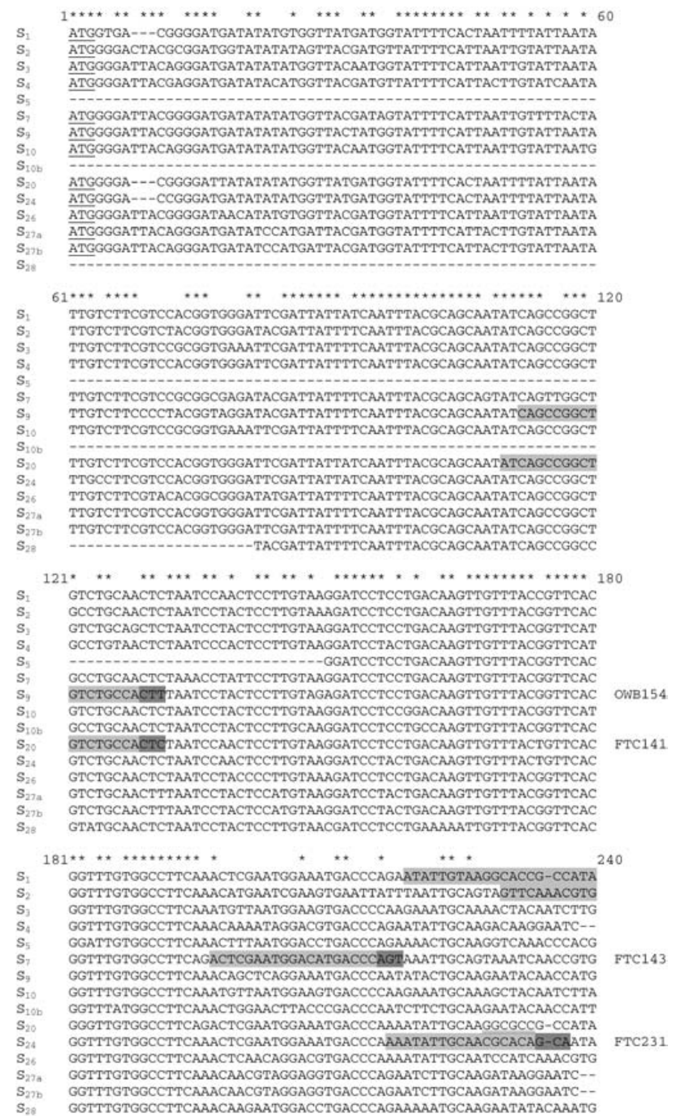

Fig. 1 Alignment of nucleotide sequences encoding apple S-alleles and location of the annealing sites of allele-specific primers. Only part of the cDNA sequences is shown, i.e. the region from the protein translation initiation site (ATG) to the stop codon (*underlined*) and part of the 3′ UTR. Conserved residues are denoted by an *asterisk*. The location of the single intron is indicated by a *triangle* above the alignment. Allele-specific primer positions (see names at the right) are highlighted in *gray*, and their orientation is indicated by the *darker color* surrounding the three nucleotides at the primer's 3′-end. Similarly, alternative primers to identify specific alleles (see text) are *single underlined* (*dotted line* and *full line* at their 3′-end) and named between *brackets*. Restriction enzyme sites used to differentiate amplification products are *double underlined*. Sequences at the 5′- or 3′-end that were not determined or internal gaps that are introduced during the CLUSTAL alignment are shown by a "-"

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        ▽
241     *          *     *    *    * * *** *** ******** *****  300
S1    -----TCATACGATAAA---AATGCTCGAACCCCAGTTGGTAATTATTTGGCCGAACGTA  FTC168
S2    ACTTATGCGAAGATACA---AAATATCCGAACCCAGTTGGAAATGATTTGGCCAAACGTA  OWB122
S3    AACCCTCAAACGATAAC---AAATCTTACAGCCCAGCTGGAAATTATTTGGCCAAACGTA  FTC177
S4    GTCGGAAGAGAGCAAA----AACACTCGAACCCCAGTTGGAAATTATTTGGCCGAACGTC
S5    GCGTCTCAAACGATAGATACATCACTCAAACCCCAGTTGGAAATTATTTGGCCGAACGTA
S7    GATGCTCAGAAGTTAGG---AAATCTGACAACTCAGTTGGAAATAATTTGGCCGAACGTA
S9    AATTCTACTAAGATAGC---AAATCTGACAGCCCGGTTGGAAATTATTTGGCCCAACGTA  OWB155
S10   AATCCTCAAACGATAAC---AAATCTTAAAGCCCAGCTGGAAATTATTTGGCCAAACGTA
S10b  ACGCCTCAGCAGATAGG---ACATATCGAAGCCCAGTTGGAAATTATTTGGCCGAACGTA
S20   -----TCAGACGATAAA---AATACTCGAACACCAGTTGGCAATTATTTGGCCGAACGTA
S24   -----TCAGACGATGAA---AATACTCGAACCCCAGTTGGTAATTATTTGGCCGAACGTA
S26   ACTTCTCATATGCTAAA---AAATATCCAAGCCCAGTTGGAAATTATTTGGCCGAACGTA
S27a  ATCGAACGAGAGCAAA----AGCACTCGAACCCCAGTTGGAAATTATTTGGCCGAACGTA
S27b  ATCGAACGAGAGCAAA----AGCACTCGAACCCCAGTTGGAAATTATTTGGCCGAACGTA
S28   AATTCTCAGAAGATAGG---AAATATGGCAGCCCAGTTGGAAATTATTTGGCCGAACGTA

301 * *****      ****      *******    * * * ***    ***** ***    ** 360
S1    CTCAATCGAAACGATCATGAAGGCTTCTGGCGTAAACAGTGGGATAAACATGGCTCTTGT  FTC169
S2    TTCAATCGAAAAAATCATTTAGGCTTCTGGAATAGAGAGTGGAACAAACACGGCGCCTGT
S3    CTCAATCGAAAGGCTCATGCACGCTTCTGGCGTAAACAGTGGCGTAAACATGGCACCTGT
S4    CTCGATCGAACCAATCATACAGGCTTCTGGCGTAGACAGTGGAAAAAACATGGCACCTGT
S5    TTCAATCGAGCCGATCATGAAAGCTTCTGGCAAAAACAGTGGGACAAACATGGCACCTGT
S7    TACAATCGAACCGATCATATAAGCTTCTGGGATAAACAGTGGAACAAACATGGCACCTGT
S9    CTCGATCGAACCGATCATATAACCTTCTGGAATAAACAGTGGAACAAACATGGCAGCTGT
S10   CTCAATCGAAGGGCTCATGTACGCTTCTGGCGTAAACAGTGGCGTAAACATGGCGCCTGT  FTC12 (227)
S10b  TTCAATCGAACCAATCATTTGGTCTTCTGGAATAAACAGTGGAACAAACATGGCAGCTGT  FTC222
S20   CTCAATCGAAACGATCATGAAGGCTTCTGGCGTAAACAGTGGGAGAAACATGGCTCCTGT
S24   CTCAATCGAAACGATCATGAAGGCTTCTGGCGTAAACAGTGGGAGAAACATGGCTCCTGT
S26   CTCAATCGAACAGATCATATAGGCTTCTGGGAAAGACAATGGAAAAAACATGGCAGCTGT
S27a  CTCGATCGAACCAATCATACAGCCTTCTGGCGTAGACAGTGGATAAAACATGGCACTTGT
S27b  CTCGATCGAACCAATCATACAGCCTTCTGGCGTAGACAGTGGATAAAACATGGCACTTGT
S28   CTCAATCGAACTGATCATGTAGGCTTCTGGGAAAGAGAGTGGCTCAAACATGGCACCTGC  FTC229

361*      **  ****     ** *      * ** **     ** *** **  ******** *420
S1    GCGTCTTCCCCAATACAGAACCAGAAGCATTACTTTGATACAGTAATCAAAATGTACACA
S2    GGGTATCCCACAATACGGAACGACTTGCATTACTTTCAAACAGTAATCAAAATGTACATA
S3    GGGTACCCCACAATAGCGGACGACATGCATTACTTTAGCACAGTAATCGAAATGTACATA
S4    GGGTATCCCACAATACAGAACGAGAATGATTACTTTGAAACAGTAATCAAAATGTACATA  FTC5
S5    GGGTCTCCCACAATAATAGACAAGAACCATTACTTTGAAACAGTAATCAGAATGTACATA  FTC10
S7    GGACATCCCACAATAATGAACGACATTCATTACTTTCAAACAGTAATCAAAATGTACATA  FTC144
S9    GGGCATCCCGCAATACAGAACGACATGCATTATTTGCAAACAGTAATCAAAATGTACATA
S10   GGGTACCCCACAATAGCGGACGACATGCATTACTTTAGCACAGTAATCGAAATGTACATA
S10b  GGGTATCCCATAATAAATGACGAAATACAATACTTTGAAACTGTAATCAAAATGTACATA
S20   GCGTCTTCCCCAATACAGAACCAGAAGCATTACTTTGATACAGTAATCAAAATGTACACA
S24   GCGTCTTCCCCAATACAGAACCAGAAGCATTACTTTGATACAGTAATCAAAATGTACACA
S26   GGGCGTCCCGCAATAACGAACGAGGTGGATTACTTTCAAACAGTAATCAAAATGTACATA
S27a  GGGTATCCCACAATACAGAACGAGAATGATTACTTCGAAACAGTAGTCAAAATGTACATA  FTC5
S27b  GGGTATCCCACAATACAGAACGAGAATGATTACTTCGAAACAGTAGTCAAAATGTACATA  FTC5
S28   GGGTATCCCACAATAAGGGACGACATGCATTACTTAAAAACAGTAATCAAAATGTACATA

421*    **** *****  * **     ** **   * *   ** *   **   * **         480
S1    ACCCAGAAACAAAACGTCTCTGAAATCCTCTCAAAGGCGAATATAAAACCTGGTAGGAAA
S2    ACCCAGAAACAAAACGTCTCTGATATCCTCTCAAAGGCCAAGATTGAACCGGATGGAAAT
S3    ACCAAGAAACAAAATGTCTCTGAAATCCTCTCAAAGGCGAAAATTAAACCGGAGAAGAAA  (FTC178)
S4    ACCGAGAAACAAAACGTCTCTCGAATCCTCTCAAATGCAAAGATTGAACCAGACGGGCAA
S5    ACCGAGAAGCAAAACGTCTCTTATATCCTATCCAAAGCCAATATCAATCCGGATGGTAAA
S7    ACCCAGAAACAAAACGTCTCTAAAATCCTCTCAAGGGCGAAGATTGAACCGGAGGGRAAA
S9    ACCCAGAAACAAAACGTCTCTGAAATCCTCTCAAAGGCGAAGATTGAACCGGTGGGGAAA
S10   ACCAAGAAACAAAACGTCTCTGAAATCCTCTCAAAGGCGAAGATTAAACCGGAGGGGAGA  FTC228 (223)
S10b  ATCAAGAAACAAAACGTCTCTAAAATCCTCTCAAGGGCCAAGATTAAACCGGAGGGGAAA
S20   ACCCAGAAACAAAACGTCTCTGAAATCCTCTCAAAGGCGAATATAAAACCGGGTAGGAAA
S24   ACCCAGAAACAAAACATCTCTGAAATCCTCTCAAAGGCGAATATAAAACCGGGTAGGAAA  FTC232
S26   ACCCAGAAACAAAATGTATCCAAAATCCTCTCAAAAGCGAAGATTGAACCAGAGGGGCGA
S27a  ACTGAGAAACAAAACGTCTCTAAAATCCTCTCAAATGCAAAGATTGAACCGGACGGGATA
S27b  ACCGAGAAACAAAACGTCTCGAAAATCCTCTCAAATGCAAAGATTGAACCGGACGGGATA
S28   ACCCAGAAACAAAACGTCTCTGCAATTCTCGCAAAGGCGATGATTCAACCGAACGGGCAA
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Fig. 1 (continued)

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481  **          *   **  **         ****** *       **   *     * *  *** 540
S1    AACAGGCCACTGGTGGACATTGAAAATGCCATACGCAATGTTATCAACAATATGACACCA  (FTC233)
S2    ATCAGGACACAGAAGGAAATTGTAGATGCCATAAGAAAAGGTATCCATGGTAAGGAACCA  OWB123
S3    TTCAGGACACGGGACGACATTGTGAATGCCATAAGCCAAAGTATCGACTATAAGAAACCA
S4    AGCAGACCGCTGGTGGACATTGAAAATGCCATACGCAACGGTACCCACAATAAGAAACCG
S5    GGCAGGACACGGAAGGATATTCAAATTGCCATACGCAATAGTACCAACGATAAGGAACCA  (FTC239)
S7    CCCAGGAAACAGGTAGATATTGTAAATGCCATACGCAAAGGTACAAACGATAAGGAACCA
S9    TTCAGGACACAGAAGGAAATTGAAAAGGCCATACGCAAAGGTACCAATAATAAGGAACCC
S10   TTCAGGACACGGGACGACATTGTAAATGCCATAAGCCAAAGTATCGACTATAAGAAACCA
S10b  AACAGGACACGGACGGAAATTATAAACGCCATAAGCATTAGTACCAACAATATGACACCA  FTC224
S20   AACAGGACACTGGTGGATATTGAAAATGCCATACGCAATGTTATCAACAATATGACACCA
S24   AACAGGACACTGGTGGATATTGAAAATGCCATACGTAATGTTATCAACAATATGACACCA
S26   ATCAGGATGCTGAAGGATATTGAAGATGCCATACGCAATGGTACCAACAATAAGAAACCA  FTC14
S27a  AAGAGAACACTGGCGGACCTTGAAATTGCCATACGCAGTGGTACTGACAACAAGAAACCA
S27b  AAGAGAACACTGGCGGACCTTGAAATTGCCATACGCAGTGGTACGGACAACAAGAAACCA
S28   AACAGGTCATTGGTGGATATTGAAAATGCCATACGCAGTGGTACCAACAATATGAAACCA

541 *  *******  * *  ** *    *            * ***** ****** *   * **600
S1    AAATTCAAGTGCCAAAAGAATACTAGGACATCATTGACTGAATTGGTTGAGGTTGGTCTT
S2    AACCTCAAGTGCCAAAAGAATACTCAGA------TGACTGAATTGGTTGAGGTCACTCTT
S3    AAACTCAAGTGCAAGAACAATAATCAGA------TAACTGAATTGGTTGAGGTCGGTCTT
S4    AAATTCAAGTGCCAAAAGAATAATGGGG------TGACTGAATTGGTTGAGATCACTCTT
S5    AAACTCAAGTGCCAAACGAAGAATGGGA------TAACTGAATTGGTTGAGGTCTCTCTT
S7    AAACTCAAGTGCCAAAAGAATAACCAGG------TGACTGAATTGGTTGAGGTCACTCTT
S9    AAACTCAAGTGCCAAAAGAATAGTCAGA------GGACTGAATTGGTTGAGGTCACTATT
S10   AAACTCAAGTGCAAGATCAATAATCAGA------CAACTGAATTGGTTGAGGTCGGTCTT
S10b  AAACTCAAGTGCCAAAAGAATAATGGGA------CGATTGAATTG---------------
S20   AAATTCAAGTGCCAAAAGAATACTAGGACATCATTGACTGAATTGGTTGAGGTCGGTCTT
S24   CAATTCAAGTGCCAAAAGAATACTAGGACATCATTGACTGAATTGGTTGAGGTCGGTCTT
S26   AAACTCAAGTGCCAAAAGAATAGTAGGA------TGACTGAATTGGTTGAGGTCACTCTT
S27a  AAATTCAAGTGCCAAAAGAAGAGAAGGG------TGACTGAATTAGTTGAGGTCACTCTT
S27b  AAATTCAAGTGCCAAAAGAAGAGAAGGG------TGACTGAATTAGTTGAGGTCACTCTT
S28   AAATTCAAGTGTCAAAAGAATACTAGGACA--A-CGACGGAATTGGTTGAGGTCACTCTT

601**  *  **      *  *      *****  **** ***                     **660
S1    TGCAGTGATAGCAACTTAACGCAGTTCATAAATTGCCCCCGCCCATTTCCACAAGGATCA
S2    TGCAGCGATGGCAACTTAAAGCAGTTCATAGATTGCCCCCACCATTTTCCAAATGGATCA
S3    TGCAGCGATAACAACTTAACGCAGTTCATAGATTGCCCCCGCCCATTTCCACAAGGATCA
S4    TGCAGCGATAAAAACAGAGCACATTTCATTGATTGCCCCAATCCCTTTCTACCCGGATCA  OWB249
S5    TGCAGCAATTACTTCGGAAAAAATTTCATAAATTGCCCCAACAAAACTCCAGGAAAAACA
S7    TGCAGTAATCGCAACCTAACGGGGTTCATAAATTGCCCCCGCCATATTCCAAATGGATCA
S9    TGTAGCGATCGCAACTTAAACCAGTTCATAGATTGTCCCCGCCCTATTTTAAATGGATCA
S10   TGCAGCGATAACAACTTAACGCAGTTCATAAATTGCCCCAACCCATTTCCTCAAGGATCA
S10b  ------------------------------------------------------------
S20   TGCAGCGATAGCAACTTAACGCAGTTCATAAATTGCCCCCGCCCATTTCCACAAGGATCA
S24   TGCAGCGATAGCAACTTAACGCAGTTCATAAATTGCCCCCGCCCATTTCCACGAGGATCA
S26   TGCAGAGATAGCAACTTAACACAGTTCATAAATTGCCCCAGCCCAATTCTACCAGGATCA
S27a  TGCAGCGATAAAAACAGAGCACATTTCATAGATTGCCCCAACCCCTTCCAACCAGGGTCA  OWB249
S27b  TGCAGCGATAAAAACAGAGCACATTTCATAGATTGCCCCAACCCCTTCCAACCAGGATCA  OWB249
S28   TGCCGTGATAGAGACTTAACGAAGTTCATAAATTGCCCCCAACCA---CCACAAGGATCA  FTC230

661*        ** **    *     * * *  ******                        720
S1    CGGAATTTCTGCCCCACCA---ATATTCAGTATTAAGAGCGCGGCTAGCTAATGTGTGTG
S2    CGACATAACTGCCCCACCAATCATATTCTGTATTAAGAGCCCG--CGGCTAGATAGTATA
S3    CCATTTTTCTGCCCCACCAATAATATTCAGTATTAAGAGCGCAGCTAGAGTATTGCATAT
S4    CCATATTTGTGCCCCAACATCAGTATCCGGTATTAA------------------------
S5    CGATATTCCTGTCCTACCAATGATATCCATTATTAAGAGCGCGGCTAGCTTGTGTGCGTG  FTC11
S7    CGATATTCCTGCCCCACCAAAAATATTCTGTATTAAGAGCGCTGCTAGCTATATATATAG
S9    CGATACTACTGCCCCACCAATAATATTCTGTATTAAGAGCGCGGCTAGCTAGTGTGTGTG
S10   CCATATTTCTGCCCCACCAATAATATTCGGTATTAA------------------------
S10b  ------------------------------------------------------------
S20   CGGTATTTCTGCCCCACCA---GTATTCAGTATTAAGAGCACGGCTAGCTAGTGCGTCTG  FTC142
S24   CGGTATTTCTGCCCCACCA---ATATTCAGTATTAAGATCGCGGCTAGCTAGTGTGTGTG
S26   CCATTTCTCTGCCCCGCCAA---TATTCGGTATTAAAAGCACGGCTAGCTAGGATATATA  FTC9
S27a  CCATATTTATGCCCCACCAACAATATCCTGTATTAAGAGCGCGGCTATCTAGTATATATA
S27b  CCATATTTATGCCCCACCAACAATATCCTGTATTAA------------------------
S28   CGATATCTCTGCCCCGCCG---ATGTTCAGTATTAATTAAGAGCGCGGCTAGCCACAATT
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Fig. 1 (continued)

sponded with the cloned S-allele (underlined sequences in Fig. 1; details below). Of the 13 primer pairs proposed in this work, only those for S_2 (Broothaerts et al. 1995), S_5, S_9 (Janssens et al. 1995), $S_4/S_{27a}/S_{27b}$, S_{26} (Verdoodt et al. 1998) and S_{20} (Van Nerum et al. 2001) have been described before. The primer pairs for S_1, S_3, S_7, S_{10} and S_{24} have been changed from previous publications to increase their specificity or amplification efficiency. The S_{20}-primers were further shown to allow the identification of S_6 (provisional result) and another allele from the group $S_{14/17/21}$ in addition to S_{20}. The remaining two S-alleles, "S_{10b}" and S_{28}, have not been discriminated in our S-genotype analysis before, and the corresponding primer pairs are described here for the first time.

S-allele-specific PCR analysis

Following the requirement to name the allelic sequences identified in accordance with the original classification system of Kobel (1939), we examined most of the old apple varieties for which Kobel assigned an S-genotype. Using the primer pairs selected for each S-allele and the optimal conditions for their use (Table 1), amplification products were obtained for most of the cultivars that expressed the corresponding S-phenotype (Table 2). Similarly, the absence of a PCR product denoted that the cultivar did not bear the S-allele tested. To ensure that

Table 1 Nucleotide sequences and conditions for S-allele-specific PCR analysis in apple

S-allele[a]	Primers	Sequence 5′-3′	PCR programme[c]	Size of PCR product (bp)[d]
S_1	FTC168 FTC169	ATATTGTAAGGCACCGCCATATCAT GGTTCTGTATTGGGGAAGACGCACAA	Standard	+/–530
S_2	OWB122 OWB123	GTTCAAACGTGACTTATGCG GGTTTGGTTCCTTACCATGG	Standard	449
S_3	FTC177 FTC226	CAAACGATAACAAATCTTAC TATATGGAAATCACCATTCG	A 57 °C	500
S_4	FTC5 OWB249	TCCCACAATACAGAACGAGA CAATCTATGAAATGTGCTCTG	Standard, *Taq*I	274 (197+77)
S_5	FTC10 FTC11	CAAACATGGCACCTGTGGGTCTCC TAATAATGGATATCATTGGTAGG	Standard	346
S_6[b]	FTC141 FTC142	ATCAGCCGGCTGTCTGCCACTC AGCCGTGCTCTTAATACTGAATAC	E 45 sec	+/–850
S_7	FTC143 FTC144	ACTCGAATGGACATGACCCAGT TGTCGTTCATTATTGTGGGATGTC	Standard	302
S_9	FTC154 FTC155	CAGCCGGCTGTCTGCCACTT CGGTTCGATCGAGTACGTTG	Standard	343
S_{10}	FTC12 FTC228	CCAAACGTACTCAATCGAAG ATGTCGTCCCGTGTCCTGAATC	Standard	209
"S_{10b}" (S_{23})	FTC222 FTC224	CAATCGAACCAATCATTTGGT GGTGTCATATTGTTGGTACTAATG	Standard	237
S_{20}	FTC141 FTC142	ATCAGCCGGCTGTCTGCCACTC AGCCGTGCTCTTAATACTGAATAC	E 45 sec, *Nar*I	+/–920 (800+120)
S_{24}	FTC231 FTC232	AAATATTGCAACGCACAGCA TTGAGAGGATTTCAGAGATG	Standard	+/–580
S_{26}	FTC14 FTC9	GAAGATGCCATACGCAATGG TTTAATACCGAATATTGGCG	A 55 °C	194
S_{27a} (S_{16})	FTC5 OWB249	TCCCACAATACAGAACGAGA CAATCTATGAAATGTGCTCTG	Standard, *Taq*I	274 (243+31)
S_{27b} (S_{22})	FTC5 OWB249	TCCCACAATACAGAACGAGA CAATCTATGAAATGTGCTCTG	Standard, *Taq*I	274 (199+31+44)
S_{28} (S_{19})	FTC229 FTC230	TCTGGGAAAGAGAGTGGCTC TTTATGAACTTCGTTAAGTCTC	Standard	304
$S_{14/17/21}$[b]	FTC141 FTC142	ATCAGCCGGCTGTCTGCCACTC AGCCGTGCTCTTAATACTGAATAC	E 45 sec, *Nar*I	+/–920 (920)

[a] S-alleles are shown with their old number and, in brackets, the new annotation proposed here
[b] The unique identification of S_6 is only provisional; $S_{14/17/21}$ refers to the identification of either of the alleles from the group S_{14}, S_{17} and S_{21}; remark that the primers used to identify these alleles are the same as those for S_{20}
[c] For the description of the standard PCR programme see Materials and methods; any deviation from the standard parameters is indicated (A = annealing temperature; E = extension time); if the PCR product requires further digestion, the restriction endonuclease employed is indicated
[d] "+/–" indicates that the size was estimated from migration through an agarose gel (PCR product includes an intron of unknown sequence); if applicable, size of the digestion products is indicated in brackets

the primer pairs were selective for a single S-allele (unless otherwise stated), the cultivars assayed were screened against all S-alleles in 13 independent PCR reactions (note that three S-alleles are identified in the S_4/S_{27a}/S_{27b} reaction and "three" other in the reaction encompassing S_6/S_{20}/$S_{14/17/21}$). No false amplification of different alleles was observed for the primer pairs tested. Most of the "Kobel varieties" are not commonly grown anymore and can only be retrieved from apple variety collections held at different locations. We, therefore, also analysed many more recent varieties by the same method, the results of which will be published separately. Following their S-genotype assignment, some of the more common varieties were selected as witness cultivars for worldwide reference (Table 3, see below). The S-allele-specific fragments amplified from DNA of the proposed reference cultivars are shown in Fig. 2.

In the following sections, our results on S-allele analysis in apple are described for each of the 15 S-alleles individually. Wherever appropriate, reference is given to the corresponding S-RNases detected through protein separation.

S_1: the S_1-allele, originally called Sf, has been cloned from Fuji (Sassa et al. 1996). At the nucleotide level, the S_1 coding sequence is 92% identical to S_{20} and S_{24}. Using the primers originally designed to selectively amplify the S_{24}-allele (Verdoodt et al. 1998), all three related alleles were amplified (data not shown). We therefore designed new primers for S_1. As it was extremely difficult to find even short sequence regions in S_1 that differed from all other S-alleles, we choose primers of which at least the 3′ nucleotide was specific for S_1 (FTC168/FTC169). This prevented the S_1-primers to amplify the corresponding region in S_{20} and S_{24}. The sequence spanned by the primer pair included 155 bp of

Table 2 S-genotype analysis of apple cultivars with investigated incompatibility phenotypes (Kobel et al. 1939)

Cultivar	Proposed S-allele genotype deduced from		
	Incompatibility phenotype (Kobel et al. 1939)	Stylar RNase analysis (Bošković and Tobutt 1999)	Allele-specific PCR analysis (this work)[a]
Diploids			
Berner Rosen	S_1S_2	S_1S_2	S_1S_2
(Rote) Sauergrauech	S_1S_3	S_1S_3	S_1S_3
King of the Pippins (= Goldparmäne)	S_1S_3	S_1S_3	S_1S_3
White Transparent (= Weisser Klarapfel)	S_1S_5	S_1S_5	S_1S_5
Ontario	S_1S_8	S_1S_8	S_1S_x
Transparent von Croncels	S_2S_3	S_2S_3	S_2S_3
Champagner Reinette	S_2S_4	S_2S_4	S_2S_4
Danziger Kantapfel	S_2S_7	S_2S_7	S_2S_7
Oberrieder Glanzreinette	S_3S_6	S_3S_6	$S_3S_{(6)}$
Oetwiler	S_3S_6	S_3S_6	S_3S_6
Wellington Reinette	S_8S_9	S_8S_9	S_9S_x
Adam's Pearmain	$S_{10}S_{11}$	$S_{10A}S_{11}$	$S_1S_3S_{10}$
Triploids			
Kanada Reinette	$S_1S_2S_3$	$S_1S_2S_3$	$S_1S_2S_3$
Blenheim Orange (= Goldrein. von Bl.)	$S_1S_3S_4$	$S_1S_3S_{17}$	$S_1S_3S_{14/17/21}$
Jacques Lebel (=Jakob Lebel)	$S_1S_3S_4$	$S_1S_3S_{14}$	$S_1S_3S_{14/17/21}$
Schöner von Boskoop	$S_2S_3S_5$	$S_2S_3S_5$	$S_2S_3S_5$
Menznauer Jägerapfel	$S_3S_5S_9$	$S_3S_5S_{18}$	$S_3S_5S_y$
Stäfner Rosen	$S_3S_7S_8$	$S_3S_7S_8$	$S_3S_7S_x$
Brünnerling	$S_5S_7S_{10}$	$S_5S_7S_{10}$	$S_5S_7S_{10}$
Ribston Pippin	$S_1S_9S_x$ (x=8,10,11 of >11)	$S_1S_9S_{21}$	$S_1S_9S_{14/17/21}$
Gravenstein	$S_4S_{10/11}S_x$ (x>11)	$S_4S_{13}S_{20}$	$S_4S_{20}S_x$
Winterzitronenapfel (= Citron d'Hiver)	$S_3S_xS_y$ (x, y >9)	$S_3S_5S_{12}$	$S_3S_5S_{10}$
Bohnapfel	$S_9S_xS_y$ (x, y >11)	$S_9S_{16}S_{19}$	$S_9S_{27a}S_{28}$
Kaiserapfel	$S_1S_xS_y$ (x, y = 5,10 of 11)	$S_1S_{10}S_{15}$	$S_1S_{10}S_y$

[a] S_x denotes an S-allele different from all S-alleles assayed; in the case of Oberrieder Glanzreinette, the presumed S_6-fragment was inferred from the result of Oetwiler, but this awaits further confirmation; S_y may correspond to S_6, S_{10b}, $S_{14/17/21}$, S_{20}, S_{28} (not determined) or an S-allele other than the ones assayed here

Table 3 Proposed re-numbering of S-alleles in apple and identification of corresponding witness cultivars

S-allele (old name)[a]	S-allele (new)[b]	GenBank no.	Sequence cloned from[c]	Reference cultivar[d]
S_1, Sf	S_1	D50837	Fuji[1]	Fuji (S_1S_9)
S_2, Sa	S_2	U12199	Golden Delicious[2]	Golden Delicious (S_2S_3)
S_3, Sb	S_3	U12200	Golden Delicious[2]	Golden Delicious (S_2S_3)
S_4	S_4	AF327223	Gravenstein[3]	Gloster (S_4S_{19})
S_5	S_5	U19791	Queen's Cox[4]	Gala (S_2S_5)
S_7, Sd	S_7	AB032246 U19792	Akane[5] Idared[4]	Idared (S_3S_7)
S_9, Sc	S_9	U1979 D50836	Queen's Cox[4] Fuji[1]	Fuji (S_1S_9)
S_{10}, Si, *S_{25}*	S_{10}	AB052683	Discovery[6], Maypole[6], McIntosh[7], Prima[3], Telamon[3]	McIntosh ($S_{10}S_{(25)}$)
S_{27a}, *S_{16}*	S_{16}	AF016919	Baskatong[8]	Baskatong ($S_{16}S_{26}$),
S_{27b}, *S_{22}*, *S_{23}*, *S_{25}*	S_{22}	AF327222	Alkmene[3], Delbard Jubilé[3], Merlijn[3]	Alkmene (S_5S_{22})
S_{28}, Se, S_{30}, S-I, *S_{19}*	S_{19}	AB035273 AB017636	Delicious[9] Starking Delicious[10]	Delicious (S_9S_{19})
S_{20}, Sg	S_{20}	AB019184	Indo[11]	Mutsu ($S_2S_3S_{20}$)
"S_{10b}"	S_{23}	AF239809	Granny Smith[12]	Granny Smith (S_3S_{23})
S_{24}, Sh	S_{24}	AB032246 AF016920	Akane[5] Braeburn[8]	Braeburn (S_9S_{24})
S_{26}	S_{26}	AF016918	Baskatong[8]	Baskatong ($S_{16}S_{26}$)

[a] Current annotation for S-alleles based on the determination of the DNA sequence (in normal text) or on the identification of the protein on electrophoresis gels (in italics)
[b] Unique S-allele numbers proposed in this paper
[c] References: 1 Sassa et al. 1996; 2 Broothaerts et al. 1995; 3 Van Nerum et al. 2001; 4 Janssens et al. 1995; 5 Kitahara et al. 2000; 6 Broothaerts unpublished and Richman et al. 1997; 7 Kitahara and Matsumoto 2002; 8 Verdoodt et al. 1998; 9 Matsumoto et al. 2000; 10 Okuno 2000, GenBank submission; 11 Matsumoto et al. 1999; 12 Schneider et al. 2001
[d] This is either the cultivar from which the S-allele was cloned or another common cultivar, if available

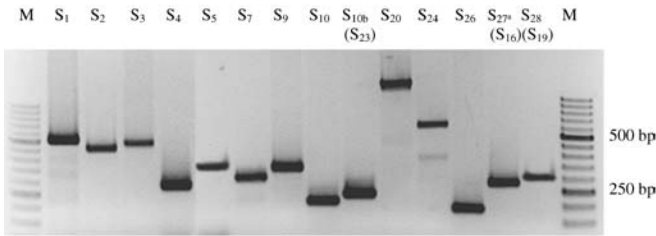

Fig. 2 Electrophoretic separation of amplification products for each of the S-alleles in apple. Every lane shows the outcome of a single allele-specific PCR reaction as indicated (without digestion), using the reference cultivars shown in Table 3 as a genomic DNA source. *M* denotes the 50-bp DNA ladder (150–750 bp)

the S_1 exon sequence and approximately 375 bp of an unknown intron sequence. Using the specified amplification conditions, an S_1-amplification product was found for all five diploid and five triploid cultivars to which Kobel assigned the S_1-allele, and was absent from all other cultivars assayed by the author (Table 2). There was one exception, however, i.e. Adam's Pearmain (A.P.), where the S_1-primers produced an amplification product, while Kobel assigned the genotype as $S_{10}S_{11}$. To rule out the possibility that our primers annealed to another allele which was unknown at the time, a second reverse primer was designed (FTC 233). Using FTC168 and FTC233, specific amplification products were obtained for all varieties that previously were shown to bear S_1, including A.P. Also Fuji, from which the S_1-allele was cloned, showed positive reactions with both primer pairs. We have tested four different A.P. accessions (obtained from Brogdale, UK; Hatton, UK; Balsgard, Sweden; and Gembloux, Belgium) and found the same result in every case. Furthermore, these accessions also revealed the presence of the S_{10}-allele (see further), and are probably genetically identical. Using RNase activity staining, Bošković and Tobutt (1999) previously reported the identification of S_{11} in A.P. The S_{11}-RNase was detected only in this variety, and was barely distinguishable from S_1 under any IEF/NEPHGE conditions tested (pI difference of 0.03 units). Moreover, the occurrence of the S_{11}-allele has not been reported in the literature from any cultivar other than A.P. All these elements together, and the risk inherently associated with the assignment of S-alleles solely based on protein mobility shifts (see Van Nerum et al. 2001), seemed to indicate that either S_{11} does not exist or is extremely similar to S_1.

S_2: using the "S_2-specific" primers, an amplification product was found in all six varieties that expressed the S_2-phenotype, while being absent from all other varieties. The S_2-cDNA was cloned from Golden Delicious and the sequence has been published in Broothaerts et al. (1995).

S_3: in 12 varieties that have the S_3-allele phenotype, a PCR product was obtained using the "S_3-specific" primers. Additionally, A.P. (presumed $S_{10}S_{11}$) was found to contain this fragment. The S_3 and S_{10} coding sequences are highly identical, and the corresponding gene products are difficult to distinguish on protein gels (see Bošković and Tobutt 1999). Therefore, the primers used to amplify S_3 were carefully designed in order to prevent annealing to complementary S_{10}-sequences. If S_{10} would be co-amplified from A.P. in addition to S_3, Brünnerling ($S_5S_7S_{10}$) should have revealed the same result, which was not the case [note that Gravenstein ($S_4S_{10/11}S_x$) was bearing neither S_{10} nor S_{11}, see below]. Employing an alternative S_3-specific reverse primer, FTC 178, revealed the same results. Note that the previously described primers for S_3, which included a reverse primer binding to a sequence in the intron (Broothaerts et al. 1995), may be less-specific because the intron sequence is unknown for many S-alleles. We have, therefore, designed new primers which anneal to known exon sequences. Although FTC177 spans part of the exon/intron boundary (6 bp at the 5′ end of the primer being complementary to the 3′ end of exon 1), it was shown to bind to exon 2 and amplify the correct sequence; however, the S_3-primers required a reduced annealing temperature (57 °C instead of 60 °C).

S_4: using PCR primers designed to amplify both S_4 and S_{27} (Verdoodt et al. 1998), PCR fragments were amplified from Champagne Reinette (S_2S_4) and Gravenstein ($S_4S_{10/11}S_x$), in addition to Bohnapfel ($S_9S_xS_y$; x, y >11). After digestion with *Taq*I, distinctive digestion products were obtained for the two first varieties (200 + 80 bp) in comparison with the latter one (240 + 30 bp). We concluded that the 200 + 80 bp fragments were derived from S_4, and the other from a new allele (S_{27}), not discriminated by Kobel (see S_{27} below). As a confirmation, we cloned the S_4-coding sequence from Gravenstein (Van Nerum et al. 2001). Two 3n varieties that were genotyped by Kobel as $S_1S_3S_4$ (Jacques Lebel, J.L., and Blenheim Orange, B.O.) did not reveal the S_4-specific amplification product, although they contained S_1 and S_3. The absence of S_4 in J.L. and B.O. was confirmed by S-RNase analysis (Bošković and Tobutt 1999) and was discussed therein.

S_5: Kobel assigned S_5 to only one diploid and three triploid varieties, which were all found to reveal PCR products using the corresponding PCR primers. The same PCR fragment was obtained for Winterzitronenapfel ($S_3S_xS_y$; x, y >9), which should therefore contain the S_5-allele, in contrast to Kobel's suggestion (x, y >9). Bošković and Tobutt (1999) also detected the S_5-RNase in this variety.

S_7: by PCR, the S_7-allele was successfully detected in Danziger Kantapfel (S_2S_7), Stafner Rosen ($S_3S_7S_8$) and Brünnerling ($S_5S_7S_{10}$), fitting Kobel's pollination results. The primers previously described to amplify S_7 (Janssens et al. 1995) were often found to be unreliable and were therefore replaced by the ones described here. The partial S_7-sequence, determined by Janssens et al. (1995) and cloned from Idared, matched the complete sequence of the Sd-cDNA cloned from Akane (Kitahara et al.

2000), except for one nucleotide (R (=A or G) at position 477 in Fig. 1).

S_9: three varieties reported to bear S_9 all produced the S_9-specific PCR fragment, while this fragment was absent from the other varieties studied by Kobel. Menznauer Jägerapfel ($S_3S_5S_9$), however, did not reveal the S_9-fragment, an observation that was confirmed by Bošković and Tobutt (1999). The Cox S_9-cDNA sequence (Janssens et al. 1995) was identical to the Sc-sequence retrieved from Fuji (Sassa et al. 1996), except for a few GT/AT repeats in the 3′-UTR.

S_{10}: among the varieties studied by Kobel there are only two varieties to which S_{10} was assigned, i.e. A.P. ($S_{10}S_{11}$) and Brünnerling ($S_5S_7S_{10}$). Additionally, S_{10} or S_{11} was attributed to Gravenstein, and S_5, S_{10} or S_{11} could be present in Kaiserapfel (Kobel's results were inconclusive in both cases). Using PCR, the S_{10}-fragment was amplified from A.P., Brünnerling and Kaiserapfel, but not from Gravenstein. Again, the absence of S_{10} in Gravenstein was confirmed through protein analysis (Bošković and Tobutt 1999). The S_{10} coding sequence was cloned from five different cultivars, i.e. Maypole, Discovery (Broothaerts et al., unpublished data, but included in the phylogenetic analysis in Richmann et al. 1997), Prima, Telamon (Van Nerum et al. 2001) and McIntosh (named "Si", Kitahara and Matsumoto 2002). The nucleotide sequences for the S_3- and S_{10}-cDNAs are >96% identical, showing only single or double nucleotide substitutions dispersed over the sequence. To ensure that the S_{10}-primers were selective for the S_{10}-sequence, four different S_{10}-specific primers were employed in this analysis, including two forward (FTC12 and FTC227) and two reverse (FTC228 and FTC223) primers. All four possible combinations of these primers amplified S_{10}, but not S_3, confirming their specificity. It was remarked that the previously described reverse primer for S_{10} (Van Nerum et al. 2001) contained an error (an extra C should be included at position –3); even with the error adjusted, this primer was considered less specific for S_{10}. During the preparation of this paper, an alternative PCR/digestion identification method for S_{10} was published (Kitahara and Matsumoto 2002).

"S_{10}" from Granny Smith (here provisionally called "S_{10b}"): Schneider et al. (2001) amplified and sequenced the second allele of Granny Smith (which already contained S_3) and named it "S_{10}", following the publication of the S-genotype of this variety as S_3S_{10} by Bošković and Tobutt (1999). However, the presumed S-genotype of Granny Smith was shown by our analysis to be wrong, i.e. S_{10} was clearly lacking and, hence, the sequence cloned from this variety cannot be named S_{10}. We have designed specific primers for amplification of the Granny Smith "S_{10b}" allele, and, because of its absence in the Kobel varieties, we found it to differ from all other S-alleles known through their phenotype or gene product. We, therefore, assigned a new number to this allele, i.e. S_{23}, a number that became available after its withdrawal as a distinct allele (see below). The S_{23}-allele appeared extremely rare even in common apple varieties. It was, however, detected in the upcoming variety Pink Lady (S_2S_{23}), which is reported to be the product of a cross between Golden Delicious (contributing S_2) and Lady Williams (S-alleles unknown), both of which are not known to be related to Granny Smith. The origin of Granny Smith itself is unknown.

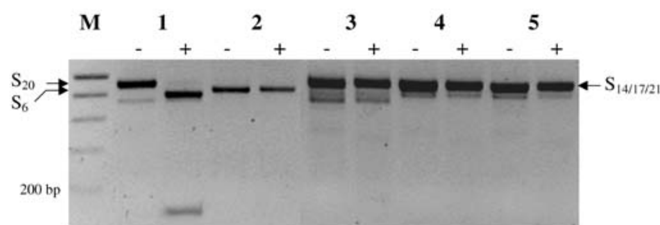

Fig. 3 Discrimination of S_{20}, S_6, and $S_{14/17/21}$ through PCR amplification without (-) and with subsequent restriction digestion by *Nar*I (+). *1* Gravenstein (S_{20}, 920 bp, digested into fragments of 800 and 120 bp), *2* Oetwiler (S_6, 850 bp), *3* Jacques Lebel (S_{14}, 920 bp), *4* Blenheim Orange (S_{17}, 920 bp) and *5* Ribston Pippin (S_{21}, 920 bp). *M* denotes the 200-bp DNA ladder (200–1000 bp)

S_{20}: Matsumoto et al. (1999) cloned an S-allele from Indo and named it Sg. Allele-specific primers for Sg were developed and the allele was shown to correspond to Tobutt's S_{20}-RNase identified in Gravenstein and Mutsu, the latter having Indo as one of its parents (Van Nerum et al. 2001). The size of the PCR product obtained for S_{20} (920 bp), including 589 bp of exon sequence and approximately 330 bp of unknown intron sequence, demanded the use of a longer extension time during PCR (45 s in stead of 30 s). An amplification product of the correct size was also obtained from Ribston Pippin ($S_1S_9S_x$), and from J.L. and B.O. (both $S_1S_3S_4$ according to Kobel). The absence of S_4 in the two latter cultivars was already reported by Bošković and Tobutt (1999), and instead the authors suggested the occurrence of S_{14} (J.L.) and S_{17} (B.O.). Ribston Pippin was reported to carry the new allele S_{21}. In order to confirm the distinction between S_{14}, S_{17}, S_{21} and S_{20}, all of which were amplified with the same primers, we digested the PCR product with *Nar*I. This restriction enzyme recognizes a 6-bp sequence that was present in S_{20}, but not in any of the other alleles aligned in Fig. 1 (note that the sequences of S_{14}, S_{17} and S_{21} have not been determined). The results are shown in Fig. 3. The 920-bp S_{20}-product in Gravenstein was correctly digested in fragments of approximately 800 and 120 bp. In contrast, no digestion occurred in the PCR products obtained from J.L., B.O. and Ribston Pippin, indicating that these cultivars do not bear S_{20}. As we cannot discriminate among them by PCR or digestion, their occurrence is here referred to as $S_{14/17/21}$. Interestingly, the S_{20}-primers (weakly) amplified a fragment of approximately 850 bp from Oetwiler, a cultivar that was reported by Kobel as S_3S_6. This fragment, being approximately 70 bp smaller than the correct S_{20}-product, should represent the S_6-allele. The S_6-product was also not cut by *Nar*I, as shown in Fig. 3. As the S_6-sequence is unknown, the assignment of the S_6-ampli-

fication product is only provisional. Its weak amplification may indicate that the primers employed are not completely matching the annealing site(s). Similar fragments were also obtained from McIntosh and its bud mutant Wijcik, and from Tydeman's Early Worcester (McIntosh x Worcester Pearmain). Bošković and Tobutt (1999) proposed the 2nd allele in these cultivars to be distinct from other S-alleles and named it S_{25}. However, the identity of the S_{25}-RNase seemed rather problematic in the light of recent findings (Van Nerum et al. 2001).

S_{24}: the S_{24}-cDNA was originally derived from Braeburn (Verdoodt et al. 1998) and the corresponding protein has been detected (Bošković and Tobutt 1999). Kitahara et al. (2000) cloned a nearly identical sequence from the variety Akane, named Sh, which only differed from S_{24} at two positions in the 3′-UTR (at –1 and –4 relative to the polyA-tail). Using S_{24}-specific primers, the S_{24}-allele appeared absent from Kobel's S-gene pool, but was found in a number of more common varieties such as Discovery and Worcester Pearmain. Remark that the original primers designed for S_{24} (Verdoodt et al. 1998) co-amplified S_{20}-sequences and should be replaced with the more specific primers reported here.

S_{26}: the S_{26}-cDNA was derived from the crabapple variety Baskatong and was already shown to differ from the other S-alleles (Verdoodt et al. 1998). The allele was named S_{26} in the absence of data to relate it to any of the other 25 S-proteins discriminated by K. Tobutt (personal communication). Further analysis confirmed its extremely rare occurrence among apple varieties. It was, however, detected in *Malus floribunda* 821, a common source of scab resistance genes in breeding programmes, and could therefore have been introgressed in domestic apples.

S_{27a} and S_{27b}: the second allele of Baskatong was named S_{27}(Verdoodt et al. 1998), and later shown to correspond to S_{27a} (Van Nerum et al. 2001). The S_4/S_{27a}/S_{27b}-primers amplified both S_{27} (a and b) and S_4, which could be easily discriminated by restriction digestion with *Taq*I, as was clearly shown by Van Nerum et al. (2001; note that the primers described herein contained a 5′-extension for cloning purposes which was omitted here). Here we found that S_{27a} was also present in Bohnapfel ($S_9S_xS_y$; x,y >11) and should, therefore, relate to Kobel's S_x or S_y and also correspond to the S-RNases S_{16} or S_{19} that were assigned to this variety by Bošković and Tobutt (1999). The related S_{27b}-allele, differing from S_{27a} by four nonfunctional point mutations, had been cloned from Alkmene, Delbard Jubilé and Merlijn (Van Nerum et al. 2001), three cultivars for which Bošković and Tobutt (1999) had invented three new S-alleles, S_{22}, S_{23} and S_{25}, respectively. It has to be stressed that at the protein level, S_{27a} and S_{27b} are identical, and they express the same S-specificity, as was shown in the pollination study of Van Nerum et al. (2001).

S_{28}: recently, a new S-allele was cloned from a sport of Red Delicious and named S_{28}, because the relationship with any of the other S-alleles reported in the literature was not determined (Schneider et al. 2001). Using specific primers, we found S_{28} to be the third unknown S-allele of Bohnapfel, hence corresponding to either the S_{16} or S_{19}-RNase (see above). Besides Delicious, one of its offspring, Gloster, also contained the S_{28}-allele. Matsumoto and Kitahara (2000), who also cloned the Se-allele (= S_{28}) from Delicious, previously described other allele-specific primers for the specific identification of this allele. Comparing the sequences of these primers with the S-allele alignment shown in Fig. 1 confirms that the Se-primers are S_{28}-specific and could be used as an alternative for those described here.

An S-allele should encode an RNase gene product

The interpretation of the data from ribonuclease zymograms of many cultivars had brought Bošković and Tobutt (1999) to discriminate 14 additional new S-alleles, named S_{12} to S_{25}. Some of these new S-alleles, i.e. S_{22} and S_{23}, have since been withdrawn as distinct alleles (Van Nerum et al. 2001). Additionally, the "S_{25}-RNase" attributed to several unrelated cultivars was found to represent different alleles, i.e. S_{10} in Telamon, S_{27b} in Merlijn and maybe S_6 in McIntosh and progeny (recent data, however, indicate that the second McIntosh allele differs from S_6 in Oetwiler; S. Matsumoto, personal communication). This would mean that either the RNase zymograms were misinterpreted, or the cultivars assayed by Tobutt's team were different from the ones we tested. Our data were gathered from trees growing at the orchard of the Fruitteeltcentrum K.U. Leuven at Rillaar, and we believe they are true-to-type. Here we additionally found that S_{16} and S_{19} in Bohnapfel corresponded with S_{27a} and S_{28} respectively. The S_{16}-RNase was reported to have a pI of 9.90, only differing with max. 0.1 pH unit from that of S_{22} and S_{23}, which have both previously been shown to be identical to S_{27b}. Because they encode identical proteins, we would expect the RNases S_{16}, S_{22} and S_{23} to migrate to the same region in the gel. In contrast, S_{19} has a pI of 9.63 and should, therefore, relate to the S_{28}-allele. The correspondence between alleles and RNases described in the literature has been compiled in Table 3. We propose the use of a unique identification number for each of the S-alleles, including S_{27a} and S_{27b}. A few S-alleles have, therefore, received a new annotation, i.e. S_{27a} is named according to its gene product S_{16} and S_{27b} then becomes S_{22}; similarly, S_{28} now becomes S_{19}; finally, S_{10b} is called S_{23}, a number that became available because of its identity with a number of other S-alleles. From the 14 new S-alleles introduced by Bošković and Tobutt (1999), S_{23} was rejected and three others, S_{16}, S_{22} and S_{19}, were linked to their nucleotide sequences, which had originally received another number.

There remain a few discrepancies between our results and those reported by Bošković and Tobutt (1999). Our discovery of S_{10} in Winterzitronenapfel was not unexpected, since Kobel did not perform the cross with A.P. which should have revealed its presence. Nevertheless,

Bošković and Tobutt (1999) annotated the S_{12}-RNase band to this cultivar, which was running at a position quite different from that of S_{10}. Furthermore, the distinction between S_{10} and S_{10A} (in A.P.) was not made in our results, and we actually detected three S-alleles, S_1, S_3 and S_{10}, in A.P. Similarly, we could not differentiate between the presumed S_{11} in A.P. and S_1, of which the gene products were running very close to each other. There also remains at least one S-allele that was not identified by our PCR method, i.e. S_{13} in Gravenstein, which represents the third allele in this variety (actually, it may correspond to the lower band seen in Fig. 3, which was co-amplified with the S_{20}-primers). Likewise, S_{15} and S_{18} in the cultivars Menznauer Jägerapfel and Kaiserapfel, respectively, cannot be identified because their nucleotide sequences are unknown. Although we have no evidence to doubt on the unique S-genotype proposed for these cultivars by Bošković and Tobutt (1999), it should be remarked, however, that the latter cultivars were not tested for the presence of S_{10b}, S_{20}, S_6, $S_{14/17/21}$ and S_{28} due to the lack of plant material for these cultivars at the time this analysis was done.

Discussion

Despite the importance of apple as a crop, not much information exists on the S-genotypes of varieties. This contrasts to the situation in sweet cherry where a number of pollen-incompatibility groups have been defined (recently revised by Wiersma et al. 2001 and Sonneveld et al. 2001). In contrast to sweet cherry and other *Prunus* species, where the incompatibility reaction is strong and hence easily scored, the interpretation of pollination studies in apple is much-less straightforward. As a result, the proposed compatibility relationships between cultivars are often not in agreement with the true self-incompatibility genotypes. Following the identification and sequence analysis of individual S-alleles in apple, we have developed a DNA-based approach to discriminate the S-alleles operating in the apple genome. Since the first report (Janssens et al. 1995), the method has been extended and modified several times (Verdoodt et al. 1998; Van Nerum et al. 2001) and was used for S-genotyping (Sakurai et al. 1997, 2000; Matsumoto and Kitahara 2000; Kitahara and Matsumoto 2002), to assess homozygosity (Verdoodt et al. 1998), to study pollen flow within an orchard (Janssens et al. 1996; Schneider et al. 2001), in genome mapping (Maliepaard et al. 1999) and to confirm cultivar identity during multiplex PCR (Broothaerts et al. 2001). Variations of the original protocol have also been described for Japanese pear and almond (Ishimuzu et al. 1999; Tamura et al. 2000). In this report, two new apple S-alleles, reported in the literature, were included in the analysis, and a number of further improvements to primers and amplification conditions were suggested. This allowed us to selectively identify a total of 15 different S-alleles distributed within the domesticated apple species. Additionally, the identification of S_6, which is not known from its nucleotide sequence, was proposed, as well as the detection of one of the alleles of the group encompassing S_{14}, S_{17} and S_{21}. The results obtained with this approach and its current design were compiled in this paper.

Overall, there was a good correspondence between the results of our S-genotyping method and those proposed by Kobel et al. (1939). Except for S_8 and perhaps S_{11} (see below), of which the nucleotide sequences have not been determined, all other S-alleles were correctly detected in the diploid cultivars that revealed the corresponding incompatibility phenotype. Nevertheless, we had to modify several primer pairs in comparison with previous reports in order to avoid mispriming or inconsistent amplification results. A special remark should be made in relation to the S_6-allele. Using S_{20}-primers, we observed a weak PCR product of a size differing from that of the true S_{20}-fragment in the varieties Oetwiler (S_3S_6), McIntosh/Wijcik and Tydeman's Early Worcester. In all these varieties we only detect a single second S-allele (different for all of them), hence we may conclude that the co-amplified product seen after amplification with S_{20}-primers represents a new S-allele. This fragment should presumably correspond to Tobutt's S_{25}-RNase assigned to these varieties and, more importantly, to S_6 assigned by Kobel. If it is confirmed that S_6 and S_{25} are distinct alleles, which seems to be the case (S. Matsumoto, personal communication), future work should find a way to discriminate them through PCR analysis.

Among the triploid cultivars, a few discrepancies were discovered between the S-genotype assigned by pollination study and our analysis method. First, in both J. L. and B. O., the presence of the S_4-allele was rejected by our data, and was replaced by an allele of the $S_{14/17/21}$ group. Similarly, the third allele in Ribston Pippin (S_{21}) gave the same amplification/restriction pattern. As all three RNases were clearly discriminated by NEPHGE, they should represent three different alleles. We have not found any other cultivar, however, that contains one of these alleles. Secondly, in Winterzitronenapfel ($S_3S_xS_y$), Kobel decided that x and y both were of a number >9, while we, as well as Bošković and Tobutt, detected S_5 in this variety. For the third allele, our finding of S_{10}, while not in conflict with Kobel's suggestion of $y > 9$, disagrees with that of Bošković and Tobutt (1999), who discriminated a new allele, S_{12}. Again, S_{12} has not been reported from any other cultivar and is hardly discriminated from S_2. In fact, the zymogram for Belle de Boskoop ($S_2S_3S_5$) is completely identical with the one of Winterzitronenapfel, except for a slight difference under one of the three NEPHGE conditions tested. One has to consider, therefore, if the assignment of S_{12} might have been the result of a mislabeling of plant material. Finally, in Gravenstein, the suggestion of the presence of "S_{10} or S_{11}" was not confirmed. Kobel's suggestion was exclusively based on the incompatibility reaction observed when Gravenstein was pollinated with pollen from A.P., which in his eyes was $S_{10}S_{11}$. However, from our data,

the incompatibility composition of A.P. is highly confusing, which could have explained the wrong S-allele deduction for Gravenstein. The existence of an S_{11}-allele in A.P. is uncertain because we amplified both the S_1- and S_3-specific sequences from this cultivar, in addition to S_{10}. Remarkably, the S_{11}-allele has never been reported from any other apple cultivar that was examined for S-RNase content, incompatibility phenotype or the presence of S-allele-specific products. Moreover, on the protein level, the presumed S_{11}-RNase migrates very close to S_1. Because the slight difference observed between S_1 and S_{11} may have been caused by variations in glycan extension (Woodward et al. 1992), dependent on the genetic background of the S-RNase, the analysis of seedlings of A.P. could indicate if the difference persists. Bošković and Tobutt (1999) found the presumed S_{10}-RNase band in A.P. running slightly faster than S_3, which was already slightly faster than S_{10} in other cultivars. They denoted the second band in A.P. as "S_{10A}" to indicate its distinct position compared to S_3 and S_{10}. However, because the S_3-RNase produces a plurality of bands in the same gel region where the S_{10}-RNase is positioned, and because the latter appears as a rather faint band, it remains difficult to clearly detect the S_3- and S_{10}-RNase band in a cultivar that contains both S_3 and S_{10}. This might also have been the reason for the wrong assignment of the S-genotypes of Telamon, Granny Smith and perhaps Winterzitronenapfel by Bošković and Tobutt (1999). Obviously, the discovery of three different S-alleles in a 2n cultivar remains intriguing. Triploids generally have few viable pollen grains. From Kobel's data, presumably revealing plenty of pollen tubes growing through the pistil if used as the pollen parent, it seems unlikely that A.P. is a real triploid cultivar. It would be interesting to analyse the A.P. case more carefully, i.e. by pollination and by sequencing of the amplification products, in order to find out if the third allelic sequence found here is present in the genome at the S-locus or at a non-S-locus position. Similarly, the difference between S_1 and S_{11}, and between S_{10} and S_{10A}, if confirmed, should be resolved in further studies.

Besides the S-alleles discriminated by Kobel et al. (1939), several other alleles have been discovered in recent years. For the S_{20} and S_{24}-allele, the data from S-allele-specific PCR matched those obtained by RNase analysis, i.e. S_{20} was found in Gravenstein and Mutsu, and S_{24} in Braeburn and Tydeman's Early Worcester. For the "S_{10b}"-allele cloned from Granny Smith, there exist no doubt that Bošković's conclusion to assign the S_3S_{10}-genotype to this cultivar was wrong. Both alleles are not particularly similar in sequence, hence it would be surprising that the corresponding RNase bands would have migrated close to one another. It appears, thus, that the gene product of this allele was not discriminated by zymogram analysis. We have proposed to name it S_{23}, a number that became available after the rejection of the suggested S-genotype of Delbard Jubilé. We previously proved through sequence analysis that S_{22}, S_{23} and S_{25} (in Merlijn) were all identical with S_{27b}. Tobutt had already suggested that the second allele in Delbard Jubilé (S_{23}) seemed similar to one of the Baskatong alleles, i.e. S_{27a} (personal communication). Here we also found S_{16} in Bohnapfel to be related to the latter allele and suggested, therefore, to re-name S_{27a} according to its gene product S_{16}. Likewise, S_{27b} was changed to S_{22} (of Alkmene). S_{16} and S_{22} are thus two S-alleles that have the same functionality. They have received a distinct number to accommodate the fact that the genetic sequences are different and may further evolve independently. Also S_{28} from Delicious is not a new allele, but relates to the S_{19}-RNase from Bohnapfel, which is barely discriminated from the S_5- and S_7-bands. The use of our PCR method to distinguish these alleles should, however, highly facilitate their correct assignment in future studies. Finally, evidence for the existence of S_{15} and S_{18} has only been obtained from RNase zymogram analysis, as the single cultivars to which these alleles were assigned have not been thorougly analysed for the S-alleles with a number >10, except for S_{24}. In contrast to these alleles which were reported from single cultivars, the S_8-RNase, another allele of which the sequence is still unknown, has been found in other cultivars such as James Grieve (Bošković and Tobutt 1999). It has to be admitted, though, that some alleles seem to occur at an extremely low frequency, as was also found for S_{23} ("S_{10b}") and S_{26}.

Because of the multiple and sometimes highly confusing annotations of apple S-alleles that have appeared in the literature during the past 8 years, I have tried to compile all the data reported and proposed unique numbers to each of the alleles. Four S-alleles were re-numbered, i.e. "S_{10b}", S_{27a}, S_{27b} and S_{28} became S_{23}, S_{16}, S_{22} and S_{19} respectively (Table 3). The alleles previously identified alphabetically have also been linked with a numbered allele, and I suggest to use the numerical data in future referencing. Instead of the 29 alleles reported earlier (S_1 to S_{28} and "S_{10b}"), the number of S-alleles that are operating in domestic apple rather seems to be restricted to 22–24 different alleles. Of these, 15 have been unequivocally identified by their nucleotide sequences. All 15 can be analysed through allele-specific PCR, and 13 of them, excluding S_{23} and S_{26}, also by RNase separation (S_{16} and S_{22} encode identical RNase proteins and cannot, however, be discriminated). A further S-allele, S_6, can be detected by both RNase analysis and presumably PCR amplification, but its sequence has not been determined. Six additional alleles can only be distinguished by their RNases, i.e. S_8, S_{13}, S_{14}, S_{15}, S_{17}, S_{18} and S_{21}, although S_{14}, S_{17} and S_{21} can be identified by S-allele PCR as a group ($S_{14/17/21}$). Finally, there remain two alleles, S_{11} and S_{12}, of which the nature is uncertain on the basis of the evidence provided above. If additional evidence is presented that these alleles are distinct from known alleles, they should persist; if not, the numbers may be re-used for new alleles of the S-gene. Importantly, new discoveries in S-genotyping should be checked against the database of previously assigned S-alleles. To improve the accessibility of research groups all over the

world to cultivars with a known S-genotypes, we have identified a number of widespread witness cultivars. Such witness material is available for the 15 S-alleles that were discriminated in our work. It would be helpful if similar widespread witness cultivars were identified for the remaining S-alleles that are currently only known by their gene products.

References

Bošković R, Tobutt KR (1999) Correlation of stylar rybonuclease isoenzymes with incompatibility alleles in apple. Euphytica 107:29–43

Broothaerts W, Janssens GA, Proost P, Broekaert WF (1995) cDNA cloning and molecular analysis of two self-incompatibility alleles from apple. Plant Mol Biol 27:499–511

Broothaerts W, Wiersma PA, Lane WD (2001) Multiplex PCR combining transgene and S-allele control primers to simultaneously confirm cultivar identity and transformation in apple. Plant Cell Rep 20:349–353

Golz JF, Oh H-Y, Su V, Kusaba M, Newbigin E (2001) Genetic analysis of *Nicotiana* pollen-part mutants is consistent with the presence of an S-ribonuclease inhibitor at the S-locus. Proc Natl Acad Sci USA 98:15,372–15,376

Ishimizu T, Inoue K, Shimonaka M, Saito T, Terai O, Norioka S (1999) PCR-based method for identifying the *S*-genotypes of Japanese pear cultivars. Theor Appl Genet 98:961–967

Janssens GA, Goderis IJ, Broekaert WF, Broothaerts W (1995) A molecular method for S-allele identification in apple based on allele-specific PCR. Theor Appl Genet 91:691–698

Janssens GA, Van Haute AM, Keulemans J, Broothaerts W, Broekaert WF (1996) PCR analysis of self-incompatibility alleles in apple applied to leaves, seed embryos and in vitro shoots. Acta Hort 484:403–407

Kitahara K, Matsumoto S (2002) Sequence of the S_{10} cDNA from McIntosh apple and a PCR-digestion identification method. HortScience 37:187–190

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 über die Befruchtungsverhältnisse der Apfel- und Birnsorten. Landw Jb Schweiz 53:160–191

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

Maliepaard C, Alston FH, van Arkel G, Brown LM, Chevreau E, Dunemann F, Evans KM, Gardiner S, Guilford P, van Heusden AW, Janse J, Laurens F, Lynn JR, Manganaris AG, den Nijs APM, Periam N, Rikkerink E, Roche P, Ryder C, Sansavini S, Schmidt H, Tartarini S, Verhaegh JJ, Vrielink-van Ginkel M, King GJ (1999) Aligning male and female linkage maps of apple (*Malus pumila* Mill.) using multi-allelic markers. Theor Appl Genet 97:60–73

Matsumoto S, Kitahara K (2000) Discovery of a new self-incompatibility allele in apple. HortScience 35:1329–1332

Matsumoto S, Kitahara K, Komori S, Soejima J (1999) A new S-allele in apple, ‘Sg’, and its similarity to the ‘Sf’ allele from Fuji. HortScience 34:708–710

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

Okuno T (2000) S-RNase from *Malus domestica*, cultivar Starking Delicious. GenBank submission no. AB017636

Richman AD, Broothaerts W, Kohn JR (1997) Self-incompatibility RNases from three plant families: homology or convergence? Am J Bot 84:912–917

Sakurai K, Brown SK, Weeden NF (1997) Determining the self-incompatibility alleles of Japanese apple cultivars. HortScience 32:1258–1259

Sakurai K, Brown SK, Weeden NF (2000) Self-incompatibility alleles of apple cultivars and advanced selections. HortScience 35:116–119

Sassa H, Mase N, Hirano H, Ikehashi H (1994) Identification of self-incompatibility related glycoproteins in styles of apple (*Malus* × *domestica*). Theor Appl Genet 89:201–205

Sassa H, Nishio T, Kowyama Y, Hirano T, Koba T, Ikehashi H (1996) Self-incompatibility (*S*) alleles of the Rosaceae encode members of a distinct class of the T_2/S ribonuclease superfamily. Mol Gen Genet 250:547–557

Schneider D, Stern RA, Eisikowitch D, Goldway M (2001) Analysis of S-alleles by PCR for determination of compatibility in the Red Delicious apple orchard. J Hortic Sci Biotech 76:596–600

Sonneveld T, Robbins TP, Bošković R, Tobutt KR (2001) Cloning of six cherry self-incompatibility alleles and development of allele-specific PCR detection. Theor Appl Genet 102:1046–1055

Tamura M, Ushijima K, Sassa H, Hirano H, Tao R, Gradziel TM, Dandekar AM (2000) Identification of self-incompatibility genotypes of almond by allele-specific PCR analysis. Theor Appl Genet 101:344–349

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

Van Nerum I, Geerts M, Van Haute A, Keulemans J, Broothaerts W (2001) Re-examination of the self-incompatibility genotype of apple cultivars containing putative ‘new’ S-alleles. Theor Appl Genet 103:584–591

Wiersma PA, Wu Z, Zhou L, Hampson C, Kappel F (2001) Identification of new self-incompatibility alleles in sweet cherry (*Prunus avium* L.) and clarification of incompatibility groups by PCR and sequence analysis. Theor Appl Genet 102:700–708

Woodward JR, Craik D, Dell A, Khoo KH, Munroe SLA, Clarke AE, Bacic A (1992) Structural analysis of the N-linked glycan chains from a stylar glyco-protein associated with expression of self-incompatibility in *Nicotiana alata*. Glycobiology 2:241–250