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# Re-examination of the self-incompatibility genotype of apple cultivars containing putative 'new' S-alleles

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**Abstract** Recently, the self-incompatibility (S-) genotypes of 56 apple cultivars were examined by protein analysis, which led to the identification by Boskovic and Tobutt of 14 putative 'new' S-alleles, S12 to S25. This paper reports a re-examination of the S-genotypes of some of these cultivars through S-allele 'specific' PCR and sequence analysis. The results obtained by this analysis indicated that the number of S-alleles that are present in apple is probably smaller than the number proposed by Boskovic and Tobutt. The existence of three 'new' S-alleles (S20, S22 and S24) was confirmed. The existence of two other putative 'new' S-alleles (S23 and S25) was, however, contradicted. The coding sequences of the S-alleles that correspond to the S10 and the S25 ribonuclease bands as well as those corresponding to the S22 and the S23 ribonuclease bands were shown to be identical in sequence. Interestingly, the S-allele corresponding to the S22 and the S23 ribonuclease bands shared a high sequence identity (99% identity) with S27, which was previously cloned and sequenced from Baskatong, but which was not included in the analysis conducted by Boskovic and Tobutt. Both S-alleles only differ in point mutations, which are not translated into differences in amino-acid sequence. To our knowledge, this is the first report of two S-alleles that differ at the nucleotide level but still encode for identical S-RNases. The implications of these observations for determining the S-genotypes of plants by PCR analysis or protein analysis are discussed.

**Keywords** Apple · *Malus X domestica* Borkh. · Self-incompatibility · S-allele 'specific' PCR · Sequence analysis

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## Introduction

Self-incompatibility is a genetically controlled system in flowering plants that prevents self-fertilisation. Apple (*Malus X domestica* Borkh.), which belongs to the Rosaceae family, has a gametophytic self-incompatibility system (Kobel et al. 1939). Its self-incompatibility reaction is controlled by a single multi-allelic S-locus. When the S-allele of the pollen grain matches one of the S-alleles of the style, the growth of the pollen tube in the style is inhibited. Since the discovery of the multi-allelic S-gene that controls the self-incompatibility reaction in the pistil, several S-alleles from apple have been cloned and sequenced [S2, S3: Broothaerts et al. (1995); S5, S7, S9: Janssens et al. (1995); Sc, Sf: Sassa et al. (1996); S24, S26 and S27: Verdoodt et al. (1998); Sg: Matsumoto et al. (1999)]. As in other species that have a gametophytic self-incompatibility system, the products of this multi-allelic S-gene in apple are basic ribonucleases, named S-RNases (Broothaerts et al. 1995).

Because of the operation of a self-incompatibility system, most commercial apple cultivars must be grown in mixed planting systems to guarantee an optimal fruit production. Trees of a cross-compatible cultivar, so called pollinator trees, have to be planted in between the trees of the main cultivar to ensure successful fertilisation. Knowledge of the self-incompatibility relationship between cultivars is obviously useful for selecting suitable pollinators. Additionally, such information is advantageous in the design of breeding programmes. One way to determine the incompatibility relationship between cultivars is by performing test crosses followed by microscopic analysis of pollen tube growth in the style or the analysis of fruit and seed set. By using this method, Kobel et al. (1939) were able to infer the S-genotype of 20 cultivars and to distinguish 11 S-alleles, S1 to S11.

Crossing experiments are very laborious and time-consuming. Another drawback of this method to determine self-incompatibility relationships is that pollen tube growth is influenced by environmental and physiological

conditions. As a consequence, the results are often ambiguous. To circumvent the necessity of crossing experiments, various molecular methods for S-genotyping have been developed. In one of these methods, S-genotypes are determined by analysing S-RNase polymorphisms, which can be detected by analysing the migration mobility of S-RNases through a protein gel (Boskovic and Tobutt 1999). Using this method, Boskovic and Tobutt were able to distinguish 25 different S-RNases. Besides the 11 S-alleles that were first identified by Kobel et al. (1939), ribonuclease bands corresponding to 14 putative 'new' S-alleles were detected (S12 to S25). Following the sequence determination of several S-alleles, an alternative approach for S-genotyping was developed (Broothaerts et al. 1995; Janssens et al. 1995). In this method, the presence of an S-allele is verified by PCR amplification with S-allele 'specific' primers, often followed by restriction digestion of the amplification product.

In this paper the S-genotype of some cultivars that were proposed to contain putative 'new' S-alleles based on protein analysis (Boskovic and Tobutt 1999) were re-examined by S-allele 'specific' PCR and sequence analysis. The existence of three 'new' S-alleles was confirmed. One of these S-alleles, however, only differed in synonymous point mutations from an S-allele (S27) previously cloned from Baskatong. The existence of two other putative 'new' S-alleles inferred by Boskovic and Tobutt was contradicted. Based on the new information of this study, the S-genotype of a number of apple cultivars was revised.

## Material and methods

### Plant material

The cultivars that were analysed are listed in Table 1 with their S-genotype based on protein analysis (Boskovic and Tobutt 1999) and S-allele 'specific' PCR (Broothaerts et al. 1996; Verdoodt et al. 1998; this study). Pistils were collected in the field from flowers at the balloon stage of development, immediately frozen in liquid nitrogen and stored at –80°C. Leaves were collected in the field, frozen in liquid nitrogen, lyophilised, and stored with silica at room temperature.

### S-allele 'specific' PCR

Genomic DNA was isolated from leaves using the Genomic DNA Purification Kit (Fermentas). PCR reactions were performed as described in Broothaerts et al. (1995) (S2 and S3), Janssens et al. (1995) (S5, S7 and S9) and Verdoodt et al. (1998) (S4/S27, S24 and S26). Based on the sequence of Sg (Matsumoto et al. 1999) and S10 (Broothaerts et al., unpublished data), Sg and S10 'specific' primers were developed (respectively 5′-ATCAGCCGGCTGTCTGCCACTC-3′, 5′-AGCCGTGCTCTTAATACTGAATAC-3′, annealing temperature 67°C and 5′-CCAAACGTACTCAATCGAAG-3′, 5′-CGTGTCCTGAATCTCCCTC-3′, annealing temperature 58°C). PCR reactions were run on GeneAmp2400 thermal cyclers (Perkin Elmer) using the following amplification programme: 1 cycle of 1 min at 96°C, 35 cycles of 30 s denaturation at 94°C, 30 s annealing at a specific annealing temperature and 1 min extension at 72°C, one cycle of 10 min at 72°C. Standard PCR reactions contained 200 µM of dNTPs, 1 µM of each primer, 1.5 mM of MgCl2, 1×PCR buffer, 0.5 U of *Taq* DNA polymerase (Promega) and approximately 100 ng of genomic DNA in a total volume of 25 µl. PCR products were analysed by agarose-gel electrophoresis.

### Cloning and sequence analysis

Total RNA was isolated from pistils using the method of Eggermont et al. (1996), followed by DNaseI treatment, phenol/chloroform extraction and precipitation with sodium acetate. Reverse transcription was performed at 37°C for 60 min in a total reaction volume of 20 µl containing 1 µg of total RNA, 1×RT buffer, 10 units of Mu-MLV-RT (Eurogentec), 1 µM of the degenerated S-allele primer FTC175 (5′-ACTCTAGATGAGMTYTTAATAMHG-3′), 0.5 mM of dNTPs and 20 Units of RNase inhibitor (Promega). Four microliters of the RT-reaction was used in a 50-µl PCR reaction containing the degenerated S-allele primers OWB134 (5′-ATGGATCCGAATTCTTGAACAAAYATTATTCAATG-3′′) and FTC175 under the standard conditions mentioned above and an annealing temperature of 50°C. Both primers were designed to anneal to all known S-alleles and to amplify the complete coding sequence. Following amplification, the PCR product was purified using the Microcon PCR Purification Kit (Millipore) and subsequently digested with a restriction enzyme which cuts only the already known S-allele. The PCR products amplified from Alkmene, Delbard Jubilé, Gravenstein, Merlijn, Prima and Telamon were digested with respectively *Hind*III, *Nco*I, *Hae*II, *Kpn*I, *Nco*I and *Pst*I. Digested and undigested PCR products were separated on a 1% agarose gel. After purification with the GeneClean Kit (BI0 101) the undigested PCR fragment was inserted into the pCR2.1-TOPO vector using the TOPO-TA cloning Kit (Invitrogen). Colonies were screened by PCR using S-allele 'specific' primers, known to amplify the S-allele that was under investigation. The plasmid of three positive colonies per allele were isolated using the Wizard Plus SV Minipreps DNA purification kit (Promega). The nucleotide sequences of the cloned PCR products were determined on a LI-COR 4000L DNA sequencer by the dideoxynucleotide chain-terminating method using Infrared (IRD41)-labelled M13 primers and the Amersham ThermoSequenase Cycle Sequencing Kit. To confirm the sequence data, PCR amplification, cloning and sequence analysis was repeated once.

### Pollination tests

During the flowering period, anthers were collected from flowers at the balloon stage and allowed to dry overnight. The germination percentage of the pollen was determined by applying the pollen onto germination medium (0.2% agar, 15 ppm of boric acid, 10% sucrose) and counting the number of germinated and ungerminated pollen grains after incubation overnight at room temperature. Flowers were hand-pollinated at the balloon stage of development. To prevent cross-pollination by air or insects, pollinated branches were isolated by bags made from nylon curtains. To minimise the effects of post-fertilisation fruit losses, the fruit set was determined at fruit initiation (before the June drop). After fruit maturation, the average seed set of the apples was determined.

For the analysis of pollen tube growth in the style, five pollinated pistils were collected from each parental combination 7 days after pollination and stored in FAA medium. The pistils were rinsed with water, soaked for 0.5 h in 8 N NaOH and rinsed again with water. After incubation for at least 0.5 h in 0.05% aniline blue dissolved in potassium phosphate buffer (pH10), the individual styles were removed, squashed and monitored with a fluorescence microscope.

## Results

S20 corresponds to Sg, previously cloned from Indo

Based on protein analysis, the apple cultivar Braeburn contains a 'new' S-allele (S24) (Boskovic and Tobutt 1999). The coding sequence of S24 was previously cloned and based on its sequence, S24 'specific' primers were developed (Verdoodt et al. 1998). Besides Braeburn the apple cultivars Gravenstein and Mutsu also yielded an amplification product with these S24 'specific' primers. According to Boskovic and Tobutt (1999), however, after separation of stylar proteins of these two cultivars, the S24 ribonuclease band could not be detected. Instead, a ribonuclease band distinct from S24 and other known S-RNases was observed. Based on their analysis Boskovic and Tobutt proposed that both cultivars contain a putative 'new' S-allele, which was named S20. Recently another new S-allele (Sg) was cloned and sequenced from Indo (Matsumoto et al. 1999), a cultivar that gives an amplification product with S7 and S24 'specific' primers. Sequence comparison revealed that Sg shares high sequence identity (97%) with S24. Both S-alleles only differ in point mutations. The binding sites of the S24 'specific' primers are completely conserved in the Sg allele. Also the ScrFI restriction site, used to digest the S24 'specific' PCR product (Verdoodt et al. 1998), is conserved. At the amino-acid level the S24 and Sg RNase share 93% sequence identity. Compared to S24, S7 shares much less sequence identity with Sg (approximately 74% sequence identity at the nucleotide level) and the binding sites of the S7 'specific' primers are not conserved in Sg. Based on these data, we concluded that, besides S24, the S24 'specific primers, also amplify Sg. To be able to distinguish Sg and S24 by PCR, Sg 'specific' primers were developed. All cultivars that gave amplification with S24 'specific' primers were tested with the Sg primers. When Indo, Gravenstein or Mutsu genomic DNA was used as a template in the PCR reaction an amplification product of the expected size was obtained. In the presence of genomic DNA of the cultivar Braeburn (S9S24), by contrast, no amplification occurred (Table 1). Based on these data and the knowledge that Mutsu is a progeny from the cross Golden Delicious × Indo, we concuded that the Sg allele corresponds to S20, a 'new' S-allele which was previously identified by S-RNase analysis.

The S22, S23 and S25 ribonuclease bands, identified in respectively Alkmene, Delbard Jubilé and Merlijn, represent the same S-allele

Alkmene, Delbard Jubilé and Merlijn each yielded an amplification product with S4/S27 'specific' primers (Fig. 1). After amplification with these primers, S4 and S27 can be distinguished by digestion of the PCR product with TaqI. After digestion, the S4 fragment splits in two bands of approximately 200 and 80 base pairs, while digestion of the S27 fragment results into two bands of approximately 240 and 40 base pairs (Verdoodt et al. 1998). Digestion with TaqI revealed that the S4/S27 PCR fragment obtained from Alkmene, Delbard Jubilé and Merlijn did not correspond to the S4 fragment nor to the S27 fragment. In each of these three cultivars, two bands of approximately 40 and 200 base pairs were observed after digestion (Fig. 1). This result indicated that these cultivars probably contain a 'new' S-allele, distinct from S4, S27 and other known S-alleles. Based on protein analysis, Boskovic and Tobutt (1999) proposed

**Table 1** Proposed S-genotype of apple varieties, based on protein analysis and S-allele 'specific' PCR. nd=not determined

| Cultivar | S-genotype based on protein analysis[a] | S-genotype based on S-allele 'specific' PCR[b] |
|---|---|---|
| Alkmene | $S_5S_{22}$ | $S_5S_{27b}$[c] |
| Baskatong | nd | $S_{26}S_{27a}$ |
| Braeburn | $S_9S_{24}$ | $S_9S_{24}$ |
| Delbard Jubilé | $S_2S_{23}$ | $S_2S_{27b}$[c] |
| Elstar | $S_3S_5$ | $S_3S_5$ |
| Gravenstein | $S_4S_{13}S_{20}$ | $S_4S_g$S- |
| Indo | nd | $S_7S_g$ |
| Lobo | nd | $S_{10}S_{27b}$[c] |
| Maypole | nd | $S_{10}S_{27a}$[c] |
| Merlijn | $S_3S_{25}$ | $S_3S_{27b}$[c] |
| Mutsu | $S_2S_3S_{20}$ | $S_2S_3S_g$ |
| Prima | $S_2S_{10}$ | $S_2S_{10}$ |
| Telamon | $S_3S_{25}$ | $S_3S_{10}$ |

[a] S-genotypes determined by Boskovic and Tobutt (1999)
[b] S-genotypes determined by this work or previously described in Broothaerts et al. (1996) or Verdoodt et al. (1998)
[c] S27a and S27b are two S-alleles that only differ at the nucleotide level. They can be distinguished from each other and from S4 by digestion of the S4/S27 'specific' PCR product with *Taq*I

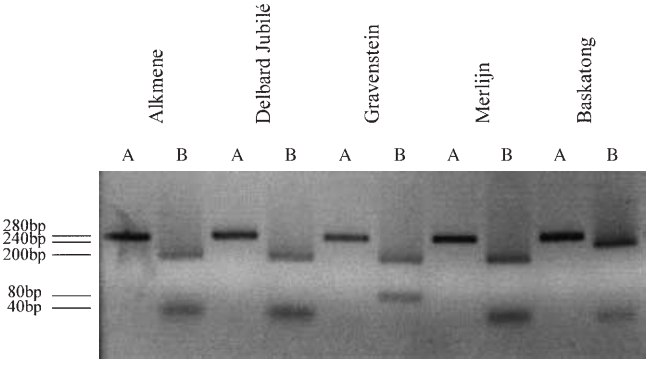


**Fig. 1** PCR amplification products from different cultivars using S4/S27 'specific' primers (*lanes A*), followed by digestion with *Taq*I (*lanes B*). The sizes of the different bands are shown on the left side of the figure. After PCR amplification a band of approximately 280 bp is obtained. After digestion with *Taq*I the S4 fragment in Gravenstein splits into two bands of approximately 200 bp and 80 bp, while the S27 fragment in Baskatong splits into two bands of approximately 240 bp and 40 bp (Verdoodt et al. 1998). The amplification product obtained from Alkmene, Delbard Jubilé and Merlijn does not correspond to the S4 fragment nor to the S27 fragment. After digestion with *Taq*I, the amplification product splits into two bands of approximately 200 bp and 40 bp

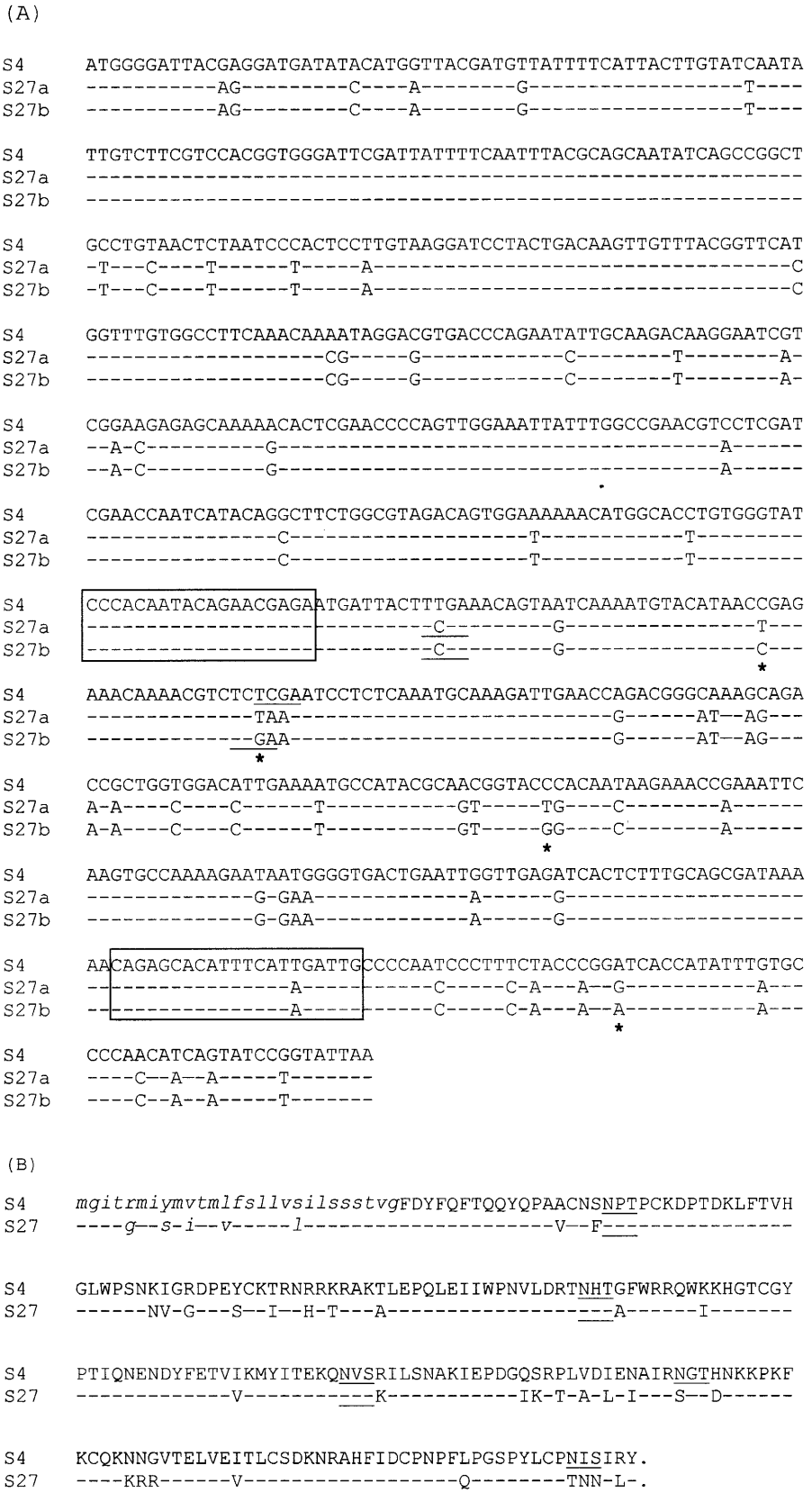


**Fig. 2** (**A**) Alignment of the coding sequence of S4, 27a and S27b, derived from respectively Gravenstein, Baskatong and Alkmene/Delbard Jubilé/Merlijn. Conserved nucleotides are indicated by –. The bindings sites of the S4/S27 'specific' primers are *boxed*. The *Taq*I restriction sites that are used to distinguish S4, S27a and S27b following PCR amplification are *underlined*. S27a and S27b only differ in four point mutations, which are marked with an *asterisk*. (**B**) Alignment of the amino acid sequence of S4 and S27 (the derived amino-acid sequence of S27a and S27b are identical and therefore named S27). – indicates amino acids that are conserved in both S-RNases. The putative signal peptide is shown in *lowercase italic letters*. Potential N-glycosylation sites, based on the N-Xaa-S/T recognition site, are *underlined*

that Alkmene, Delbard Jubilé and Merlijn each contain a distinct 'new' S-allele, respectively S22, S23 and S25 (Table 1). To verify if the S22, S23 and S25 ribonuclease bands indeed represent three distinct S-alleles, the coding sequences of the S-alleles corresponding to the ribonuclease bands were cloned and sequenced. Sequence comparison revealed that the corresponding S-alleles were identical in sequence (see below), in contrast to what had previously been proposed by Boskovic and Tobutt (1999).

## S27a and S27b, two alleles that encode identical protein sequences but differ at the nucleotide level

The coding sequence of the three S-alleles that were amplified by the S4/S27 'specific' primers are aligned in Fig. 2A. S4 was cloned from Gravenstein, a cultivar in which S4 was originally detected by Kobel et al. (1939). S27 (named S27a in Fig. 2A) was previously cloned from Baskatong (Verdoodt et al. 1998). Sequence comparison confirmed that the 'new' S-allele, derived from Alkmene, Delbard Jubilé and Merlijn (named S27b in Fig. 2A), is distinct from S4 and S27. The 'new' S-allele contains two *Taq*I restriction sites between the S4/S27 'specific' primer bindings sites, while S27 and S4 contain only one *Taq*I restriction site in this region. The positions of the *Taq*I restriction sites were in agreement with the sizes of the digestion products obtained by S-allele 'specific' PCR followed by restriction digestion (Fig. 1). Interestingly, the 'new' S-allele appeared to share a remarkably high sequence identity with S27 (99%). Both S-alleles only differ in four point mutations and these mutations do not lead to differences in amino-acid sequence. As the S-RNases encoded by these two S-alleles are identical, the S27 allele and the 'new' S-allele were named respectively S27a and S27b. At the nucleotide level, S27a and S27b both show approximately 91% sequence identity with S4. Their derived amino-acid sequence shows 84% sequence identity with S4. The differences between both S-RNases are spread throughout their protein sequence (Fig. 2B). Based on the N-Xaa-S/T recognition site, S4 and S27 contain respectively five and three potential N-glycosylation sites. The sequences of the S4 and the S27b cDNA were deposited under the GeneBank accession numbers AF327223 and AF327222.

To confirm that S27a and S27b are functionally identical, controlled pollinations were carried out. Lobo was pollinated with pollen from the cultivars Maypole, Lobo and Delbard Jubilé. Based on S-allele 'specific' PCR the S-genotypes of these cultivars were proposed to be respectively S10S27a, S10S27b and S2S27b (Table 1). As expected, self-pollination led to a low fruit (12%) and seed set (on average 3.7 seeds per fruit) while pollination with the semi-compatible cultivar Delbard Jubilé resulted in a fruit (92%) and seed set (on average 9.1 seeds per fruit), which was significantly higher. Following pollination with pollen from the cultivar Maypole, the fruit (3%) and seed set (on average 3.6 seeds per fruit) was comparable to those following self-pollination. This result indicated that Maypole and Lobo are cross-incompatible. Through microscopic analysis of the pollen tube growth in the styles, it was shown that the low fruit and seed set following self-pollination and pollination with pollen of the cultivar Maypole was not a result of insufficient pollination or a low germination rate. Seven days after pollination, the number of pollen tubes in the upper, the middle and the basal part of 25 styles were counted. In the upper part of the style the number of pollen tubes was comparable for each pollination, while at the base of the style there was a clear distinction between self-pollination and cross-compatible pollination. Following pollination with Lobo or Maypole no pollen tubes were detected at the base of the style, while pollination with pollen of Delbard Jubilé resulted in pollen tubes that reached the base in every style examined (data not shown).

## Telamon and Merlijn have a distinct S-genotype

According to Boskovic and Tobutt (1999), Merlijn and Telamon have identical S-genotypes (S3S25). Based on their analysis both cultivars contain the 'new' S-allele S25. When we analysed these cultivars by S-allele 'specific' PCR, an inconsistency was revealed. When genomic DNA of Merlijn was used as a template, an amplification product was obtained with S4/S27 'specific' primers and not with S10 'specific' primers, while Telamon gave amplification with S10 'specific' primers and not with S4/S27 'specific' primers (Table 1). In both cultivars an amplification product was obtained with S3 'specific' primers. These data indicated that the S25 ribonuclease bands detected in Telamon and Merlijn probably represent different S-alleles. To verify if the S25 ribonuclease band detected in Telamon indeed corresponds to a 'new' S-allele distinct from the S-allele of Merlijn, the complete coding sequence of this S-allele was cloned. Sequence comparison revealed that the S-allele from Telamon shares 80% sequence identity with the S-allele from Merlijn. The derived amino-acid sequences of both S-alleles share 67% sequence identity.

That both Telamon and Merlijn have distinct S-genotypes was further confirmed by controlled pollination. Telamon was pollinated with pollen from the cultivars Merlijn, Telamon and Elstar. Based on S-allele 'specific' PCR the S-genotypes of these cultivars were proposed to be respectively S3S27b, S3S10 and S3S5 (Table 1). As expected, self-pollination led to a low fruit (5%) and seed set (on average 2.7 seeds per fruit), while pollination with the semi-compatible cultivar Elstar resulted in a high fruit (76%) and seed set (on average 7.5 seeds per fruit). Following pollination with pollen from the cultivar Merlijn, a fruit (75%) and seed set (on average 7.0 seeds per fruit) was obtained that was comparable to the fruit and seed set following pollination with Elstar, which indicates that Merlijn and Telamon are cross-compatible.

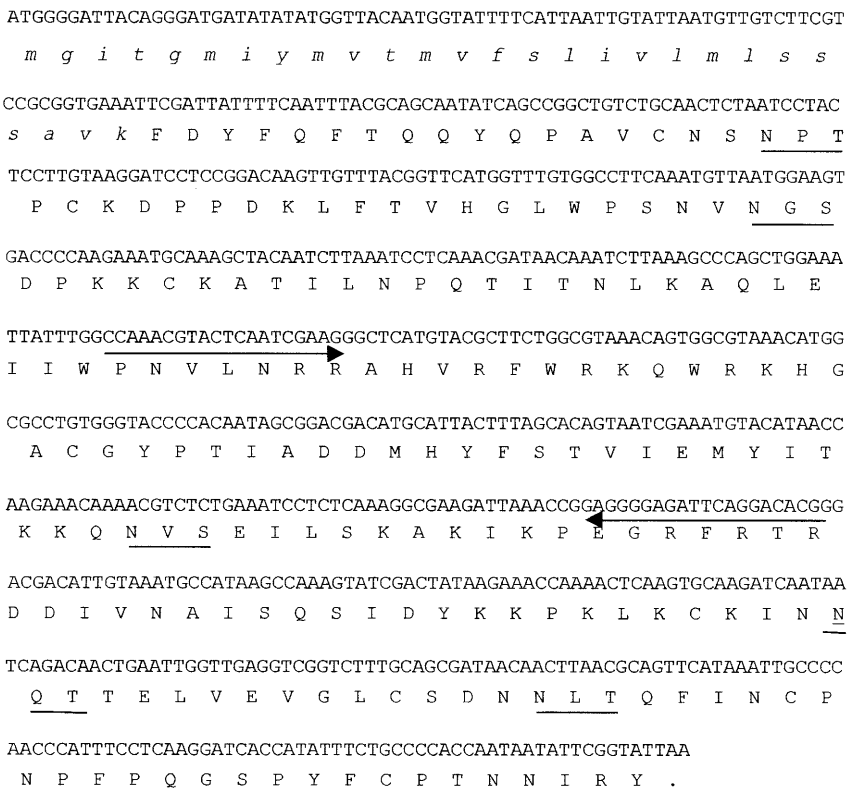


**Fig. 3** The coding sequence and derived amino-acid sequence of S10 from Telamon and Prima. Deduced amino-acid residues are shown below the nucleotide sequence. The putative signal peptide is shown in *lowercase italic letters* and the putative N-glycosylation sites are *underlined.* *Arrows* mark the binding sites of the S10 'specific' primers

The S10 and S25 ribonuclease bands, identified in respectively Prima and Telamon, represent the same S-allele

Because the S25-allele from Telamon was amplified with S10 'specific' primers, we compared the coding sequence of this S-allele with that of the S10 allele. S10 was cloned from Prima, a cultivar in which a ribonuclease band that corresponds to S10 was detected (Boskovic and Tobutt 1999). Sequence comparison revealed that, in contrast to what was expected, the coding sequences of both S-alleles were identical. The complete coding sequence of S10 from Prima and Telamon and its derived amino-acid sequence is shown in Fig. 3. Based on the N-Xaa-S/T recognition site the corresponding ribonuclease contains five potential N-glycosylation sites. S10 was previously already isolated from Maypole and Discovery (Broothaerts et al., unpublished data). The latter sequences were identical to the one cloned from Prima and Telamon in this study. The sequence of the S10 cDNA was deposited under GeneBank accession number AF327221.

## Discussion

Previously, two molecular methods for S-allele genotyping of apple cultivars, a protein- and a DNA-based method, have been developed. In the protein-based method (Boskovic and Tobutt 1999) S-genotypes are assigned by analysing differences in S-RNase migration through a protein gel, while in the DNA-based method (Janssens et al. 1995) the S-genotype is determined by PCR amplification of genomic DNA with S-allele 'specific' primers, often followed by restriction digestion. Not only in apple but also in other members of the Rosaceae family, like Japanese pear (Hiratsuka et al. 1998; Ishimizu et al. 1999), sweet cherry (Boskovic et al. 1997a; Tao et al. 1999) and almond (Boskovic et al. 1997b; Tamura et al. 2000), similar methods for S-genotyping are being used. However, both approaches for S-genotyping have some drawbacks, which were observed during this study.

In the protein-based method the assumption is made that, in the absence of other evidence, indistinguishable S-RNase bands represent the same S-allele and bands that show consistent electrophoretic differences, even when the difference is only slight, represent different S-alleles. In their paper Boskovic and Tobutt (1999) already mention that this assumption might not always be correct, although so far no such examples were known. During the course of this study some cases in which this assumption was not applicable were revealed. The coding sequences of the S-alleles that correspond to the S10 and the S25 ribonuclease bands, detected in respectively Prima and Telamon, appeared to be identical. Also the S22 and S23 ribonuclease bands, detected in respectively Alkmene and Delbard Jubilé, were shown to represent the same S-allele. After isoelectric focusing, in both cases only a slight electrophoretic difference between the two ribonucleases was observed (Boskovic and Tobutt 1999). A small electrophoretic difference between proteins with an identical amino-acid sequence

can be explained by potential differences in their glycan chains. Micro-heterogeneities of glycan chains have also been used to explain why some S-RNases in pear gave double bands after separation by two-dimensional gel electrophoresis (Ishimizu et al. 1996). Previously, it has been shown that the specificity of the self-incompatibility reaction lies in the amino-acid sequence and not in the glycan chains (Karunanandaa et al. 1994). Transformation of *Petunia inflata* with a mutant S-gene that codes for a non-glycosylated S-RNase resulted in plants that were still able to specifically reject pollen bearing this S-allele. The identical amino-acid sequence therefore indicates that the ribonucleases are probably also functionally identical. Interestingly, the apparently identical S25 RNase bands detected in Telamon and Merlijn appeared to represent different S-alleles. To our surprise the S25 ribonuclease band from Merlijn appeared to correspond to the same S-allele as the S22 and the S23 ribonuclease bands detected in respectively Alkmene and Delbard Jubilé. As the difference between the pI value of the S25 protein and those of the two other S-RNases was large (pI value of 8.50 compared to 9.85 and 9.80, Boskovic and Tobutt 1999), this result was quite surprising. The large difference in electrophoretic mobility between these proteins with identical amino-acid sequence indicates that the difference in their glycan chains must also be large. Alternatively, the discrepancy between our result and the result obtained by Boskovic and Tobutt concerning the S-genotype of Merlijn might be attributed to misinterpretation of the RNase zymogram or mislabelling of plant material. That Merlijn and Alkmene should have one S-allele in common was confirmed by parentage analysis. Microsatellite analysis revealed that Merlijn is an offspring of Alkmene and Idared (Kenis and Keulemans, unpublished results).

As mentioned above, the DNA-based method also has some drawbacks, which were observed during the course of this study. The primers originally designed to specifically amplify S24 appeared to amplify also Sg, an S-allele that was recently cloned and sequenced from Indo. Sequence comparison revealed that the S24 primer binding sites are completely conserved in the Sg sequence. Also our S1/S24 primers, originally designed to specifically amplify S1 but later shown to co-amplify S24, seemed to amplify Sg (data not shown). Based on the sequence of Sg and that of other known S-alleles, new Sg 'specific' primers were developed. Using the S1/S24/Sg, the S24/Sg and the Sg 'specific' primers, it is possible to distinguish these three S-alleles. To allow a more straightforward detection of S1 and S24, new S1 and S24 'specific' primers should be developed. The fact that it can not be excluded that a primer pair, designed to specifically amplify a particular S-allele, also amplifies another yet unknown but similar S-allele, forms the major drawback of the method of S-allele 'specific' amplification. By restriction digesting the amplification product the risk to falsely assign an S-allele can be reduced but not eliminated. Sequence comparison revealed that not only are the S24 primer binding sites completely conserved in Sg but so too is the *Scr*FI restriction site used to digest the amplification product. As the sequences of more S-alleles are known, the chance that an S-allele 'specific' primer pair also amplifies another unknown S-allele becomes smaller. Besides reducing the risk to falsely assign an S-allele, restriction digestion of the amplification product also makes it possible to detect potentially 'new' S-alleles. By analysing different cultivars with S4/S27 'specific' primers and digesting the PCR products with *Taq*I, a putative 'new' S-allele was detected. Sequence comparison revealed, however, that this S-allele only deviates in four point mutations from S27, previously cloned and sequenced from Baskatong, and that these point mutations do not lead to differences in amino-acid sequence. That both S-alleles are functionally identical was confirmed by controlled pollination. To our knowledge this is the first report of two S-alleles that differ at the nucleotide level, but still encode for identical S-RNases. The existence of such S-alleles is in agreement with the hypothesis that new S-alleles are generated by an accumulation of point mutations, which may or may not lead to differences in amino-acid sequence (Després et al. 1994). Although such S-alleles are probably rare, care must be taken when assigning new S-alleles solely based on differences in nucleotide sequence. Before assigning new S-alleles based on absence of a restriction site or absence of amplification, confirmation by other data is necessary.

Finally, we can conclude that by this study and the study conducted by Verdoodt et al. (1998) the existence of three of the 14 'new' S-alleles proposed by Boskovic and Tobutt (S20, S22 and S24) was confirmed. The derived amino-acid sequence of one of these S-alleles (S22) was shown to be identical to S27, an S-RNase which was not included in the analysis conducted by Boskovic and Tobutt (1999). The existence of two other putative 'new' S-alleles (S23 and S25) was withdrawn. Because the electrophoretic differences between several S-RNases identified by Boskovic and Tobutt appeared to be small, it is conceivable that also other putative 'new' S-alleles do not exist. The number of S-alleles present in apple might therefore be smaller than the number proposed by Boskovic and Tobutt. This assumption is even more conceivable as many of the proposed 'new' S-alleles were only shown to occur in one cultivar.

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