Theor Appl Genet (2000) 101:344–349 © Springer-Verlag 2000

M. Tamura · K. Ushijima · H. Sassa · H. Hirano
R. Tao · T.M. Gradziel · A.M. Dandekar

Identification of self-incompatibility genotypes of almond by allele-specific PCR analysis

Received: 21 June 1999 / Accepted: 15 November 1999

Abstract In almond, gametophytic self-incompatibility is controlled by a single multiallelic locus (*S*-locus). In styles, the products of *S*-alleles are ribonucleases, the S-RNases. Cultivated almond in California have four predominant *S*-alleles (S^a, S^b, S^c, S^d). We previously reported the cDNA cloning of three of these alleles, namely S^b, S^c and S^d. In this paper we report the cloning and DNA sequence analysis of the S^a allele. The S^a-RNase displays approximately 55% similarity at the amino-acid level with other almond S-RNases (S^b, S^c, and S^d) and this similarity was lower than that observed among the S^b, S^c and S^d-RNases. Using the cDNA sequence, a PCR-based identification system using genomic DNA was developed for each of the S-RNase alleles. Five almond cultivars with known self-incompatibility (SI) geno-types were analyzed. Common sequences among four *S*-alleles were used to create four primers, which, when used as sets, amplify DNA bands of unique size that corresponded to each of the four almond *S*-alleles; S^a (602 bp), S^b (1083 bp), S^c (221 bp) and S^d (343 bp). All PCR products obtained from genomic DNA isolated from the five almond cultivars were cloned and their DNA sequence obtained. The nucleotide sequence of these genomic DNA fragments matched the corresponding *S*-allele cDNA sequence in every case. The amplified products obtained for the S^a- and S^b-alleles were both longer than that expected for the coding region, revealing the presence of an intron of 84 bp in the S^a-allele and 556 bp in the S^b-allele. Both introns are present within the site of the hypervariable region common in S-RNases from the Rosaceae family and which may be important for *S* specificity. The exon portions of the genomic DNA sequences were completely consistent with the cDNA sequence of the corresponding *S*-allele. A useful application of these primers would be to identify the *S*-genotype of progeny in a breeding program, new varieties in an almond nursery, or new grower selections at the seedling stage.

Key words Almond · *Prunus dulcis* · *S*-allele · Self-incompatibility · S-RNase

Communicated by J. Dvórak

M. Tamura · T.M. Gradziel · A.M. Dandekar (✉)
Department of Pomology, University of California,
1 Shields Avenue, Davis, CA 95616, USA
e-mail:amdandekar@ucdavis.edu
Tel.: +1 530-752-7784, Fax: +1 530-752-8502
e-mail: amdandekar@ucdavis.edu

K. Ushijima · H. Sassa · H. Hirano
Kihara Institute for Biological Research
and Graduate School of Integrated Science,
Yokohama City University, Yokohama 244–0813 Japan

R. Tao
Graduate School of Agriculture, Kyoto University,
Kyoto 606–8502 Japan

Introduction

Self-incompatibility (SI) is a widespread inherited reproductive phenomenon in flowering plants that prevents self-fertilization through rejection of pollen from same plant. This trait promotes outcrossing while preventing inbreeding, and thus is an important evolutionary mechanism that maintains genetic variability among plant populations. There are two distinct mechanisms that mediate SI: gametophytic self-incompatibility (GSI) or sporophytic self-incompatibility (SSI) (Nettancourt 1977). In GSI pollen germinates but pollen-tube elongation is terminated within the style whereas in SSI, the incompatibility takes place at the stigmatic surface and pollen fails to germinate normally (Ebert et al. 1989). *Prunus* species exhibit GSI which is controlled by a single *S* locus having several codominant *S*-alleles. In the case of GSI in the Solanaceae, Rosaceae and Scrophulariaceae, the *S*-alleles encode specific ribonucleases (S-RNases) that block self pollen-tube growth through the style (McClure et al. 1989; Huang et al. 1994; Lee et al. 1994; Murfett et al. 1994, 1995; Kao and McCubbin 1996).

Self-incompatiblity produces individuals that are heterozygous for SI alleles because the SI alleles act to prevent the formation of zygotes that are homozygous at the *S*-locus. This enforces selection for other features that fa-

cilitate pollen transfer between individuals, and leads to the evolution of outcrossing syndromes in such plants. In breeding programs, progeny will only be obtained if the parents do not share identical SI alleles. Therefore, knowledge of SI genotypes of parents is essential for efficient breeding. Almond *(Prunus dulcis)*, the focus of this study, is self-incompatible with cross-pollination being essential for fruit set. Cultivated almond in California has four predominant *S*-alleles (S^a, S^b, S^c and S^d) that can be assembled into seven cross incompatibility groups (CIGs) depending on the combination of individual *S*-alleles (Kester et al. 1994). The determination of the *S*-genotype of segregating progeny in a cross is a time-consuming task that involves growing plants to maturity and testing for fruit set with pollen from various known *S*-genotypes. The identification of individual *S*-alleles could provide a faster means of identification not only of progeny but also of new cultivars and selections.

S-RNases encoded by the *S*-alleles in almond have been isolated and their N-terminal amino-acid sequences determined (Tao et al. 1997). Also, Boskovic et al. (1997) separated S-RNases using non-equilibrium pH-gradient electrophoresis, and identified some *S*-alleles of almond. These techniques, though useful, can only be used with mature plants. One of our foci has been to identify *S*-genotypes at the seedling stage using genomic DNA. To do this, we needed the DNA sequences of the four *S*-alleles. Earlier we reported the cDNA cloning and DNA sequence determination of three of the four *S*-alleles (S^b, S^c, and S^d) of almond (Ushijima et al. 1998b). In this study: (1) the cDNA for the remaining *S*-allele, S^a, was obtained and its DNA sequence was determined, (2) specific primer sets were created that can be used to generate *S*-allele-specific PCR products that are polymorphic for each of the predominant *S*-alleles, and (3) partial genomic DNAs encoding S-RNases were characterized.

Materials and methods

Isolation of total stylar RNA

Total RNA was isolated from freeze-dried styles of 'Mission' (S^aS^b) almond at the balloon stage of flower-bud development using the hot phenol method (Vries et al. 1988).

Cloning of cDNA encoding S^a-RNase

cDNA encoding S^a-RNase was amplified from total stylar RNA of 'Mission' (S^aS^b) by 3′RACE with the gene-specific primer AS-1 and an adapter primer as described previously (Ushijima et al. 1998b). The 5′ coding region, which was not contained in the 3′RACE clone, was amplified by 5′RACE using the primer Pru-C5 and the adapter primer BSRACE-2 as described previously (Ushijima et al. 1998b). PCR fragments amplified by RACE were cloned and sequenced as described previously (Ushijima et al. 1998a).

Table 1 Primer combinations for *S*-allele-specific PCRs

Aimed *S*-allele amplification	Forward primer	Reverse primer
S^a and S^b	AS1II	AmyC5R
S^c and S^d	AlSc1 AlSd2	AmyC5R

DNA isolation

Total DNA was isolated from young almond leaves of five cultivars each belonging to a different CIG ('Nonpareil': S^cS^d, 'Monterey': S^bS^d, 'Mission': S^aS^b, 'Ne Plus Ultra': S^bS^c, 'Thompson': S^aS^c) using a modified CTAB method. About 50 mg of leaf tissue was homogenized in liquid nitrogen with 500 µl of ice-cold isolation buffer [10% PEG6000, 0.35 M sorbitol, 0.1 M Tris-HCl (pH 8.0), 0.5% spermidine, 0.5% spermine, 0.5% mercaptoethanol]. After centrifugation and removal of the supernatant, the pellet was resuspended in 250 µl of lysis buffer [0.35 M sorbitol, 0.1 M Tris-HCl (pH 8.0), 0.5% spermidine, 0.5% spermine, 0.5% mercaptoethanol] containing 1/10 vol of 10% lauroyl sarcosine. After incubation for 10 min at room temperature, 500 µl of CTAB extraction buffer [2% CTAB, 1.4 M NaCl, 1% PVP-10, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0), 1% mercaptoethanol] was added, mixed and incubated for 30 min at 60°C. Then 500 µl of chloroform-isoamyl alcohol (24:1) was added and the supernatant was precipitated by isopropanol. After rinsing the pellet with 76% ethanol-10 mM sodium acetate and dissolving in TE, it was treated with RNase and incubated for 30 min at 37°C. DNA was precipitated by ethanol, rinsed with 75% ethanol and dissolved in TE.

PCR analysis

PCR was conducted using about 20 ng of DNA, 2.5 µl of 10× PCR buffer, 0.12 mM of dNTP mix, 0.125 µM of each primer and 0.2 µl of *Taq* polymerase (5 unit/µl, Promega, Wis., USA) in a 25-µl reaction mixture. Cycling parameters were 5-min initial denaturation at 94°C, 30 cycles of 1-min denaturation at 94°C, 1-min of annealing at 53°C and 2-min of extension at 72°C, and then, a 5-min final extension.

Four primers were synthesized using *S*-allele cDNA sequence information (Ushijima et al. 1998b); AS1II (forward, 5′-TATT TTCAATTTGTGCAACAATGG-3′), AmyC5R (reverse, 5′-CAA AATACCACTTCATGTAACAAC-3′), AlSc1 (forward, 5′-CAG ACACTTAATCAATTCCAG-3′), and AlSd2 (forward, 5′-CCAC ATGGCGTGGGATACCTCG-3′). AS1II and AmyC5R are common sequences between the four S-RNases, and also represent conserved regions within Rosaceae S-RNases (C1 and C5). AlSc1, and AlSd2 correspond to specific regions of S^c- and S^d-alleles, respectively. Two primer sets were used for PCR: (1) AS1II and AmyC5, (2) AlSc1, AlSd2 and AmyC5R (Table 1).

Cloning and DNA sequence of PCR products

Each PCR product was cloned using TOPO TA Cloning kit (Invitrogen, Calif., USA) and their sequences were determined and analyzed by MacDNAsis (Hitachi Software, Calif., USA).

Results and discussion

Cloning of the S^a-allele

Following 5′ and 3′ RACE, a 811-bp cDNA sequence encoding the full-length S^a-RNase allele (DDBJ/EMBL/

```
                      10         20          30  C1      40         50
Sa    1 MAMLKLSLAF HVLAFVLFLC FTMSTGSYQY FQFVQQWPPT TCAVSKQPCY   50
Sb    1 MAMLKSSPAF LLVAFAFFLC FIMSTGSYVY FQFVQQWPPT NCRVRIKRPC   50
Sc    1 MGMLKSSLAF LVLGFAFFFC YVMSSGSYDY FQFVQQWPPT NCRVRMKRPC   50
Sd    1 ---------- --------LC FIMSTGSYVY FQFVQQWPPT TCRLSSK-PS   50
                                        AS1II
                      60  C2      70         80          90 HV       100
Sa   51 QNP--PSIFT IHGLWPSNYS KKAWVANCTR TRFNNSLAPK LEA-KLKISW  100
Sb   51 SNPRPLQYFT IHGLWPSNYS NPTKPSNCNG SQFNFTKVSP KMRVKLKRSW  100
Sc   51 SNPRPLQYFT IHGLWPSNFS NPTKPSNCNG TKFDARKVYP EMRSDLKISW  100
Sd   51 NQHRPLQRFT IHGLWQGNYS NPRKPSNCNG SQFNFMKVYP QLRTKLKRSW  100

                     110        120 C3       130        140        150
Sa  101 PNVENANYTE FWEREWNKHG TCSEQTLDQE EYFQRSHDIW NAYNITNILK  150
Sb  101 PDVESGNDTR FWEGEWNKHG TCSEGSLNQM QYFERSHEMW YSFNITEILK  150
Sc  101 PDVESGNDTK FWEDEWNKHG TCSEQTLNQF QYFERSHEMW MSYNITEILK  150
Sd  101 PDVEGGNDTK FWEGEWNKHG TCSERTLNQM QYFEVSHAMW RSYNITNILK  150

         RC4         160        170        180        190        200
Sa  151 KANILPN-GA IWNYSDIVSP IKTVTRKMPA LRCKPDPTKP KNHKISHQLL  200
Sb  151 NASIVPHPTQ TWKYSDIVAP IKTATKRTPV LRCKPDPAQN KSGPK-TQLL  200
Sc  151 NASIVPHPAK TWTYSDIVSP IKAATGRTPL LRCKYDN--- -----NTQLL  200
Sd  151 DAHIVPNPTQ RWKYSDIVSP IKTATGRTPT LRCKTDPAMP N----NSQLL  200

         C5          210        220        230        240        250
Sa  201 HEVVLCLHYK GRALIDCNRT A-CDNNLKIL FQ........ ..........  250
Sb  201 HEVVFCYEYH ALKQIDCNRT AGCWNNVDIK FQ........ ..........  250
Sc  201 HEVVFCYGYK AIKQIDCNRP G-CKNKIDIK FQ........ ..........  250
Sd  201 HEVVFCYGYN AKLHIDCNRT AGCRNHIDIL FQ........ ..........  250
        AmyC5R
```

Fig. 1 Amino-acid sequence alignment of four S-RNases from almond. The five conserved regions (C1, C2, C3, RC4 and C5) and a hypervariable region (HV) are *boxed*. AS1II and AmyC5R primer regions are *underlined*. *Arrows* indicate the position of introns in S^a and S^b. S^b, S^c and S^d-RNase: DDBJ/EMBL/GenBank Nucleotide Sequence Databases, Accession numbers AB011469, AB011470, AB011471 (Ushijima et al. 1998b). S^a-RNase: Accession number AB026836

Table 2 Amino-acid sequence similarity (%) among the S-RNases of almond

Type	S-RNases			
	S^a	S^b	S^c	S^d
S^a	–	57.0	54.2	57.0
S^b		–	76.2	75.2
S^c			–	68.7

GenBank AB026836) was obtained. The deduced amino acid sequence perfectly matched our previously reported N-terminal amino-acid sequence of S^a-RNase as separated on 2D gels (Tao et al. 1997). Comparisons were then made between the alignment of the amino-acid sequence of S^a-RNase with that of the other three previously reported S-RNases from almond (Ushijima et al. 1998b) to evaluate the amino-acid sequence differences (Table 2, Fig. 1). The similarity of the amino-acid sequence of S^a with S^b, S^c, and S^d was 54–57%, whereas the similarity between S^b, S^c, and S^d was 68–76%. The lower S^a similarity was also observed at the nucleotide level. A 65–67% identity of nucleotide sequences was observed between $S^a \times S^b$, $S^a \times S^c$, $S^a \times S^d$, while a 76–87% nucleotide identity was observed between $S^b \times S^c$, $S^b \times S^d$, and $S^c \times S^d$. Among the four *S*-alleles, S^a had less similarity with the S^b, S^c and S^d-alleles.

According to Ushijima et al. (1998b), the amino-acid sequence alignment of S-RNases from 12 *S*-alleles belonging to four different members of the Rosaceae family (almond; S^b, S^c, S^d, sweet cherry; S^2, S^3, S^6, Japanese pear; S^2, S^4, S^5, and apple; S^2, S^c, S^f) revealed five conserved regions. Four of the five conserved regions were similar to those reported by Ioerger et al. (1990) for S-RNase belonging to the Solanaceae (C1, C2, C3 and C5). Our results indicate that all S-RNases in almond have the same five conserved regions (C1, C2, C3, RC4 and C5) and a hypervariable region (HV), which are common structural features of the S-RNases of the Rosaceae (Fig. 1).

S-allele-specific PCR

PCR analysis was conducted using *S*-allele-specific primer sets based on S^a–S^d RNase cDNA sequences to identify the corresponding *S*-alleles in almond. Primer sequences for the AS1II forward primer and the AmyC5R reverse primer were based upon the common sequences of the four almond *S*-alleles within the conserved regions C1 and C5, respectively. However only two bands instead of the expected four were amplified. A 0.6-kbp PCR product was observed in the cultivars with the S^a-allele, 'Mission' (S^aS^b) and 'Thompson' (S^aS^c); while an amplicon of 1.1 kbp was observed in the cultivars with S^b-allele; 'Monterey' (S^bS^d), 'Mission' (S^aS^b) and 'Ne Plus Ultra' (S^bS^c). These results indicated that the 0.6-kbp band represented the S^a-allele (S^a-band), and the 1.1-kbp band represented the S^b-allele (S^b-band) (Fig. 2). Because the coding regions of S^a- and S^b-alleles between AS1II and AmyC5R were 518 bp and 527 bp, respectively (Fig. 3), the existence of an intron in the genomic DNA between AS1II and AmyC5R was suggested.

To identify the S^c- and S^d-alleles, we synthesized two forward primers based on the S^c and S^d gene-specific sequences (designated AlSc1 and AlSd2, respectively). By

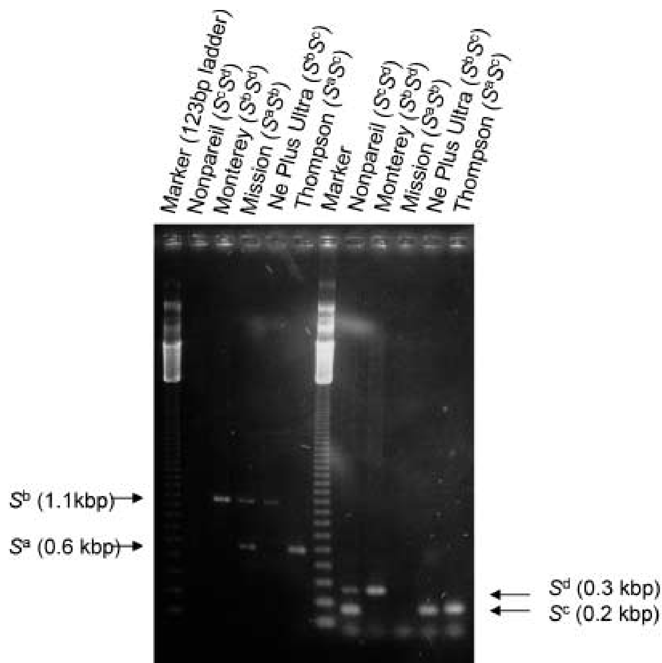

Fig. 2 PCR analysis of five cultivars of almond; Nonpareil (S^cS^d), Monterey (S^bS^d), Mission (S^aS^b), Ne Plus Ultra (S^bS^c) and Thompson (S^aS^c). M DNA size markers/ 123-bp ladder. *Left lanes* primer set was [AS1II+AmyC5R]. *Right lanes* primer set was [AlSc1+AlSd2+AmyC5R]

using three primers (AlSc1, AlSd2 and AmyC5R) in the same PCR reaction, approximately 0.2-kbp and approximately 0.3-kbp products were amplified. The approximately 0.2-kbp product was obtained in the cultivars with an S^c-allele: 'Nonpareil' (S^cS^d), 'Thompson' (S^aS^c) and 'Ne Plus Ultra' (S^bS^c); while the approximately 0.3-kbp product was obtained in the cultivars with the S^d-allele: 'Monterey' (S^bS^d) and 'Nonpareil' (S^cS^d). Therefore, the 0.2-kbp band was considered to correspond to the S^c-allele (S^c-band) and the 0.3-kbp band to the S^d-allele (S^d-band) (Fig. 2). Since the coding region of S^c- and S^d-alleles were 221 bp and 343 bp, respectively (Fig. 3), amplified genomic DNAs of S^c- and S^d-RNases were considered to have a short, or no, intron.

The three sets of primers produced unique bands of a determinant size that corresponded to each of the four predominant *S*-alleles of almond, providing a rapid way of identifying the *S*-genotype directly from genomic DNA. However, we needed to confirm that the bands amplified by these primers using genomic DNA from the five different almond cultivars did indeed represent the four individual *S*-alleles.

Characterization of genomic DNA encoding S-RNases

Amplicons obtained from almond genomic DNA were sequenced to compare with cDNA sequences encoding each of the four S-RNases (S^a: GenBank AB026836, S^b: AF148466, S^c: AF148467, and S^d: AF148468).

The DNA sequence of the 602-bp amplicon (S^a-product) and the 1083-bp amplicon (S^b-product) from 'Mission' (S^aS^b) were determined (Tamura et al. 1999; GenBank AF148465, AF148466). The S^a-amplicon from 'Thompson' (S^aS^c), and the S^b-amplicon from 'Monterey' (S^bS^d) and 'Ne Plus Ultra' (S^bS^c) were also sequenced. Amplified products were both longer than would be expected from the corresponding coding region in S^a or S^b, and this indicated the potential existence of introns (Fig. 3). One intron of 84 bp in the S^a-allele and one intron of 556 bp in the S^b-allele were found within the hypervariable region (HV), which is common in rosaceous S-RNases (Sassa et al. 1996; Ushijima et al. 1998a) (Fig. 4). HV regions of S-RNases in the Solanaceae are responsible for the determination of self-recognition specificity (Matton et al. 1997). Therefore, it is interesting that, in almond, a HV region includes the insertion of an intron that varies in size in the two *S*-alleles and these regions may characterize the structure of individual alleles. DNA sequences of both putative intron regions of the S^a- and S^b-alleles had features consistent with that observed in typical intron regions. The intron regions have a higher AT content, 69% and 78% for S^a and S^b, respectively, whereas the AT contents of coding regions for all four alleles vary between 55 and 59%. Additionally, both S^a and S^b introns contain essential sequences necessary for the splicing reaction, i.e., splice junctions (GT, AG) and branch-point sequences (Fincham 1994) (Fig. 4). The exon portions of the genomic DNA sequences, i.e., the 518-bp sequences of S^a from 'Mission' and 'Thompson' and the 527-bp of S^b from 'Mission', 'Monterey' and 'Ne Plus Ultra', were consistent with the corresponding coding region of the cDNA sequence of S^a or S^b, respectively.

The genomic sequences of the S^c-amplicon and S^d-amplicon in 'Nonpareil' matched perfectly with the corresponding cDNA sequences encoding S^c-RNase and S^d-RNase, respectively (GenBank AF148467, AF148468), and also S^c-bands of 'Ne Plus Ultra' and 'Thompson', and S^d-bands of 'Monterey' and 'Nonpareil'.

Although the primers used in PCR-amplification contained a sequence common to all four almond *S*-alleles, only the S^a- and S^b-alleles were amplified. It seems plausible that introns may also be present in the S^c- and S^d-alleles at a similar, but not necessarily identical, location and that these intron sequences, or their structure, may interfere with the PCR reaction. This explanation is supported by the fact that no PCR-amplification was observed when PCR reactions for the S^c- and S^d-alleles covered the site of the putative intron region, using forward primers 72-bp and 43-bp upstream of AlSc1 and AlSd2, respectively (data not shown).

We have identified a unique set of primers that can be used for the specific amplification of individual almond *S*-alleles using a rapid PCR technique. This approach is useful in evaluating *S*-genotypes in segregating progeny, and new cultivars and introductions, using DNA from seedlings. Recently, similiar approaches have been used to identify *S*-alleles in apple (Janssens at al. 1995;

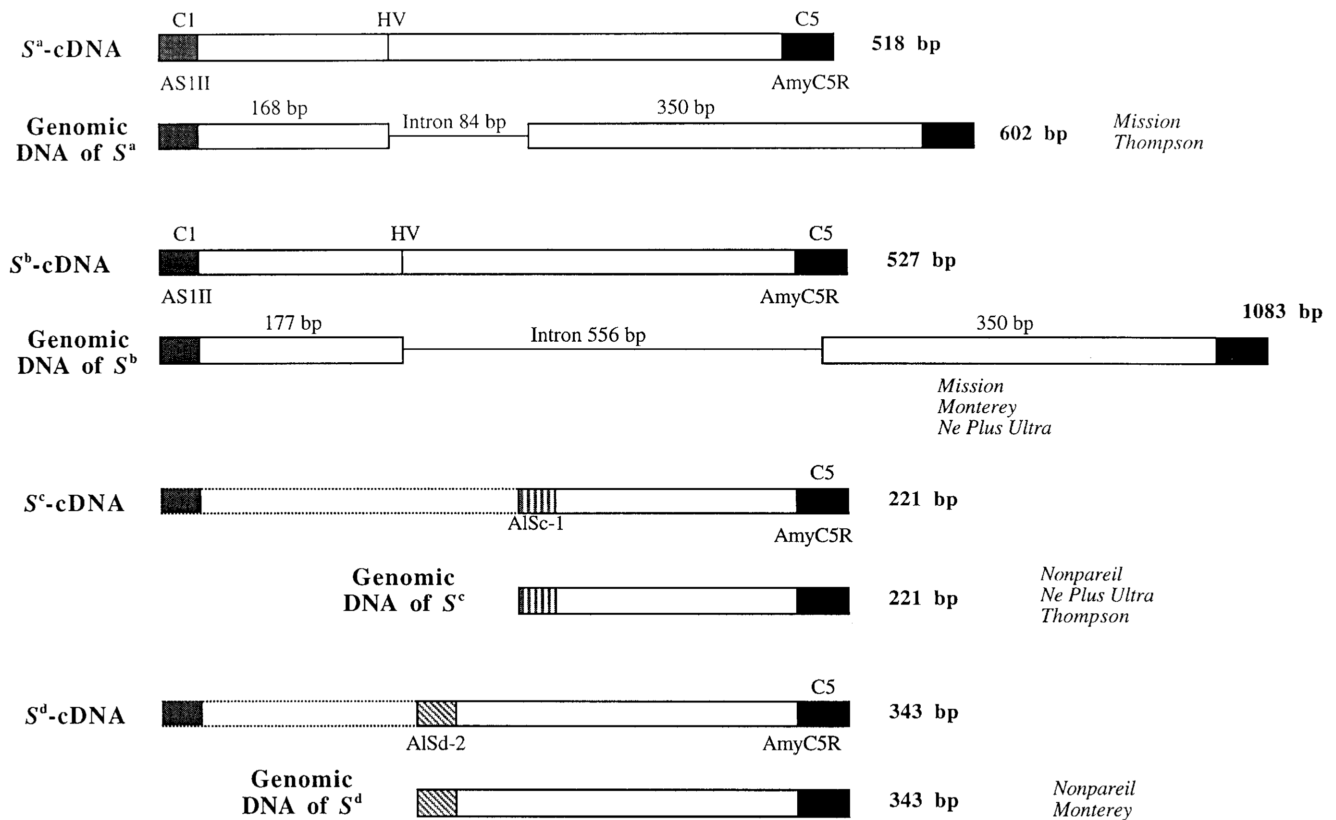

Fig. 3 Schematic representation of the structures of PCR-amplified products of genomic DNA of the four *S*-alleles (S^a, S^b, S^c, S^d) and their corresponding cDNAs in almond. Location of the primers used in PCR analysis, length of PCR, and the C1, C5 and HV regions are indicated. Introns are represented by the *bars* between two boxes. The DNA sequences of the PCR fragments corresponding to genomic DNA encoding each of the *S*-alleles was registered on DDBJ/EMBL/GenBank Nucleotide Sequence Databases, Accession numbers: AF148465 (S^a), AF148466 (S^b), AF148467 (S^c), AF148468 (S^d)

Fig. 4 DNA sequences of introns in the S^a- and S^b-alleles

S^a (84bp):
GTATGAATTGGCTCTTTGTTTTTCTAGTTACTCTTTAGTTTTTTGTATTTTCCTCACAATAGATTTATTGCTTGGATGT
TGCAG

S^b (556bp):
GTATGTATTATTTCAAATTTTTTTTTTTCACTTACTCTTTAGCTTTTATTGTTTTTATTTTTTGCACAAATACTCTTT
AGCATTTTAGTTTTTAGAAAATTAGATTGTCGTATGAAGATTTAAAATTTAATTAAAATTTTTTTTCAATAAGCCTT
GGGGGTTAGATATAAAATTTTGGTGTTGGTTTAGTTTTTTTAATTATTATTTTTTTAATGTTGGTTCTTAGTTAGACA
CATTATTTTGAAAATATAGTCAAGTTCAAAATGGNACGTATATTTATATTTATATGCATATTAATGTACTTGCGAAA
ATATGATGGATCTGCTCATCTAATTACATGACCTACCATTTTGTCCTAATGTATATATGCAAAAAATTTGTTAGAAG
AAAAAGCAAGGCTATAATATATTATTGAAGGGTTGAACTCAAAAATTCAATTTTAATGCTNAGATTTNAAGGAAAA
CAAAANAAAAAAAAATCTTTATTTCAANGAAATGAAAATCCTAATTTATTCNATTTTATTCCAAATTTAACTTTTT
TTCTTGGAAAAAATAAG

Verdoodt et al. 1998), sweet cherry (Tao et al. 1999) and Japanese pear (Ishimizu et al. 1999). Apart from *S*-alleles, PCR techniques have allowed the detection of various markers in fruit trees, such as gender (Hormaza et al. 1994; Gill et al. 1998) and disease resistance (Yang et al. 1997; Hemmat et al. 1998). Clearly, the major advantage here is the ability to evaluate as many traits as possible at the seedling stage in fruit trees that have a long juvenile phase.

References

Boskovic R, Tobutt KR, Batlle I, Duval H (1997) Correlation of ribonuclease zymograms and incompatibility genotypes in almond. Euphytica 97:167–176

Ebert PR, Anderson MA, Bernatzky R, Altschuler M, Clarke AE (1989) Genetic polymorphism of self-incompatibility in flowering plants. Cell 56:255–262

Fincham JRS (1994) Genetic analysis. Blackwell Science UK, pp 102–110

Gill GP, Harvey CF, Gardner RC, Fraser LG (1998) Development of sex-linked PCR markers for gender identification in *Actinidia*. Theor Appl Genet 97:439–445

Hemmat M, Weeden NF, Aldwinckle HS, Brown SK (1998) Molecular markers for the scab resistance (Vf) region in apple. J Am Soc Hort Sci 123:992–996

Hormaza JI, Dollo L, Polito VS (1994) Identification of RAPD markers linked to sex determination in *Pistacia vera* using bulked segregant analysis. Theor Appl Genet 89:9–13

Huang S, Lee H-S, Karunandaa B, Kao T-H (1994) Ribonuclease activity of *Petunia inflata* S proteins is essential for rejection of self-pollen. Plant Cell 6:1021–1028

Ioerger TR, Clarke AG, Kao T-H (1990) Polymorphism at the self-incompatibility locus in *Solanaceae* predates speciation. Proc Natl Acad Sci USA 87:9732–9735

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

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

Kao TH, McCubbin AG (1996) How flowering plants discriminate between self and non-self pollen to prevent inbreeding. Proc Natl Acad Sci USA 93:12059–12065

Kester DE, Gradziel TM, Micke WC (1994) Identifying pollen incompatibility groups in California almond cultivars. J Am Soc Hort Sci 119:106–109

Lee H-S, Huang S, Kao T-H (1994) S proteins control rejection of incompatible pollen in *Petunia inflata*. Nature 367:560–563

Matton DP, Maes O, Laublin G, Xike Q, Bertrand C, Morse D, Cappadocia M (1997) Hypervariable domains of self-incompatibility RNases mediate allele-specific pollen recognition. Plant Cell 9:1757–1766

McClure BA, Haring V, Ebert PR, Anderson MA, Simpson RJ, Sakiyama F, Clarke AE (1989) Style self-incompatibility gene products of *Nicotiana alata* are ribonucleases. Nature 342: 955–957

Murfett J, Atherton TL, Mou B, Gasser CS, McClure BA (1994) S-RNase expressed in transgenic *Nicotiana* causes *S*-allele-specific pollen rejection. Nature 367:563–566

Murfett J, Bourque JE, McClure BA (1995) Antisense suppression of S-RNase expression in *Nicotiana* using RNA polymerase II- and III-transcribed gene constructs. Plant Mol Biol 29: 201–212

Nettancourt D de (1977) Incompatibility in angiosperms. Springer, Berlin Heiderberg New York

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

Tamura M, Gradziel TM, Dandekar AM (1999) Cloning of genomic DNA sequences encoding almond (*Prunus dulcis*) S-RNase genes (Accession No. AF148465, AF148466, AF148467, AF148468). Plant Physiol 120:1206

Tao R, Yamane H, Sassa H, Mori H, Gradziel TM, Dandekar AM, Sugiura A (1997) Identification of stylar RNase associated with gametophytic self-incompatibility in almond (*Prunus dulcis*). Plant Cell Physiol 38:304–311

Tao R, Yamane H, Sugiura A, Murayama H, Sassa H, Mori H (1999) Molecular typing of S-alleles through identification, characterization and cDNA cloning for S-RNases in sweet cherry. J Am Soc Hort Sci 124:224–233

Ushijima K, Sassa H, Hirano H (1998a) Characterization of the flanking regions of the *S*-RNase genes of Japanese pear (*Pyrus serotina*) and apple (*Malus domestica*). Gene 211:159–167

Ushijima K, Sassa H, Tao R, Yamane H, Dandekar AM, Gradziel TM, Hirano H (1998b) Cloning and characterization of cDNAs encoding S-RNases in almond (*Prunus dulcis*): primary structural features and sequence diversity of the S-RNases in Rosaceae. Mol Gen Genet 260:261–268

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

Vries S de, Hoge H, Bisseling T (1988) Isolation of total and polysomal RNA from plant tissues, In: Glevin SB, Schilperoot RA, Verma DPS (eds) Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht The Netherlands, pp 1–13

Yang HY, Korban SS, Kruger J, Schmidt H (1997) A random amplified polymorphic DNA (RAPD) marker tightly linked to the scab-resistance gene in apple. J Am Soc Hort Sci 122:47–52