Theor Appl Genet (2001) 103:1115–1122

C. Channuntapipat · M. Sedgley · G. Collins

Sequences of the cDNAs and genomic DNAs encoding the *S1, S7, S8,* and *Sf* alleles from almond, *Prunus dulcis*

Received: 18 October 2000 / Accepted: 8 March 2001

Abstract Partial genomic and cDNA sequences of the RNase alleles *S1*, *S7*, *S8* and *Sf* were obtained from *Prunus dulcis* cvs 'Ne Plus Ultra', 'Ferragnes' and 'Nonpareil' 15–1, and IRTA Selection 12–2. Total DNA was extracted from leaves, and cDNA was prepared from total RNA extracted from styles. The partial cDNA sequences of the *S1* allele from 'Ferragnes', and the *S7* and *S8* alleles from 'Nonpareil' 15–1, matched those reported in the literature for the alleles *Sb*, *Sc* and *Sd* respectively. The sequences of the *S1*, *S7*, *S8* and *Sf* alleles found in genomic DNA contained introns of 562, 1,530, 2,208 and 689 bp respectively. The exon/intron splice junction sites of all alleles followed the GT/AG consensus sequence rule, and the sequences were found to be highly conserved.

Keywords Almond · *Prunus dulcis* · Self-incompatibility · *S*-allele sequences

Introduction

Gametophytic self-incompatibility occurs in the family Rosaceae (Sedgley 1994; Newbigin 1996), which includes a number of ornamental and fruit-tree crops such as *Malus*, *Potentilla*, *Prunus*, *Pyrus*, *Rosa* and *Rubus*. A single gene at the *S* locus encodes an allelic series of stylar glycoproteins with ribonuclease activity, known as *S*-RNases, and pollen grains expressing the *S*-alleles encoded by their haploid genome are rejected by pistils that express the same *S*-allele (Ishimizu et al. 1998). In almond, the inhibition of pollen-tube growth occurs in the style (Pimienta et al. 1983).

Communicated by H.C. Becker

C. Channuntapipat · M. Sedgley · G. Collins (✉)
Adelaide University, Department of Horticulture,
Viticulture and Oenology, Waite Campus,
Glen Osmond, South Australia 5064, Australia
e-mail: graham.collins@adelaide.edu.au
Fax: +618-8303-7116, Tel. +618-83037295

Most almonds will not set fruit unless pollinated by cultivars of different incompatibility genotypes, and thus the pollination group is a significant agronomic character affecting the choice of pollinator cultivars. However, a limited number of almond cultivars grown world-wide show self-fertility (Dicenta and Garcia 1993), and the *S*-allele for this character, designated *Sf*, appears to be derived from the species *Prunus webbii* that grows wild in regions of southern Italy (Bošković et al. 1999). Many almond breeding programmes aim to produce self-fertile cultivars that would be suitable for monoculture orchards, and less dependent on the activity of bees for pollination (Batlle et al. 1997).

Crossa-Raynaud and Grasselly (1985) used the numerical nomenclature *S1*, *S2*, *S3*, etc. to describe the almond *S*-alleles, whereas Kester et al. (1994a) used an alphabetical nomenclature *Sa*, *Sb*, *Sc*, etc., and divided 27 cultivars into six incompatibility groups. About 93% of the common commercially important almond cultivars grown in California, including 'Mission', 'Nonpareil', 'Thompson', 'Carmel', 'Merced' and 'Monterey', occur in these six groups (Kester et al. 1994a, b). On the basis of incompatibility observations, Nonpareil was assigned *S7S8* by Crossa-Raynaud and Grasselly (1985), and *ScSd* by Kester et al. (1994a). Kester et al. (1994b) equated *Sd* with *S8*, and inferred that *Sc* could be equated with *S7*. Table 1 shows the reported *S*-alleles for some selected almond cultivars, including those elucidated by Bošković et al. (1997) using isozyme analysis.

The primary aim of this research was to compare the sequences for the *S1, S7* and *S8* alleles obtained from genomic DNA with those of cDNA prepared from stylar RNA to confirm the relationship of *Sb*, *Sc* and *Sd* to *S1*, *S7* and *S8* respectively. A second aim was to identify the sequence for the *Sf* gene from the genomic DNA of a homozygous selfing progeny developed within the almond breeding programme at the Institut de Recerca i Tecnologia Agroalimentàries (IRTA), Mas Bové, Spain.

Table 1 Reported *S*-alleles for selected almond cultivars

Cultivar	*S*-alleles	References	
		S-alleles	Sequences (refer to Table 3)
– 'Ne Plus Ultra'[a]	*S1S7*	Bošković et al. (1997)	–[b]
	SbSc	Kester et al. (1994a)	Partial sequence of *Sb* from genomic DNA (Tamura et al. 2000)
– 'Mission'	*SaSb*	Kester et al. (1994a)	Complete sequence for *Sb* from mRNA (Ushijima et al. 1998)
– 'Nonpareil' 15–1[a]	*S7S8*	Crossa-Raynaud and Grasselly (1985); Bošković et al. (1997	–[b]
	ScSd	Kester et al. (1994a)	Complete sequence for *Sc* from mRNA (Ushijima et al. 1998) Partial sequence for *Sd* from mRNA (Ushijima et al. 1998)
– Selection 12–2[a]	*SfSf*	I. Batlle and P. Arus (personal communication)	–[b]
– 'Tuono'	*S1Sf*	Crossa-Raynaud and Grasselly (1985); Bošković et al. (1997)	Partial sequence for *Sf* from genomic DNA (Ma and Oliveira 1999)
– 'Ferragnes'[a]	*S1S3*	Crossa-Raynaud and Grasselly (1985); Bošković et al. (1997)	–[b]

[a] Used in this study [b] –, no report

Material and methods

Plant material

Leaves of the almond cultivars 'Ne Plus Ultra' and 'Nonpareil' 15–1 were collected from the Waite Campus, Adelaide University, South Australia. Leaves of IRTA Selection 12–2, reported to be derived from selfing 'Lauranne' (Bošković et al. 1999), were collected from IRTA, Mas Bové, Spain. When flowers were at the balloon stage, styles and stigmas without ovaries of 'Nonpareil' 15–1 and 'Ferragnes', were collected from the Waite Campus, Adelaide University, and the Loxton Research Centre, South Australia, respectively. All plant material was frozen in liquid nitrogen and stored at –80°C until used.

Isolation of nucleic acid

Genomic DNA was extracted from leaves using the technique of Mekuria et al. (1999). About 1.5 g were ground to a fine powder in liquid nitrogen using a pre-chilled mortar and pestle, and added to 7.5 ml of extraction buffer [3% (w/v) cetyltrimethyl ammonium-bromide (CTAB), 1.4 M sodium chloride, 10 mM ethylenediamine tetraacetic acid (EDTA), and 1.0 M TRIZMA base, pH 8.0] supplemented with 15 µl of 2-mercaptoethanol and 15 mg of PVP-40 (Sigma Chemical Co.). The mixture was incubated at 60°C for 30 min and then extracted with an equal volume of chloroform:isoamyl alcohol (24:1) for 10 min. After centrifugation at 2,500 g for 20 min, the upper aqueous phase was mixed with a 2/3 volume of cold isopropanol. DNA strands were spooled, cleaned with washing buffer (76% ethanol containing 10 mM NH_4Ac), and dissolved in 1 ml of TE buffer (10 mM Tris HCl, 0.1 mM EDTA, pH 8.0). RNA was digested with DNase-free RNase A and proteinaceous material precipitated with NH_4Ac, followed by centrifugation. DNA was precipitated by adding 2 vol of ethanol in the presence of 0.3 M NaAc, pH 5.2, and, after centrifuging, dissolved in TE buffer. The quantity and quality of the purified DNA were assessed by the absorbances at 230, 260 and 280 nm (Mekuria et al. 1999).

RNA preparation

Total RNA was prepared as described by Shi et al. (1997). Fifty styles and stigmas, without ovaries, were ground to a fine powder in liquid nitrogen and mixed with 400 µl of extraction buffer (0.1 M LiCl, 0.1 M Tris HCl, pH 8.0, 0.01 M EDTA, and 1% SDS); 400 µl of phenol (saturated with Tris HCl, pH 8.0) was added and vortexed for 1 min. Then, 400 µl of chloroform was added, vortexed for 1 min, and the mixture centrifuged at 17,300 g at room temperature for 10 min. The aqueous phase was removed, an equal volume of 4 M LiCl added, and incubated at –20°C for 3–4 h. The pellet of RNA was collected by centrifuging at 17,300 g at 4°C for 15 min, dissolved in 400 µl of sterile water, and re-precipitated with 2.5 vol of ethanol in the presence of 0.3 M NaAc (pH 5.2). RNA was recovered by centrifuging at 14,000 rpm at 4°C for 15 min, washed twice with cold 70% ethanol, and dissolved in 50 µl of 0.1 mM EDTA, pH 7.0. The quality and quantity of the purified RNA were assessed after electrophoresis on 1.5% agarose gels in TBE running buffer.

First-strand cDNA synthesis

Total RNA (0.7 µg) was annealed to oligo$(dT)_{18}$ primer (13 ng/µl) in a volume of 10.5 µL by heating at 70°C for 10 min, followed by quick-chilling on ice, and briefly centrifuging. The product was mixed with 8.5 µl of reverse transcription reaction mixture [1×RT buffer (Life Technologies), 0.01 M dithiothreitol (DTT), 0.001 M dNTPs, and 2 U/µl of RNase Inhibitor (Roche Diagnostic Corporation)], mixed gently and incubated at 42°C for 2 min. Then 200 U of Superscript II RNase H^- Reverse Transcriptase (Life Technologies) was added, mixed by pipetting gently, and the mixture incubated at 42°C for 50 min. The reaction was terminated by heating at 70°C for 15 min.

Primers

For the amplification of *S1*, *S7* and *Sf* alleles from genomic DNA, the first pair of primers (ConF and ConR; Table 2) was designed based on conserved regions in the published sequences of the *S*-RNase alleles *Sb* (AB011469), *Sc* (AB011470) and *Sd* (AB011471) of almond (Ushijima et al. 1998). For the *S8* allele,

Table 2 Primers used in initial amplification of genomic DNA, and double-stranded cDNAs encoding *S*-RNases

Primers	Sequences (5′→3′)	Descriptions	PCR	
			Genomic DNA	cDNA
ConF	GTG CAA CAA TGG CCA CCG AC	Conserved sequence (forward) for 1st sequence from genomic DNA of *S1*, S*7* and *Sf*, and whole s equence from cDNA of *S1* and *S7*	✓	✓
ConR	TAC CAC TTC ATG TAA CAA CTG AG	Conserved sequence (reverse) for 1st sequence from genomic DNA of *S1*, *S7* and *Sf*, and whole sequence from cDNA of *S1*, *S7* and *S8*	✓	✓
SdF	AGC AGC AAA CCT TCC AAC C	Specific sequence for *S8* (forward) for whole sequence from cDNA	–	✓
SdF1	ATT ATG AGC ACT GGA TCT TAC GTG	Specific sequence for *S8* (forward) for 1st sequence from genomic DNA	✓	–
SdR1	AAA CAA GAT GTC AAT ATG ATT TCG	Specific sequence for *S8* (reverse) for 1st sequence from genomic DNA	✓	–

the first pair of primers (SdF1 and SdR1; Table 2) was designed based on the specific sequences from the *Sd*-RNase alleles (AB011471) of almond (Ushijima et al. 1998). Other pairs of primers were designed based on the results of the sequences obtained until the full sequences were completed.

For the amplification of *S1* and *S7* alleles from cDNA, the specific primers were ConF and ConR as described for genomic DNA. For the *S8* allele from cDNA, one specific primer was designed based on the sequence of the *Sd* allele from almond (Ushijima et al. 1998) and designated SdF, and the other primer used was ConR (Table 2).

Amplification of genomic DNA and double-stranded cDNA encoding the *S*-RNases

Genomic DNA or first-strand cDNA was amplified using appropriate primers (Table 2) in 20 µl of PCR reaction, containing 1.5 mM $MgCl_2$, 1×*Taq* DNA Polymerase buffer [67 mM Tris-HCl, pH 8.0, 16.6 mM $(NH_4)_2SO_4$, 0.45% Triton X-100, 0.2 mg/ml gelatin], 200 µM of dNTPs, 0.25 µM of each primer, and 1.1 unit of *Taq* DNA Polymerase (Life Technologies). The PCR programme consisted of an initial denaturation of 3 min at 95°C, followed by 34 cycles of 30 s at 95°C, 45 s at 55°C, 1 min at 72°C, with a final extension step of 10 min at 72°C.

Cloning and sequencing of genomic DNA and cDNA encoding the *S*-RNases

Genomic DNA and double-stranded cDNA were inserted into the pCR 2.1-TOPO 3.9 kb vector using the TOPO TA Cloning Kit (Invitrogen). The presence of the insert in plasmid DNA was confirmed both by restriction enzyme digestion with *Eco*RI, and by PCR using M13 forward (5′-GTAAAACGACGGCCAG-3′) and reverse (5′-CAGGAAACAGCTATGAC-3′) primers. The nucleotide sequences were determined using the DyeDeoxy Terminator Sequencing Kit of Applied Biosystems, and sequencing on an Applied Biosystems Model 337 A. Sequences were aligned with Clustal X (Thompson et al. 1997) and the aligned sequences were edited with BioEdit v. 4.8.1 (North Carolina State University; Hall 1999).

Results

Amplification of genomic DNA and double-stranded cDNA encoding the *S*-RNases

Using primers ConF-ConR, the PCR-amplified fragments from the *S1*, *S7* and *Sf* RNase alleles of genomic DNA were about 1,100, 2,000 and 1,200 bp respectively. The corresponding cDNA fragments for *S1* and *S7* were about 510 and 490 bp respectively. For the *S8* allele, using SdF1-SdR1 and SdF-ConR, amplified fragments were about 3,000 and 460 bp from genomic DNA and cDNA respectively. No amplified products with the correct sequences were obtained from genomic DNA with the primer pair SdF and ConR.

Partial nucleotide sequences for *S*-alleles

The partial sequences for the *S1*, *S7*, *S8* and *Sf* genes from genomic DNA were 1,072, 2,019, 2,823 and 1,205 bp respectively (Table 3; Fig. 1). Similarly, the sequences for the cDNAs from *S1*, *S7* and *S8* were 510, 489 and 465 bp (Table 3; Fig. 1).

Comparison of nucleotide sequences of *S1*, *S7*, *S8* and *Sf* alleles from genomic DNAs and cDNA

When the nucleotide sequences from three sources, stylar RNA, genomic DNA and stylar RNA reported by Ushijima et al. (1998), were aligned, the partial DNA sequences of the *S1*, *S7* and *S8* alleles from stylar RNA were identical to those for *Sb*, *Sc* and *Sd* respectively. Matching of the sequences from stylar RNA and genomic DNA revealed the presence of introns with sizes of 562, 1,530 and 2,208 bp for the *S1*, *S7* and *S8* genes respectively (Table 3). The intron size of 662 bp for *Sf*

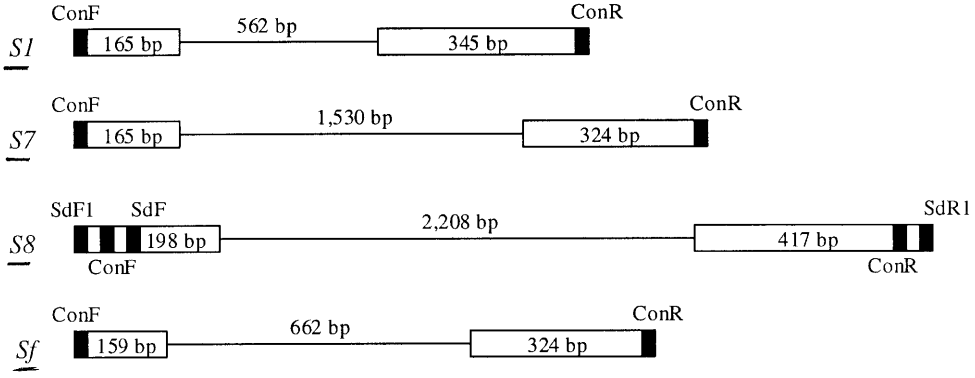

Fig 1 Scheme of structures of PCR-amplified products of genomic DNAs and cDNAs of four alleles, *S1*, *S7*, *S8* and *Sf*, in almond. Introns are representd by the bars between two boxes which are the exons

Table 3 Sequences from stylar RNA and genomic DNA compared to reported sequences

[a] AB011469 (Ushijima et al. 1998)
[b] Tamura et al. (2000)
[c] AB011470 (Ushijima et al. 1998)
[d] AB011471 (Ushijima et al. 1998)
[e] AF157009 (Ma and Oliveira 1999)
[f] No data
[g] No data but determined from the sequence derived from primer pair, SdF1-SdR1

Reported sequences			Alleles	Primers	Sequences (number of bp)			
Alleles	Number of bp				Stylar RNA	Genomic DNA		
	Stylar RNA	Genomic DNA				Whole	Exon	Intron
Sb	884[a]	556[b]	*S1*	ConF-ConR	510	1,072	510	562
Sc	751[c]	–	*S7*	ConF-ConR	489	2,019	489	1,530
Sd	731[d]	–	*S8*	SdF- ConR	465	–[f]	–	–
			S8	SdF1-SdR1	615[g]	2,823	615	2,208
			S8	ConF-ConR	–	2,706[g]	498[g]	–
Sf	–	1,208[e]	*Sf*	ConF-ConR	–	1,205	543	662

was determined by matching the partial exon sequence for *Sf* with those for the *S1*, *S7* and *S8* alleles (Table 3). Figure 2 shows the homologous regions when all sequences are aligned.

The partial sequences of the exons varied from 489 to 510 bp, and showed between 73.22 and 84.70% homology (Table 4). There was very little homology between the introns (Figs. 2 and 3).

Intron/exon structures

For each of the four alleles examined (Figs. 2 and 3), the sequences of the exon/intron splice junction regions followed the GT/AG consensus sequence rule (Thangstad et al. 1993), and the sequences adjacent to the splice junctions were highly conserved (Fig. 2).

Composition of the sequences of the *S*-alleles

Table 5 shows the nucleotide composition of partial exons and full introns in each *S*-allele. The sequences of the introns contained a high proportion of A+T nucleotides (69.93–77.40%), whereas the partial sequences of the exons contained a higher proportion of C+G (43.35–45.86%) compared to the introns (22.60–32.33%) (Table 5).

Table 4 Similarity among partial exons of *S* alleles (*S1*, *S7*, *S8* and *Sf*) amplified with primer ConF and ConR

Pairs of alleles		% Similarity
1st allele	2nd allele	
S1	*S1*	100.00
	S7	83.92
	S8	84.70
	Sf	76.32
S7	*S7*	100.00
	S8	80.83
	Sf	73.22
S8	*S8*	100.00
	Sf	76.19
Sf	*Sf*	100.00

Discussion

This study has confirmed and extended previous research on almond *S*-alleles. It was found that exons of *S1*, *S7* and *S8* in this study showed 100% homology to *Sb* (AB011469), *Sc* (AB011470) and *Sd* (AB011471) (Ushijima et al. 1998). For the *Sf* allele, the sequence obtained in the present study, including the intron, showed

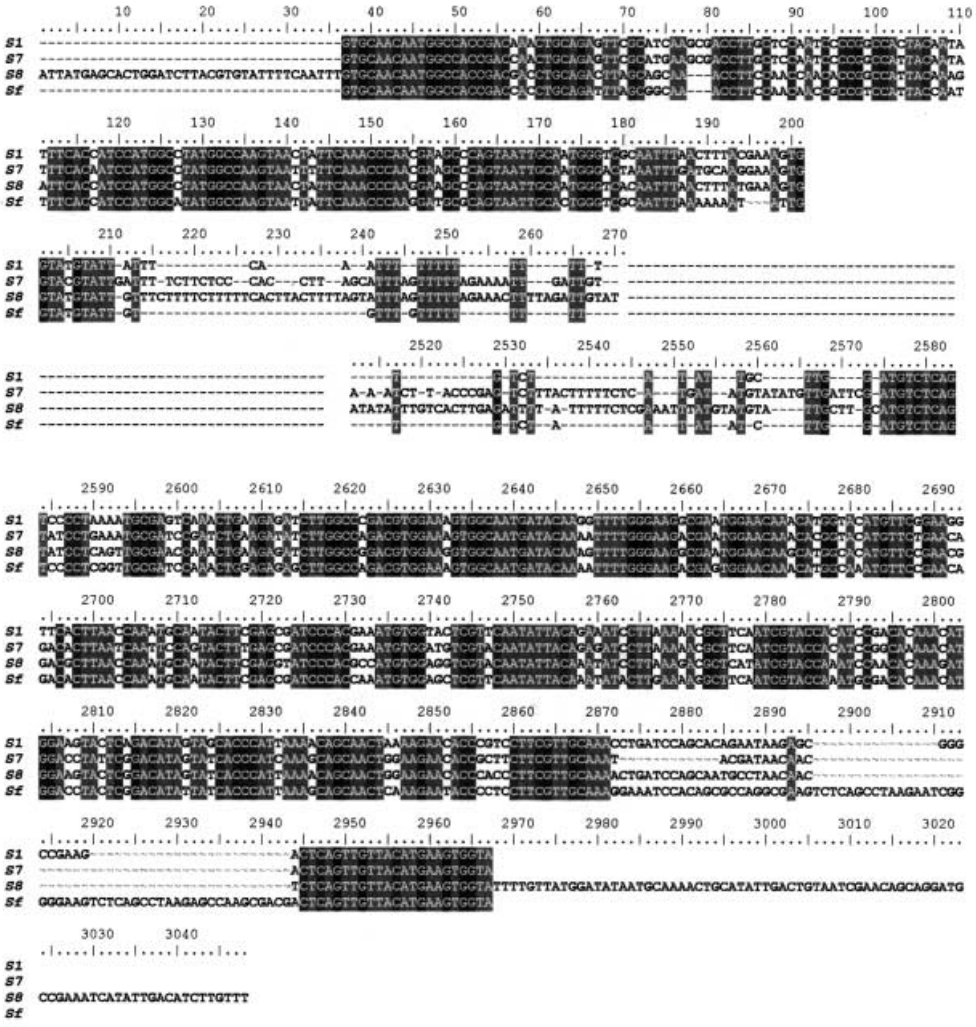

Fig 2 Sequence aligment of *S1*, *S7*, *S8* and *Sf* front exons start at 1–201 bp, introns start at 202–2.583 bp, and back exons start at 2.584–3.048

Table 5 A/T and G/C ratios of partial sequences of exons and full sequences of introns from *S1*, *S7*, *S8* and *Sf* alleles amplified with primers ConF and ConR

S-allele	Exon (partial)						Intron					
	Number of nucleotides				% Content		Number of nucleotides				% Content	
	A	C	G	T	C+G	A+T	A	C	G	T	C+G	A+T
S1	172	128	103	107	45.29	54.71	191	55	72	244	22.60	77.40
S7	165	115	97	112	43.35	56.65	488	246	214	582	30.07	69.93
S8	169	125	100	104	45.18	54.82	765	276	344	823	28.08	71.92
Sf	181	137	112	113	45.86	54.14	224	122	92	224	32.33	67.67

99.17% homology to the sequence of *Sf* (AF157009) obtained from the cultivar 'Tuono' (Ma and Oliveira 1999). Furthermore, the sequence of the intron of the *S1* allele in the present study showed 91.27% homology to the intron of the *Sb* allele from 'Mission', 'Monterey', and 'Ne Plus Ultra' reported by Tamura et al. (2000).

For amplification of DNA fragments from genomic DNA and cDNA, sequences from the conserved regions of *Sb*, *Sc* and *Sd* of almond (ConF and ConR) were used. The sequences obtained were always identical to either the *S1* allele in the case of 'Ne Plus Ultra' (*S1S7*) and 'Ferragnes' (*S1S3*), or the *S7* allele in the case of 'Nonpareil' 15–1 (*S7S8*). It was concluded that the presence of either *S1* or *S7* masked the amplification by PCR of the second allele. For this reason, primers were designed based on the sequence of a specific region of *Sd*

a

```
GTATGTATTA TTTCAAATTT TTTTTTTTTT CACTTACTCT TTAGCTTTTA TTGTTTTTAT TTTTTGCACA AATACTCTTT AGCATTTTAG TTTTTAGAAA  100
ATTAGATTGT CGTATGAAGA TTTAAAATTT AATTAAAATT TTTTTTTCAA TAAGCCTTGG GGGTTAGATA TAAAATTTTG GTGTTGGTTT AGTTTTTTTA  200
ATTATTATTT TTTTAATATT GGTTCTTAGT TAGACACATT ATTTTGAAAA TATAGTCAAG TTCAAGATGG TACGTATATT TATATTTATA TGCATATTAA  300
TGTACTTGCG AAAATATGAT GGATCTGCTC ATCTAATTAC ATGACCTACC ATTTTGTCCT AATGTATATA TGCAAAAAAT TTGTTAGAAG AAAAAGCAAG  400
GCTATAATAT ATTATTGAAG GTTGAACTCA AAATTCAATT TAATGCTCAG ATTTAAAGAA ACAAAAAAAA AAAAAAATCT TATTCAAGAA TGAAAATCTA  500
ATTATCATTT ATTCAATTTA CTTTTTCTGA AAATATGTGT CTATATTGCT TGGATGTCTC AG 562
```

b

```
GTACGTATTG ATTTTCTTCT CCCACCTTAG CATTTAGTTT TTAGAAAATT GATTGTCATA TGAAGATATC ATGCTTTTCA GTAAACAGCA GCCATGACAC  100
TGGTGAAAAT TAGAAAGTTC TTCCCCTCCA TATGTTTTCA CGTTTGCTTC TCCTAGTGCT TCCCTACTTT CGGCCCTCTC TCTACCGTCC CCTCTCCCTG  200
GGCTCTCCAC AGGCACTCCA TGAACACACT CCATTCTCCA TATATCTTCC TCCCGTTAAA TAAACACCAT CCGTTCCTAT TGTCTAAACT TTAATAGAGA  300
ACAAGAAAAG GATGGAAAAA CAAATCTGGC GATGAAGTAT AATGTCACGC CAAAAAGAGA TGCGAAGAAC ATGAATGCAT TTCAATTAAA CAAATAATTT  400
CTTATTAACT TATTGTTACT AAACCCTTGG CACACACTGT GCCAATTGGT TTTTTTATTG GAAAATACTT CCCCCTTACC AGGTAAGTAT GAGCTCTTAC  500
ATTCCTGCCA AAAATCGTAT GACTGTGTGC AAAAGTTTTT CTTTTATGTT TTTGATGAAT CATTTTTGCA TATGTGTCAA AATGTGATTC GCAAGAAGGA  600
AGAGTATCCC CTTACTAGTA AGGAGGCAAG TAGTATCATT TTTTATTTAA GAATTAAGAA AGAGAATATA ACCACGACCA CAACCATATA ACATCGTGTT  700
GCATGAAAAT AGGACCCATG TTCTTATTTT GGTTTTTATT TGAACTTTTT TAAATATGAA AAAATATTAA TTTGCTATAT TATCCCTATT TAATGAAAAG  800
TTTCAATAGT TAATAAATCA ATAACTAAGG GAAATTTTTA GTATTTTGAA TGTTTTATTC TCTACTTTTT GCTTATGTAT GTAGATAAAT TCGTTAATTA  900
AGGGCAATTT TTGGTATTTT GAATGTTTTA TTCTCTGCCT TTTGCTTGTA CGCTTGAGCA TATGTGCCAA TTGGTTTTTT AAATTTTATT TTAGAATTAA 1000
AAAAGATAAT GGGTAAGTGT TCCACCAAAA TAGGACCTAT TATTTGATTT TGTTTTAAAT TTTAAATTTT GTAATATGAA AAATTGTGAA TGTTCCATAT 1100
TATCCTCATT TAATTAATAA TTTCAATTCT TAATGTTTAT ATTAACCAAT GTCATTTTCT GATATTTTGA ATGTTTCACC GTTCTCTACT TTTTGCTTCA 1200
TATATATAAA TATAGATAAA TTCAGTAACT AAGGGCAAAT TTTGGTATTT TGAATATTTT ACCACTCTCT GTATTGTGCT TCATATATAT AGATATAGAT 1300
AAAATTCATT AATTACGGTC AAATTTTGGT ATTTTGAATG TTTCACCAAT CTTTGCCTTT TGAATATATG TAGATGAACT AAAATCCAAG AAAGAAAACT 1400
TAAGTATATA ATCTAAATAG TGTACATCAA AATTAAATTC TGTATAATAC TCAGGTTTAA TGAAGAAAAA AAATCTTACC CGAGTCTTTA CTTTTTCTCA 1500
TGATATGTAT ATGTTGATTC GATGTCTCAG 1530
```

Fig 3a–d DNA sequences of introns in *S1*, *S7*, *S8* and *Sf* alleles. **a** Intron of *S1* allele. **b** Intron of *S7* allele. **c** Intron of *S8* allele. **d** Intron of *Sf* allele

(AB011471) to target *S8* more specifically in 'Nonpareil' 15–1. Specific primers, SdF and ConR for *S8*, were used effectively for amplification from cDNA but not from genomic DNA. Another set of primers specific to the *S8* allele (SdF1 and SdR1) was designed and used for amplifying genomic DNA. Similar results were obtained by Tamura et al. (2000) for amplification of *S* alleles in almond. For example, either *Sa* or *Sb* was always preferentially amplified from 'Thompson' (*SaSc*), 'Monterey' (*SbSd*) and 'Ne Plus Ultra' (*SbSc*) when specific primers for these *S*-alleles were used.

All intron lengths found in this study were longer than 500 bp. Deutsch and Long (1999) suggested that introns smaller than 50 bp were significantly less frequent than those with longer sequences, possibly resulting from a minimum intron-size requirement for effective splicing.

The sequences of the partial exons contained a higher C+G content (43.35–45.86%) than those in the introns (22.60–32.33%). Brendel et al. (1998) found that individual introns of *Arabidopsis* and maize were typically 15% more U-rich than the flanking exons, or alternatively that the exons were typically 15% more G+C-rich than their enclosed intron and there was no difference in the content of A.

Determination of the sequences of the *S*-RNase genes, especially that of *Sf*, is useful for studying the system of gametophytic self-incompatibility in almonds. In the Solanaceae, gametophytic self-incompatibility is a primitive trait and self-compatible plants arose from self-incompatible plants by alteration of an essential component required for the recognition or rejection of self-pollen (Kowyama et al. 1994). The mutation segregates with the *S*-locus and is associated with low stylar ribonuclease activity (Kowyama et al. 1994). In Japanese pear, Norioka et al. (1996) suggested that self-compatibility arose as a mutation due to a failure of expression in the stylar ribonuclease activity. Self-compatibility in almond is also associated with the absence of stylar ribonuclease activity. The mechanism of inactivation of the ribonuclease may be due to either a deletion of the corresponding genomic fragment or to the production of a defective protein (Bošković et al. 1999).

Further work will be directed towards utilising the sequences obtained in order to identify the self-incompati-

c

```
GTATGTATTG TTTCTTTTCT TTTTCACTTA CTTTTAGTAT TTAGTTTTTA GAAACTTTTA GATTGTATCT AAAGACAATA ATATACAATT TTTTTAATGA  100
TTTTTTTTTA ATCACAAATG GTCCTTCAGG TTTTCGAAAT TATCACCTTT TATTCTAAAA GTTTTTTTTG TGACACTAAT GATTTTTAAG GTTACTCTCT  200
ACACATCAAA ATGGTCATTG CCATTAGCTT CCGTCAAATT TTATGTTAAA TTGATAATGT GGCACATATG TGGAGCCTAC ACATACAATA GTATATAGTC  300
ACATGACTTT AACTATAATA TATAATATAT TTTAAAACTT AAAATCCATA TAAAAATTTT AAAAAGAAAA TTAAGTCTTT TTATAAATAT AAAATTATTA  400
AAACTAAAAA ACTAAAAATC CTGCAGCCAT CCCTCATCGC AGACCCACTC ATCGAGCACC TTAAGCCAAG GAATTCCAAG AGCTACAAGT GTTCGAGAAG  500
GAAATGGCCT CTCGATTTGC GGATTTTGAA GCCAAAGAGT TGAACTTTAA CTTGGTGATG GGAACTAAAG CCAAGGAATT GCAGGGCATT GTACAGGAGG  600
TTGAGCCAAA CAAACAAATA TTGATTGAAG AATATGACAG AGAGATGAAA TCCAACGAGC AGAAACTTGG TTCGATTCAG AAATTAGTGT TGGAGTACTC  700
GAATGCATTT GAATCGAAAA TAAAATATTT TAATTTGTTT GAGAGTGAGT CTGCGATTTG GGGAGGGGCG CTGGGAGAGG GGGAACGTAC AGGATGGTTG  800
CAGGTTTTTT TAGTTTTTTA TTTATAAAAA GACTTAATTT TCTTTTAAAT TTTTTATATG GATTTTAAGT TTTAAAATAT TTAAAATATA TTTTATATTT  900
TATTTAAAGT CACATGGTTC TATACCGTTG GATGTTTTGG GCTTCACATA TGTGCCACAT CATCAATTTA ACATAAAATT TGACGAAAGC TAAGGCAGGG 1000
ACAATTTTGA TGTGTGGAGG GTAACGTTAA ATATTATTAG TGTCACAAAA AAACTTTAGG GATAAAAGGT GATAATTTCA CAAACTTGAA AGACCATTTG 1100
TGATTAAAAA AAAAAAAGAA GCCTTTTTTT AAATTTATAC AAGTGATAAC ATACTCAAAA TTAATAAACC TTGGGTATTG GATAAATTTT GGTGTTGGTC 1200
CTAGATGCAT TATTTTTAAC TAGACTGACC TTTTTTTTTC CTCCTTGTAT TGCGATAGAG ATTTTTCTGG GGTAAATTTA CATTTATGTA TAACTAGCCC 1300
TTGGCACACG GTGTGCCAAT TAGTTTTTTA TTTTATTTTA TTGTATTTTG TTTAAGAATT AGGAGAGAGA ATCCCAAAAA AAAAAACAAG TTCTTATTTT 1400
AAATTTTTTT TATTTACTAC ATTATACTTA TTTAATGAAA TTTTTTAATA CTTAATAAAT TCATTAATAA ATGATAATTT TTGGTATTTT GAATATTTTA 1500
CGATTCTCTG TCTTTTGCTT TAGATATAAA GATAAAATAA AAAAGACACG AAAAGAAATA GTTTTTTTTT GTTGAAGTAG AATTTTCATT CTACATAAGC 1600
AATACAAGAT AAAAGAAAGG TCGTTATGCT AGTTAGTCGG CTTTAATTAG TTATAATTTT ATTTAATTAC TGGTTTTTAA TATTTTGTCC TTAAAATATT 1700
AAATTGTGGT TGTTTGATAA ACTAGTTAAC CAAGTGTATA AAAAAAAAGT CAACTAGCAA AGGACCAACT AGTGTAGTGG TTTGGAGTAT TTATTCCCTC 1800
AGGCAAGATC CTGGATTCAA GTCCTAGCAT CCGTGTTGTG CGTGTGAGTT TAATATGCTA TCGTCCCTTT CAATAGGAAA GGTCCTCCAA AAAAAAAAAA 1900
AAAAAAGGTC AACCAATGAT TCTTTTAAGA TATTAAATTT TGTGAATGTA CTATTTTATA CCTATTTAAT GTGCTTACCT ATATATAATG AATCTGCTTT 2000
TCTAATTAAA CGACCTACAA TTTTATACTG TTTGTGTATA TATTCAAAGT ATTGTACTTT ATATATATAT ATCAACAAGG TATTGTACTT AAATGAATTA 2100
AAATCAAAAT TCAACTTAAA ACCCAGATAA ATGCAAAAAA TATATATATT TGTCACTTGA GATTTTATTT TTCTCGAAAT TTATGTATGT ATTGCTTGCA 2200
TGTCTCAG 2208
```

d

```
GTATGTATTG TGTTTGTTTT TTTTTAACGT ACTATTTGGC ATTTAGTTTT TAAAAAATTA GATTGTCATC TGAAGACAAT GTACTTCTTT TTCAACAAAC  100
TTGGGTGTTA TCTAGGTGCA AATTGTAAGT ACAATTATAT TCTTAGGTAT ATTAATGCAA CTAGTCATGC ATTTATTTCA TGTGCCCTAT CTAATTTGTT  200
GACATATGTC ATTCCACTAA CTTTTAAACT TAACACTGGT AACTTTTAAT ACAATTAGAA AAACAAAAAT TAATTATTAA AGAAATAACA AAAATCCAAC  300
AGCCATCACA ACACCCCACC CACCCAATTA ACCTGGTCAG CCACCCCACC CCACTGGCGG CAAAGCCCCT CTCTTCCCAT ATCCTATTAG AAGTTAGTGG  400
GAGTGACAGG TGTCGGGGAC AGGGAGAGGG GCACAAGAAA ATTGGACTAT TTACAATCAT CTTAAAGGAC CTACCATTTT GTACTAATAC CGTACCTAAA  500
TAAATAGTCA AAATTATAAT TTAATTTAAC GCTCAGGTTT AATGAAAAAA ACAATTTATT CAAGAATGAA GATCTATCTA TATCTTAAGT CTGTACTTTT  600
TCTCAAAATC TTTAGTTTTC ACTCTTTCTA AAAAAATGTC TAATATATCT TGGATGTCTC AG 662
```

Fig 3c–d

bility groups in Australian almond cultivars, and to overcome self-incompatibility in the major commercial cultivars.

Acknowledgements The authors are grateful to Australian Almond Growers' Association for financial assistance, and providing plant material. Special thanks to Dr. Michelle Wirthensohn for extracting DNA from IRTA Selection 12–2. Thanks also to Dr. Francisco Vargas for access to trees at IRTA, Mas Bové, Spain. One of the authors (C.C.) was supported with a scholarship from the Thai government. The experiments described herein conform to the current laws governing research in Australia.

References

Batlle I, Ballester J, Boskovic R, Romero MA, Tobutt KR, Vargas FJ (1997) Use of stylar ribonucleases in almond breeding to design crosses and select self-compatible seedlings. FAO Nucis Newslett 6:12–14

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

Bošković R, Tobutt KR, Duval H, Batlle I (1999) A stylar ribonuclease assay to detect self-compatible seedlings in almond progenies. Theor Appl Genet 99:800–810

Brendel V, Carle-Urioste JC, Walbot V (1998) Intron recognition in plants. In: Bailey-Serres J, Gallie DR (eds) A look beyond transcription: mechanisms determining mRNA stability and translation in plants. American Society of Plant Physiologists, Rockville, Maryland, pp 20–28

Crossa-Raynaud P, Grasselly C (1985) Existence de groupes d' interstérilité chez l' amandier. Options Méditerr CIHEAM/ IAMZ 85/I:43–45

Deutsch M, Long M (1999) Intron-exon structures of eukaryotic model organisms. Nucleic Acids Res 27:3219–3228

Dicenta F, Garcia JE (1993) Inheritance of self-incompatibility in almond. Heredity 70:313–317

Godini A (1979) Ipotesi sulla comparsa dell'autocompatibilità nel mandorla. Sci Tec Agr 19:1–10

Hall TA (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser 41:95–99

Ishimizu T, Shinkawa T, Sakiyama F, Norioka S (1998) Primary structural features of rosaceous S-RNases associated with gametophytic self-incompatibility. Plant Mol Biol 37:931–941

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

Kester DE, Micke WC (1994b) A mutation in 'Nonpareil' almond conferring unilateral incompatibility. J Am Soc Hort Sci 119: 1289–1292

Kowyama Y, Kunz C, Lewis I, Newbigin E (1994) Self-compatibility in a *Lycopersicon peruvianum* varient (LA2157) is associated with a lack of stylar *S*-Rnase activity. Theor Appl Genet 88:859–864

Ma R, Oliveira MM (1999) AF157009. *Prunus dulcis* strain 'Tuono' a self-incompatibility associated ribonuclease gene, partial cds. National Centre for Biotechnology Information http://www.ncbi.nlm.nih.gov:80/entrez/quer

Mekuria GT, Collins GG, Sedgley M (1999) Genetic variability between different accessions of some common commercial olive cultivars. J Hort Sci Biotech 74:309–314

Newbigin E (1996) The evolution of self-incompatibility: a molecular voyeur's perspective. Sex Plant Reprod 9:357–361

Norioka N, Norioka S, Ohnishi Y, Ishimizu T, Oneyama C, Nakanishi T, Sakiyama F (1996) Molecular cloning and nucleotide sequencing of cDNAs encoding *S*-allele specific stylar RNases in a self-incompatible cultivar and its self-compatible mutant of Japanese pear, *Pyrus pyrifolia* Nakai. J Biochem 120:335–345

Pimienta E, Polita VS, Kester DE (1983) Pollen tube growth in cross- and self-pollinated 'Nonpareil' almond. J Am Soc Hort Sci 108:643–647

Sedgley M (1994) Self-incompatibility in woody horticultural species. In: Williams EG, Clarke AE, Knox RB (eds) Genetic control of self-incompatibility and reproductive development in flowering plants. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp 141–163

Shi BJ, Ding SW, Symons RH (1997) Plasmid vector for cloning infectious cDNAs from plant RNA viruses – high infectivity of cDNA clones of tomato aspermy cucumovirus. J Gen Virol 78:1181–1185

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

Thangstad OP, Winge P, Husebye H, Bones A (1993) The myrosinase (thioglucoside glucohydrolase) gene family in Brassicaceae. Plant Mol Biol 23:511–524

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 24:4876–4882

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