Theor Appl Genet (2004) 109: 954–964
DOI 10.1007/s00122-004-1656-2

ORIGINAL PAPER

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Self-incompatibility genotypes in almond re-evaluated by PCR, stylar ribonucleases, sequencing analysis and controlled pollinations

Received: 22 December 2003 / Accepted: 6 May 2004 / Published online: 26 August 2004
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Abstract As part of the almond breeding programme at IRTA, we investigated the *S* genotypes of several cultivars using a combination of RNase zymograms, testcrosses, pollen-tube growth analysis and molecular identification by PCR analysis. For some of the cultivars examined, discrepancies appeared between their *S* alleles as reported in the literature and those found in this investigation, leading to a re-evaluation of their *S* genotypes. Analysis of the stylar ribonucleases (RNases), which are known to correlate with *S* alleles, of cvs. Achaak, Ardechoise, Desmayo Largueta, Ferrastar, Gabaix, Garbí, Glorieta, Languedoc, Primorskiy and Texas revealed inconsistencies with respect to the S_5 and S_{10} alleles. However, PCR with the conserved primer pair AS1II/AmyC5R failed to detect any of these inconsistencies. When the *S* alleles from Desmayo Largueta, Gabaix, Primorskiy and Texas were sequenced, Texas and Primorskiy were found to carry the reported S_5 allele, while Desmayo Largueta and Gabaix carried a new allele, which has been tentatively denoted as S_{25} This new *S* allele, previously reported to be S_{10}, was also identified in Achaak, Ardechoise and Ferrastar. The proposed new *S* genotypes are Achaak (S_2S_{25}), Ardechoise (S_1S_{25}), Desmayo Largueta (S_1S_{25}), Ferrastar (S_2S_{25}) and Gabaix ($S_{10}S_{25}$). The *S* alleles of Garbí, Glorieta, Languedoc, Texas and Primorskiy remain as reported in the literature. Testcrosses in the field and laboratory confirmed the new *S* genotypes. One cultivar (Gabaix) could be assigned to the existing cross-incompatibility group O of unique genotypes, and two new groups were established (XVI and XVII) consisting of two cultivars each. The clarification of these *S* alleles will be useful in almond breeding programmes and for planning new commercial orchards in the future.

Communicated by H.F. Linskens

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Introduction

Self- and cross-incompatibility are commonly found among cultivars in almond [*Prunus amygdalus* Batsch or *P. dulcis* Miller (D.A. Webb)]. Incompatibility is controlled by a single multi-allelic *S* locus with gametophytic expression. As in other species of the *Rosaceae* family (apple, pear, cherry, apricot and European plum), the *S* gene acts to prevent self-fertilization through the production of specific glycoproteins with ribonuclease activity (*S* RNases) in the styles (Boskovic et al. 1997; Tao et al. 1997). Because cultivars with the same *S*-incompatible genotype are cross-incompatible, commercial orchards are planted with compatible cultivars that have overlapping flowering times to ensure a good fruit set and thus high economic yields. The introduction of the self-compatibility character into commercial cultivars is a main aim in the scion breeding programme at the IRTA-Centre de Mas Bové and several others. Since the allele that confers self-compatibility (S_f) is dominant, it can be inherited by the offspring of crosses involving one self-compatible parent. Self-compatibility has traditionally been assessed by recording fruit set following the bagging of closed flowers in the field and by observing pollen-tube growth following the self-pollination of flowers in the laboratory.

The first *S* genotypes and cross-incompatibility groups (formed by cultivars having the same self-incompatibility genotypes) were detected through cross-pollination tests carried out at INRA-Avignon, France (Crossa-Raynaud

and Grasselly 1985) and at the University of California, Davis, in California (Kester and Asay 1975; Kester et al. 1994a). Other S alleles were detected using zymograms to determine the presence of particular stylar ribonucleases (Batlle et al. 1997; Boskovic et al. 1997, 1998, 1999, 2003; Duval et al. 1998; Mnejja et al. 2002; Ortega and Dicenta 2003). Sixteen incompatibility groups involving 24 S alleles (S_1-S_{23} and S_{7A}) are reported in almond (groups I-XV and group O of cultivars with unique genotypes) (Kester and Gradziel 1996; Boskovic et al. 2003). More recently, the sensitivity of detection has been increased by using conserved or specific PCR primers to amplify S alleles by the PCR (Tamura et al. 2000; Channuntapipat et al. 2001, 2003; Ma and Oliveira 2001; Martínez-Gómez et al. 2003a; Ortega and Dicenta, 2003; López et al. 2004). Early work at IRTA using stylar ribonuclease analysis in two crosses-Glorieta (S_1S_5) × Falsa Barese (S_1S_f) (López et al. 2004) and Falsa Barese (S_1S_f) × Desmayo Largueta (S_1S_5) (Mnejja et al. 2002)-gave the expected band segregations.

During the breeding programme at Mas Bové, analysis of the stylar ribonuclease of ten cultivars revealed S genotypes that were different from those reported in the literature. The S_5 allele, assigned to Desmayo Largueta (S_1S_5) and Gabaix (S_5S_{10}) by Boskovic et al. (1997, 2003), was observed to be different to that reported for Texas (S_1S_5). Furthermore, the S_{10} allele assigned to Ardechoise (S_1S_{10}) and Ferrastar (S_2S_{10}) by Boskovic et al. (1999) and to Achaak (S_2S_{10}) by Ortega (2002) migrated to the same position as the S_5 from Desmayo Largueta but to a different position to that of S_{10} of Gabaix. In other fruit species, such as apple (Van Nerum et al. 2001; Broothaerts 2003) and cherry (Wiersma et al. 2001; Sonneveld et al. 2003), genotype discrepancies have also been found in the identification of S alleles by ribonuclease analysis.

The aim of the investigation reported here was to re-evaluate the S genotypes of ten almond cultivars in which some inconsistencies were found on the basis of stylar ribonuclease analysis using a combination of pollination tests, stylar ribonuclease analysis, allele-specific PCR analysis and the sequencing of S alleles.

Materials and methods

Plant material

The almond cultivars analysed were Achaak, Ardechoise, Desmayo Largueta, Ferrastar, Gabaix, Garbí, Glorieta, Languedoc, Primorskiy and Texas. All of these cultivars have been reported to be self-incompatible and all but one belong to cross-incompatibility groups II and O listed in Boskovic et al. (2003); the exception being Achaak whose group is as yet unreported. With the exception of Languedoc, which was supplied from the SIA germoplasm bank at Zaragoza, Spain, the trees are located at the IRTA-Centre de Mas Bové and originated from California, France, Spain, Tunisia and Ukraine (Table 1).

Table 1 Almond cultivars analysed and their reported S genotypes

Cultivar	Origin	Parentage with reported S genotypes	Reported S genotype	Cross-incompatibility group (CIG)[a]	References
Achaak	Tunisia	Unknown	S_2S_{10}	Unreported	Ortega (2002)
Ardechoise	France	Unknown	S_1S_{10}	O	Boskovic et al. (1999)
Desmayo Largueta	Spain	Unknown	S_1S_5	II	Boskovic et al. (1997); Martínez-Gómez et al. (2003a)
Ferrastar	France	Cristomorto (S_1S_2)[b] × Ardechoise (S_1S_{10})	S_2S_{10}	O	Boskovic et al. (1999); Channuntapipat et al. (2003)
Gabaix	Spain	Unknown	S_5S_{10}	O	Boskovic et al. (2003); Martínez-Gómez et al. (2003a); Channuntapipat et al. (2003)
Garbí	Spain	Cristomorto (S_1S_2)[b] open-pollinated	S_1S_5	II	Boskovic et al. (2003); Martínez-Gómez et al. (2003a)
Glorieta	Spain	Primorskiy (S_5S_9) × Cristomorto (S_1S_2)[b]	S_1S_5	II	Boskovic et al. (1997); Martínez-Gómez et al. (2003a); Channuntapipat et al. (2003)
Languedoc	France	Unknown	S_1S_5	II	Kester et al. (1994a); Boskovic et al. (1997); Martínez-Gómez et al. (2003a)
Texas (syn. Mission)	California	Languedoc (S_1S_5) open pollinated	S_1S_5	II	Kester et al. (1994a); Boskovic et al. (1997); Tamura et al. (2000); Martínez-Gómez et al. (2003a); Channuntapipat et al. (2003)
Primorskiy	Ukraine	Princesse × Nikitskiy	S_5S_9	O	Boskovic et al. (1997); Boskovic et al. (2003); Martínez-Gómez et al. (2003a); Channuntapipat et al. (2003)

[a]CIG II, Cultivars having the S_1S_5 genotype; CIG O, cultivars having unique genotypes (Boskovic et al. 2003)
[b]Cristomorto S_1S_2 genotype reported by Crossa-Raynaud and Grasselly (1985) and Boskovic et al. (1997)

DNA isolation and *S*-specific PCR

Leaves were collected from trees and seedlings and stored at −80°C until genomic DNA was extracted by the CTAB extraction method of Doyle and Doyle (1987). For PCR, the reaction contained 50-100 ng of DNA, 1× PCR buffer (10 m*M* Tris-HCl pH 8.3, 1.5 m*M* of $MgCl_2$, 50 m*M* KCl, 0.001% gelatine), 250 μ*M* of each dNTP, 0.25 μ*M* of each primer, 1 m*M* $MgCl_2$ and 1 U *Taq* polymerase (IRTA, Cabrils) in a reaction volume of 20 μl. DNA samples from cultivars not having the expected *S* alleles were added as controls. The S_1 and S_5 alleles were amplified by primer AS1II (forward, 5′-TATTTTCAATTTGTGCAA-CAATGG-3′) and AmyC5R (reverse, 5′-CAAAATAC-

Table 2 Results of the 19 pollination tests (field and laboratory) between almond cultivars with respect to the S_5 and S_{10} alleles. The pollination tests were carried out at IRTA-Mas Bové from 1985 to 2003 in order to identify the *S* genotype and determine cross-incompatibility (CI) relationships

Cross with reported parent *S* genotypes	Crossing year[a]	Cross-incompatibility					CI[b, d]	Deduced parent *S* genotypes[e]
		Field test			Laboratory test			
		Fruit set (%)	Mean fruit set (%)	CI[b]	CC[c] (%)	CI[b]		
Cross-compatible crosses								
Desmayo L. (S_1S_5) × Glorieta (S_1S_5)	2002	32.8	32.8	C	42.9	C	C	$S_1S_{25} \times S_1S_5$
	2003				25.0	C		
Glorieta (S_1S_5) × Desmayo L. (S_1S_5)	2002	23.7	13.1	C	66.7	C	C	$S_1S_5 \times S_1S_{25}$
	2003	15.6			22.2	C		
Desmayo L. (S_1S_5) × Garbí (S_1S_5)	2002	-[f]	-	-	42.9	C	C	$S_1S_{25} \times S_1S_5$
	2003	-			22.2	C		
Garbí (S_1S_5) × Desmayo L. (S_1S_5)	1985	28.3	15.6	C	-	-	C	$S_1S_5 \times S_1S_{25}$
	2002	3.0			62.5	C		
	2003	-			75.0	C		
Desmayo L. (S_1S_5) × Texas (S_1S_5)	2002	20.5	20.5	C	-	-	C	$S_1S_{25} \times S_1S_5$
Texas (S_1S_5) × Desmayo L. (S_1S_5)	2002	25.6	25.6	C	-	-	C	$S_1S_5 \times S_1S_{25}$
	2003	-			66.7	C		
Desmayo L. (S_1S_5) × Languedoc (S_1S_5)	2002	40.7	40.7	C	-	-	C	$S_1S_{25} \times S_1S_5$
Desmayo L. (S_1S_5) × Gabaix (S_5S_{10})	2003	19.8	19.8	C	4.5	I	C	$S_1S_{25} \times S_{10}S_{25}$
Gabaix (S_5S_{10}) × Desmayo L. (S_1S_5)	2003	3.2	3.2	I	20.0	C	C	$S_{10}S_{25} \times S_1S_{25}$
Ardechoise (S_1S_{10}) × Gabaix (S_5S_{10})	2003	30.8	30.8	C	13.6	I	C	$S_1S_{25} \times S_{10}S_{25}$
Cross-incompatible crosses								
Glorieta (S_1S_5) × Garbí (S_1S_5)	2002	0	0	I	0	I	I	$S_1S_5 \times S_1S_5$
Garbí (S_1S_5) × Glorieta (S_1S_5)	1987	0	0.6	I	-	-	I	$S_1S_5 \times S_1S_5$
	1997	1.3			-	-		
	2002	-			0	I		
	2003	-			0	I		
Glorieta (S_1S_5) × Texas (S_1S_5)	2002	0	0	I	0	I	I	$S_1S_5 \times S_1S_5$
Texas (S_1S_5) × Glorieta (S_1S_5)	2002	0	0.6	I	-	-	I	$S_1S_5 \times S_1S_5$
	2003	-			0	I		
Texas (S_1S_5) × Languedoc (S_1S_5)	2003	-	-	-	0	I	I	$S_1S_5 \times S_1S_5$
Garbí (S_1S_5) × Texas (S_1S_5)	2003	0	0	I	0	I	I	$S_1S_5 \times S_1S_5$
Texas (S_1S_5) × Garbí (S_1S_5)	2002	0	0	I	-	-	I	$S_1S_5 \times S_1S_5$
	2003	-			0	I		
Desmayo L. (S_1S_5) × Ardechoise (S_1S_{10})	2003	0	0	I	0	I	I	$S_1S_{25} \times S_1S_{25}$
Ardechoise (S_1S_{10}) × Desmayo L. (S_1S_5)	2003	1.1	1.1	I	0	I	I	$S_1S_{25} \times S_1S_{25}$

[a] Data for the years before 2002 were obtained from F.J. Vargas (personal communication)
[b] C, Cross-compatible; I, cross-incompatible
[c] (Number of flowers compatible with male parent/total no. of flowers tested) × 100
[d] Consensus between field and laboratory results
[e] On the basis of the results of *S* RNase, PCR, sequencing analysis and testcrosses in this work
[f] -, No data available

CACTTCATGTAACAAC-3′) (Tamura et al. 2000). 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 53°C and 1 min at 72°C, with a final extension step of 10 min at 72°C. The PCR products were separated on 1.5% (w/v) agarose gels containing 0.1% (v/v) ethidium bromide in a 0.25× NEB buffer (0.1 *M* Tris-HCl pH 8.1, 1 m*M* EDTA and 12 m*M* NaAc·3H_2O) at 90-100 V for 2 h. DNA bands were visualized under UV light and the images captured on a Kodak camera.

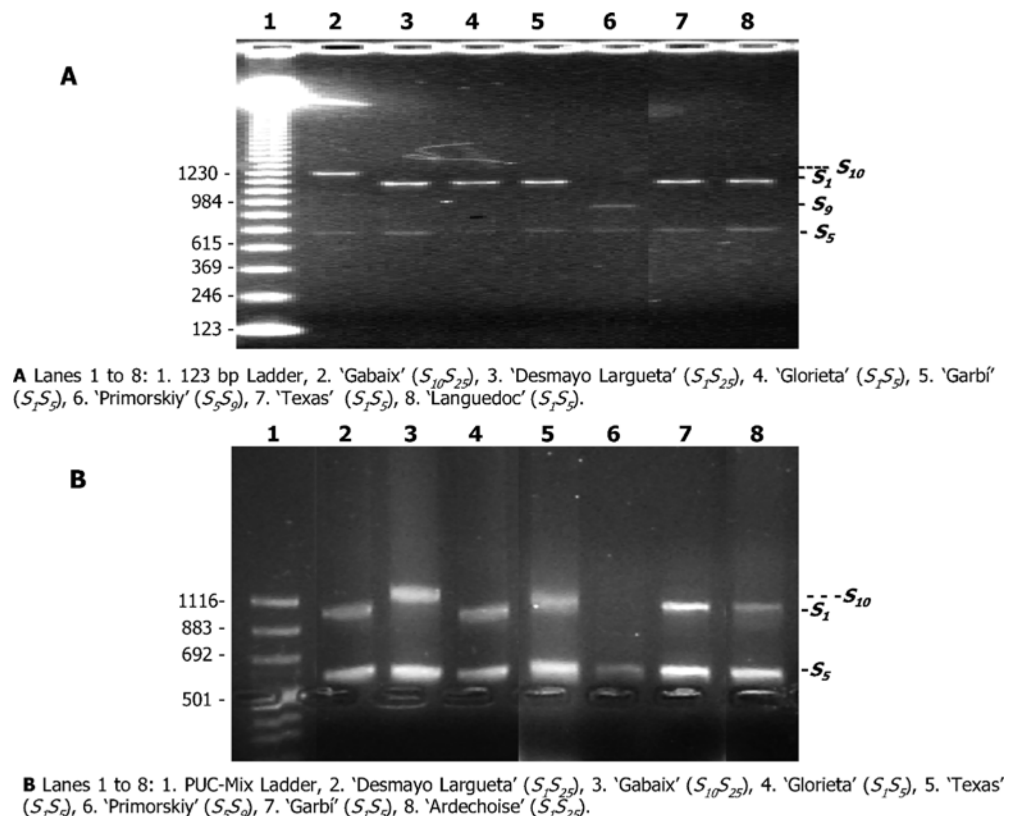
Stylar ribonuclease analysis

During a 3-year period (2001-2003) pistils were collected in the field from flowers at the balloon stage (Felipe 1977) and immediately stored at −20°C. Stylar proteins were extracted following the method of Boskovic et al. (1997), and the stylar ribonucleases were subsequently assayed as described by Boskovic et al. (1999). Cultivars with confirmed *S* alleles were included for comparison. The S_f allele was not detected on the zymograms due to the absence of detectable RNase activity (Boskovic et al. 1997).

Cloning and sequencing of the genomic fragment of the S_5 allele

The reported S_5 allele of Desmayo Largueta, Gabaix, Primorskiy and Texas was sequenced. Bands corresponding to the S_5 allele in the agarose gels were purified and cloned into plasmid vector pGEM-T according to the manufacturers instructions (Promega, Madison, Wis.). The presence of the inserts was confirmed by PCR with M13 forward and reverse primers, and plasmid DNA was isolated by the alkaline lysis method (Wizard plus SV minipreps DNA purification system, Promega). Sequencing was carried out using dye terminator cycle sequencing, with fluorescent-labelled dye terminators on a DNA sequencer (ABI/Prism 377; Perkin-Elmer Applied Biosystems, Foster City, Calif.). For each cultivar the nucleotide sequences of three clones were determined in both directions.

Genomic DNA sequences were aligned using the neighbour-joining method of the CLUSTALX (ver. 1.83, Thompson et al. 1997), edited with BIOEDIT (ver. 5.0.6, Hall 1999), and the phylogenic tree displayed with TREEVIEW (ver. 1.6.6, Page 1996). The complete sequences of S_a (S_5) (AF148465) previously reported for Texas (Tamura et al. 2000) and S_{10} (AF454003) for Gabaix (Channuntapipat et al. 2002b) were compared using BLAST at NCBI GenBank (http://www.ncbi.nlm.nih.gov/). The sequence of the four S_5 alleles was aligned against all published *S* allele sequences on the GenBank database. To estimate the degree of homology between aligned sequences, we obtained two parameters: identity (between nucleotide sequences) and similarity (between amino acid sequences).



A Lanes 1 to 8: 1. 123 bp Ladder, 2. 'Gabaix' ($S_{10}S_{25}$), 3. 'Desmayo Largueta' (S_1S_{25}), 4. 'Glorieta' (S_1S_5), 5. 'Garbí' (S_1S_5), 6. 'Primorskiy' (S_5S_9), 7. 'Texas' (S_1S_5), 8. 'Languedoc' (S_1S_5).

B Lanes 1 to 8: 1. PUC-Mix Ladder, 2. 'Desmayo Largueta' (S_1S_{25}), 3. 'Gabaix' ($S_{10}S_{25}$), 4. 'Glorieta' (S_1S_5), 5. 'Texas' (S_1S_5), 6. 'Primorskiy' (S_5S_9), 7. 'Garbí' (S_1S_5), 8. 'Ardechoise' (S_1S_{25}).

Fig. 1 PCR analysis of *S* alleles from eight almond cultivars. Genomic DNA was amplified using primer pair AS1II and AmyC5R. A DNA ladder was used for size determination

Pollination tests

Field and laboratory assessment of cross-incompatibility relationships between cultivars were made during 2002 and 2003. For the ten cultivars in which *S* genotype clarification was needed, 19 controlled pollinations were carried out in both the field and the laboratory (Table 2). Both directions of a cross were tested for most of the crosses in order to check the possibility of unilateral incompatibility, as reported for cv. Jeffries (Kester et al. 1994b). Pollen was collected from closed buds at the balloon stage (Felipe 1977) from male parents, dried and stored at 4°C. Pollen viability was checked before pollination using the fluorochromatic test procedure (FCR) (Heslop-Harrisson et al. 1984).

In the field, for each cross listed on Table 2, at least 100 carefully emasculated flowers were hand-pollinated. Fruit set (number of fruits/number of pollinated flowers) was recorded 40 days after pollination. When fruit set percentages were above 4%, the cultivars were considered to be cross-compatible (Rovira et al. 1998). Since fruit set can be affected by climatic factors in the field, the data were cross-checked with cross-compatibility and cross-incompatibility records from the database of the breeding programme at Mas Bové from 1985 to 2003.

In the laboratory, 15 flowering buds from each female parent were collected at the balloon stage, emasculated and floated in water trays. Pistils were cross-pollinated, and 72 h later, they were fixed, stained and prepared following the method described by Rovira et al. (1998). The growth of the pollen tubes through the styles was monitored with a UV fluorescence microscope. The number of pistils compatible with pollen from the male parent was divided by the total number of pistils tested, and if two or more of 12 pistils observed (>17%) showed pollen tubes reaching the ovary, the cultivars were considered to be cross-compatible.

Results

S-specific PCR analysis

A PCR product of about 600 bp, corresponding to the S_5 allele, was present in eight of the cultivars analysed (Ardechoise, Desmayo Largueta, Gabaix, Garbí, Glorieta, Languedoc, Texas and Primorskiy) (Fig. 1). Although the primer pair AS1II and AmyC5R are reported to amplify only the S_1 and S_5 alleles (Tamura et al. 2000), a PCR product corresponding to S_{10} was detected in Gabaix. Martínez-Gómez et al. (2003a) also found that this primer pair amplified a product of the same size as the S_{10} allele and, in addition, detected a product corresponding to the S_9 allele in Primorskiy (S_5S_9). In the present study, the S_9 allele was not amplified in Primorskiy nor was S_{10} in Ardechoise, although PCR products corresponding to S_1 and S_5 were present. Therefore, the PCR analysis failed to detect differences between the disputed S_5 alleles from different cultivars.

Stylar ribonuclease analysis

The zymogram (Fig. 2) shows that the positions of the purported S_5 alleles in Desmayo Largueta (S_1S_5) and Gabaix (S_5S_{10}) differed from that in Texas (S_1S_5), whereas for Garbí (S_1S_5), Glorieta (S_1S_5) and Primorskiy (S_5S_9), the positions were the same. The S_5 band from Texas appeared above but close to the S_1 band, however the S_5 band from Desmayo Largueta was observed well above the S_1 band and also above the S_{10} band of Gabaix. In addition, the zymogram shows that, for Desmayo Largueta, the position of the purported S_5 allele was the same as those for the purported S_{10} alleles of Ardechoise (S_1S_{10}) and Ferrastar (S_2S_{10}) as assigned by Boskovic et al. (1999), and of Achaak (S_2S_{10}) as assigned by Ortega (2002), but distinct from that for Gabaix (S_5S_{10}) as assigned by Boskovic et al. (2003).

Sequencing the genomic fragment of the S_5 allele

The partial sequence of the exon of the S_5 allele of the cv. Texas at IRTA was 98% identical to that previously reported for Texas by Tamura et al. (2000) (Table 3) and 99.8% homologous with that for Primorskiy. However, the exon sequence from Desmayo Largueta was only 88.4% similar to that from Texas (IRTA), whereas it showed 99.8% similarity to the exon from Gabaix (Table 3). The length of the S_5 allele sequenced from Desmayo Largueta, Gabaix, Primorskiy and Texas was 602 bp, including the 84-bp intron (Fig. 3). In all, there was a 59-bp difference between Desmayo Largueta and Texas. When the sequence of the new S_{25} allele was aligned against those recorded on the GenBank database, no match materialized. The genetic distances between the nucleotide sequences of the four cultivars are shown in a phylogenetic tree in Fig. 4. The introns of the S_5 alleles from the Texas cultivar at IRTA and those from the cv. Texas analysed by Tamura et

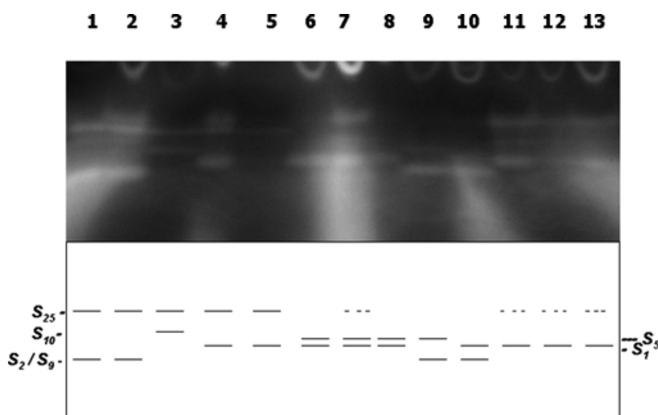


Fig. 2 Stylar ribonuclease zymogram based on NEpHGE migration and an interpretative diagram of the 13 almond cultivars. *Lanes*: *1* Achaak (S_2S_{25}), *2* Ferrastar (S_2S_{25}), *3* Gabaix ($S_{10}S_{25}$), *4* Ardechoise (S_1S_{25}), *5* Desmayo Largueta (S_1S_{25}), *6* Glorieta (S_1S_5), *7* Garbí (S_1S_5), *8* Texas (S_1S_5), *9* Primorskiy (S_5S_9), *10* Cristomorto (S_1S_2), *11* Falsa Barese (S_1S_f), *12* Tuono (S_1S_f), *13* Genco (S_1S_f). *Dotted lines* Secondary bands

al. (2000) showed a homology of 98.8%. However, with respect to the IRTA Texas and Desmayo Largueta, the homology was only 83%. The amino acid sequences for Texas (IRTA) and Desmayo Largueta showed a similarity of only 72%, whereas the similarity between Texas (IRTA) and Primorskiy was 99%, the same as between Desmayo Largueta and Gabaix.

Pollination tests

The results from the 19 pollination tests showed some inconsistencies when compared to previously reported results (Table 2). The outcome of the testcrosses were the same for both directions of each cross and are presented as cross-compatible and cross-incompatible crosses in Table 2. In some crosses, field and laboratory records were taken from more than 1 year as fruit set fluctuated from year to year.

The crosses between Desmayo Largueta and each of cvs. Glorieta, Garbí, Texas, Languedoc and Gabaix as well as the cross Ardechoise × Gabaix were cross-compatible (Table 2). Although the cross Garbí × Desmayo Largueta showed low fruit set (3.0%) in 2002, mean fruit set in the field was 15.6% and pollen tubes reached the ovary in the laboratory; therefore we considered it to be cross-compatible. The cross Desmayo Largueta × Gabaix showed 19.8% fruit set in the field and only 4.5% cross-compatibility in the laboratory. In the reciprocal cross, the low value of fruit set in the field (3.2%) was only for 1 year, and pollen tubes reached the ovary in 20% of the cross-pollinated flowers. In addition, Ardechoise proved to be cross-compatible with Gabaix in the field (30.8%) but showed a moderate level of cross-compatibility in the laboratory (13.6%). Some other crosses (Glorieta × Garbí, Glorieta × Texas, and Garbí × Texas, and their reciprocals, and Ardechoise × Desmayo Largueta) failed both in the field and in the laboratory. The cross Texas × Languedoc also found to be cross-incompatible, was only tested in the laboratory. In all of these cross-incompatible crosses not one pollen tube reached the ovary, which resulted in most crosses in 0% fruit set in the field.

Discussion

Based on the combination of stylar ribonuclease analysis, testcrosses and genetic analysis, we propose that the S allele previously designated as S_5 in Desmayo Largueta and Gabaix be re-named S_{25}. We also propose that the allele previously designated as S_{10} in Achaak, Ardechoise and Ferrastar be renamed S_{25}. This new S allele was originally named S_{25} because at the time it was first observed 24 alleles had already been reported in almond (Boskovic et al. 2003). Five S almond cultivar genotypes have been relabelled: Achaak (from S_2S_{10} to S_2S_{25}), Ardechoise (from S_1S_{10} to S_1S_{25}), Desmayo Largueta (from S_1S_5 to S_1S_{25}), Ferrastar (from S_2S_{10} to S_2S_{25}) and Gabaix (from S_5S_{10} to $S_{10}S_{25}$). However, the S genotypes of Garbí (S_1S_5), Glorieta (S_1S_5), Languedoc (S_1S_5), Texas (S_1S_5) and Primorskiy (S_5S_9), which were also analysed, remain the same. In addition, two new cross-incompatibility groups in almond (XVI and XVII) have been added to the previously reported 16 groups (I- XV and O) (Table 4). The cultivars Ardechoise and Desmayo Largueta (previously assigned to group O and II, respectively) are now moved to group XVI. Cultivar Achaak (previously unassigned) and Ferrastar (previously assigned to group O) are included in group XVII.

The first indication of the S_5 and S_{25} alleles being different was raised by their distinct band position on the zymograms after stylar ribonuclease analysis. Early misassignment of S alleles by stylar ribonuclease analysis in almond cultivars was probably caused by the complexity of zymogram interpretation and isoelectric point (pI) determination as well as by the few testcrosses made to check the predicted genotypes. Recently, following extensive work by Boskovic et al. (2003), modifications in the pI values of some S alleles previously reported (Boskovic et al. 1997, 1999) were adopted.

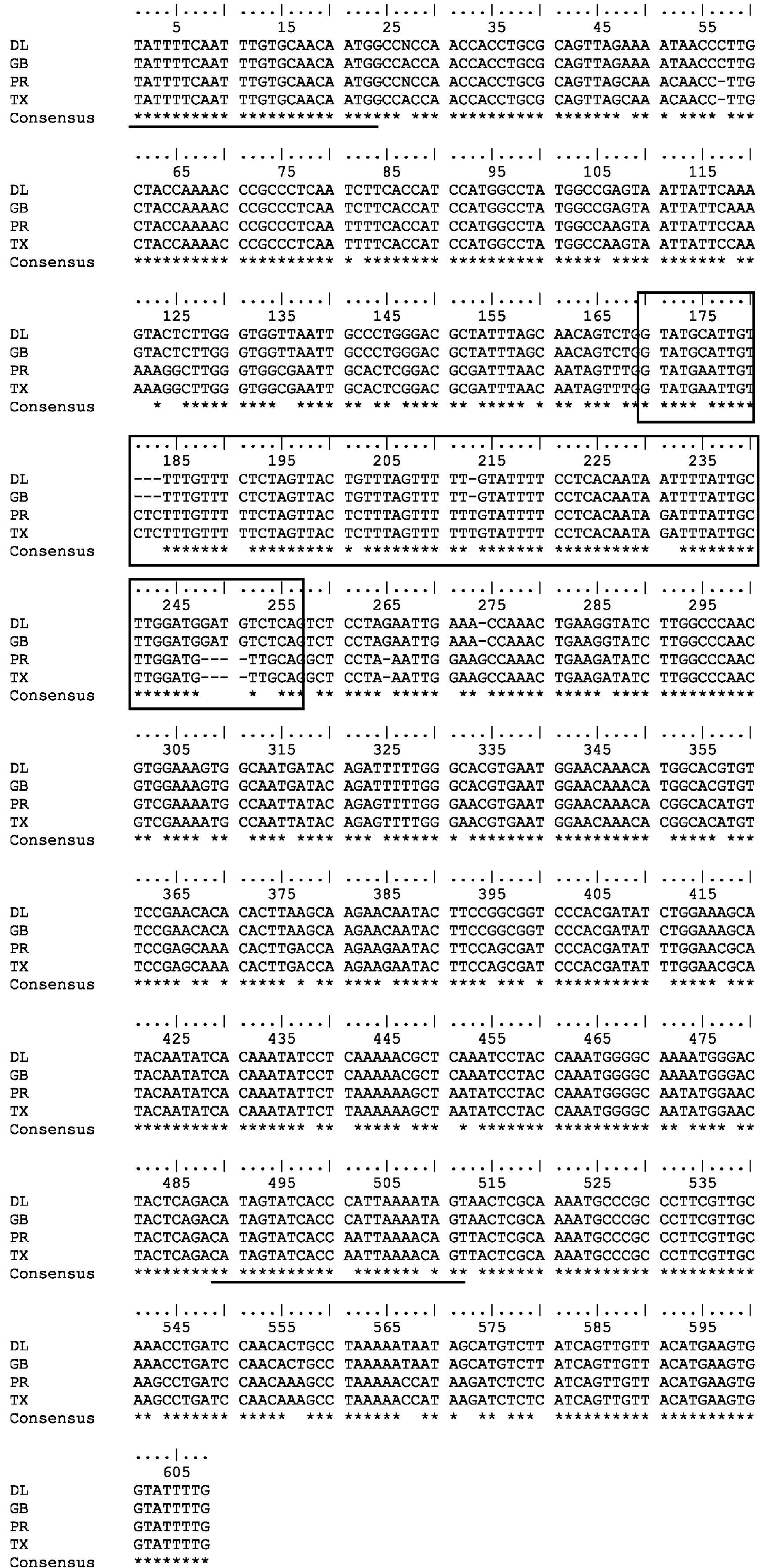
The PCR-based analysis using the S-conserved AS1II/AmyC5R primer pair designed by Tamura et al. (2000) failed to distinguish the expected S alleles from different cultivars, namely the S_5 allele from Texas (S_1S_5) and the novel S_{25} allele from Desmayo Largueta (S_1S_{25}) (Fig. 1). Thus, the S_5 and S_{25} alleles from Gabaix ($S_{10}S_{25}$), Garbí (S_1S_5), Glorieta (S_1S_5), Languedoc (S_1S_5) and Primorskiy (S_5S_9) were undistinguishable (Fig. 1), probably as a result of very small differences between nucleotide sequences. The S_5 allele from the Californian cultivar Carmel (S_5S_8) was amplified by PCR analysis using AS1II/AmyC5R

Table 3 Identity (%) matrix calculated by the neighbor-joining method as deduced from the nucleotide exons of the S_5 and S_{25} alleles in different cultivars having these alleles

Identity	S allele	Texas NCBI[a]	Texas	Primorskiy	Desmayo Largueta	Gabaix
Texas NCBI[a]	S_5	100	98.0	-	87.0	-
Texas	S_5	-	100	99.8	88.4	88.6
Primorskiy	S_5	-	-	100	88.6	88.4
Desmayo Largueta	S_{25}	-	-	-	100	99.8
Gabaix	S_{25}	-	-	-	-	100

[a]Texas NCBI: complete S_a (S_5) sequence AF148465 from Texas is reported in the GenBank (Tamura et al. 2000)

Fig. 3 Nucleotide sequence alignment of the S_5 and S_{25} alleles in four almond cultivars: *DL* Desmayo Largueta, *GB* Babaix, *TX* Texas, *PR* Primorskiy. Intron sequence is framed in a *box*. Sequences to which the AS1II and AmyC5R pair of primers get attached are *underlined* Nucleotide positions are *numbered*



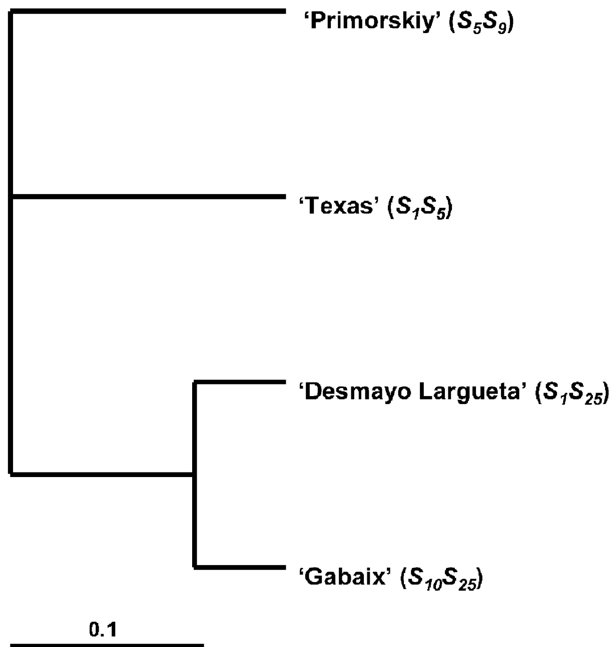


Fig. 4 Phylogenetic tree showing the estimated genetic similarity of S_5 and S_{25} alleles in almond based on the neighbor-joining method. *Bar* represents 0.1 nucleotide substitutions per site

(Martínez-Gómez et al. 2003a) and confirmed in this work (unpublished data). However, PCR analysis was useful in distinguishing the S_5S_9 and S_{10} alleles in Gabaix and in Primorskiy. The S_{10} allele from Gabaix was probably amplified since the AS1II/AmyC5R primer pair was designed from conserved regions of the S alleles. Any clarification of the differences in band amplification with respect to the S_9 allele in Primorskiy (Fig. 1) will require further work. In addition, Channuntapipat et al. (2002b) designed an S_5-specific primer (S5F/R) from intron sequences which proved successful in amplifying this allele in Glorieta and Texas (both S_1S_5) and in Primorskiy (S_5S_9) but failed in amplifying this allele in Gabaix (re-genotyped as $S_{10}S_{25}$ in this work). In contrast, Channuntapipat et al. (2003) succeeded with the same primer pair in amplifying the S_5 allele in Gabaix. As the intron nucleotide identity between the S_5 and S_{25} alleles was 83% in our work, it may be that both alleles were not always amplified using this primer. The design of a new specific primer to amplify the S_{25} allele would be useful for distinguishing it from the S_5 allele.

Our stylar ribonuclease analysis using the offspring of the crosses Glorieta (S_1S_5) × Falsa Barese (S_1S_f) (López et al. 2004) and Falsa Barese (S_1S_f) × Desmayo Largueta (S_1S_{25}) (Mnejja et al. 2002)-both sets of offspring raised at IRTA-Mas Bové-clearly showed different band positions on the zymograms between the reported S_5 allele from Glorieta (maintained as an S_5 allele in this work) and Desmayo Largueta (renamed as an S_{25} allele in this work), although they were not resolved. The results obtained from these two crosses of 63 and 54 seedlings, respectively, strongly support the new genotypes we assigned to Glorieta and Desmayo Largueta, although in the case of the cross involving the latter cultivar we recorded data using the previous assigned genotype. Results of the pollination tests in the field for these two progenies (100% self-compatible and 50% self-compatible, respectively)

Table 4 *S* genotypes of ten almond cultivars

Cultivar	Parentage with assigned genotypes in this work	Reported *S* genotype[a]		Assigned genotypes in this work	Agreement between reported and assigned genotype	Cross-incompatibility group (CIG)[b]	
		S RNase	PCR			Reported (Boskovic et al. 2003)	Assigned in this work
Achaak	Unknown	S_2S_{10}	Untested	S_2S_{25}	No	Unreported	XVII[c]
Ardechoise	Unknown	S_1S_{10}	Untested	S_1S_{25}	No	O	XVI[c]
Desmayo Largueta	Unknown	S_1S_5	S_1S_5	S_1S_{25}	No	II	XVI[c]
Ferrastar	Cristomorto (S_1S_2) × Ardechoise (S_1S_{25})	S_2S_{10}	S_2S_{10}	S_2S_{25}	No	O	XVII[c]
Gabaix	Unknown	S_5S_{10}	S_5S_{10}	$S_{25}S_{10}$	No	O	O
Garbí	Cristomorto (S_1S_2) open-pollinated	S_1S_5	S_1S_5	S_1S_5	Yes	II	II
Glorieta	Primorskiy (S_5S_9) × Cristomorto (S_1S_2)	S_1S_5	S_1S_5	S_1S_5	Yes	II	II
Languedoc	Unknown	S_1S_5	S_1S_5	S_1S_5	Yes	II	II
Texas (syn. Mission)	Languedoc (S_1S_5) open-pollinated	S_1S_5	S_1S_5	S_1S_5	Yes	II	II
Primorskiy	Princesse × Nikitskiy	S_5S_9	S_5S_9	S_5S_9	Yes	O	O

[a]Reported *S* genotype referred in Table 1
[b]CIG II (cultivars having the S_1S_5 genotype) and CIG O (cultivars having unique genotypes)
[c]Group added as a result of this work

were largely in agreement with those of the stylar ribonuclease and PCR analyses.

Sequencing analysis was carried out to distinguish between the S_5 and S_{25} alleles. These alleles were found different, with 88% nucleotide identity and 72% amino acid similarity. These differences can be explained by point mutations and deletions (Fig. 3) that occurred in one of the two sequences. Point mutations and differences in the introns, which are responsible for allele polymorphism, could have affected the final amino acid sequences and the specificity of these alleles. Since the sequences where the primer attached are common to both the S_5 and S_{25} alleles, differences between the alleles could not be detected by PCR, with the result that both alleles were sequenced. As both alleles have the same nucleotide length and are 88% identical, both could be related. Van Nerum et al. (2001) upon re-examining self-incompatibility genotypes in apple reported two alleles which differed in four nucleotide bases but still encoded for identical S RNases. In our investigation, there was a difference of 59 different nucleotide bases between the S_5 and S_{25} alleles. The glycoproteins codified by the S_5 and S_{25} alleles have different electrophoretic mobility under NEpHGE migrating conditions (Fig.2). These results, supported by the results of the pollination tests, suggest that both the S_5 and S_{25} alleles are functionally different. The 13-nucleotide difference between the S_5 sequence obtained in this work for Texas and the one reported earlier (AF148465) may be explained by errors during sequencing. Sequencing the S_{25} allele in Ardechoise, Achaak and Ferrastar should clarify if they have the same DNA sequence as the S_{25} allele reported in Desmayo Largueta.

Cultivars Garbí (S_1S_5), Glorieta (S_1S_5) and Texas (S_1S_5) set well when pollinated with Desmayo Largueta (S_1S_{25}). In addition, the cross Desmayo Largueta (S_1S_{25}) × Languedoc (S_1S_5) set fruit. As the S_1 band was clearly visible in the zymograms of these five cultivars our results support the premise that Desmayo Largueta does not share the same S_5 allele as Garbí, Glorieta, Languedoc and Texas. It would appear that the S_5 and S_{25} alleles are functionally different, which agrees with the results from the stylar ribonuclease analysis. The cross Glorieta (S_1S_5) × Desmayo Largueta (S_1S_{25}) was also carried out by Duval et al. (1998), but this failed in achieving fruit set, probably due to adverse climatic conditions during pollination (I. Batlle, personal communication), thereby supporting the initial mis-classification of Desmayo Largueta into CIG II (Boskovic et al. 1997). The crosses Desmayo Largueta (S_1S_{25}) × Gabaix ($S_{10}S_{25}$) and Ardechoise (S_1S_{25}) × Gabaix ($S_{10}S_{25}$) also set almonds well. The low fruit set percentages obtained for the crosses Garbí (S_1S_5) × Desmayo Largueta (S_1S_{25}) in 2002 (3.0%) and Gabaix ($S_{10}S_{25}$) × Desmayo Largueta (S_1S_{25}) in 2003 (3.2%) (Table 2) are in agreement with the low fruit set values that Garbí and Gabaix often show after cross-pollination with many cultivars or selections in our breeding programme, apparently due to weak flowers (F.J. Vargas, personal communication). The possibility of unilateral incompatibility was discarded, as the outcomes of the testcrosses were the same irrespective of the direction of the cross (Table 2). Since the pollination tests among Garbí, Glorieta, Languedoc and Texas showed that these cultivars were all cross-incompatible, their genotypes should be the same (S_1S_5), which is in agreement with their identical banding patterns in the RNase zymograms. Data for two of these crosses from the IRTA breeding programme (data not shown) support the results obtained in this work (Table 2). The failure of the testcrosses Ardechoise (S_1S_{25}) × Desmayo Largueta (S_1S_{25}) and the reciprocal support the premise that Ardechoise shares the S_1S_{25} genotype with Desmayo Largueta, which was also determined by stylar ribonuclease analysis (Fig. 2).

Cultivars Desmayo Largueta and Gabaix are known to originate from the same geographic zone (Priorat, Tarragona) (F.J. Vargas 1975). Viruel (1995) found a 69% similarity between these two cultivars when he compared almond cultivars by RFLP analysis. The presence of the S_{25} allele in Gabaix ($S_{10}S_{25}$) could be explained on the basis of the latter being genetically related to Desmayo Largueta, which is a plausible assumption as their morphological and agronomical characteristics are similar (F. J. Vargas, personal communication) and they share the same S_{25} allele. The cultivar Garbí (S_1S_5) obtained at IRTA-Mas Bové is known to be an open-pollinated seedling of Cristomorto. Based on our confirmation that Garbí and Primorskiy share the S_5 allele, we suggest that Garbí most likely originated from Primorskiy, as both cultivars were placed nearby in the breeding plot. Garbí also shows a large number of features similar to this Ucranian cultivar such as late flowering and low cropping (Vargas, personal communication). Isozyme records of 13 loci are consistent with the possibility that Gabaix and Desmayo Largueta are related and that Garbí originates from Primorskiy (Arús et al. 1994 and unpublished results). Using SSR analysis Martínez-Gómez et al. (2003b) found that Texas and Languedoc appeared to be more closely related to the European cultivars than to the Californian ones. This relatedness was further supported by following the revision of the S genotypes of almond cultivars, when it was noted that some European cultivars like Primorskiy and its derivatives Glorieta and putatively Garbí carried the S_5 allele. The American cultivars Languedoc (S_1S_5) and Texas (S_1S_5) also appeared in the cross-incompatibility group II that consisted primarily of European cultivars (Boskovic et al. 2003).

The new S_{25} allele assigned to Ferrastar (S_2S_{25}) is consistent with the new genotype also suggested for Ardechoise (S_1S_{25}) given that Ferrastar comes from the cross Cristomorto × Ardechoise (Table 1). This new S_{25} allele corresponds to an allele S_{10} previously reported for Ardechoise (S_1S_{10}) and Ferrastar (S_2S_{10}) by Boskovic et al. (1999) using stylar ribonuclease analysis. Channuntapipat et al. (2002b) designed an S_{10}-specific primer (S10F/R) from the partial intron sequences of an allele isolated from Gabaix (previously reported to be S_5S_{10}), which was putatively assigned as S_{10} by Channuntapipat et al. (2002a). This primer failed in amplifying this allele in Ferrastar, which is consistent with the re-labelled S_2S_{25}

genotype proposed in this investigation, and unexpectedly amplified the S_1 allele. However, Channuntapipat et al. (2003) using the same S10F/R primer later assigned the S_2S_{10} genotype to Ferrastar. Available data from Grasselly (1972) corroborated that the S_{25} allele found in Ardechoise in this work (previously named S_{10}) is functionally different from the S_5 allele of Texas, since the cross Ardechoise (S_1S_{25}) × Texas (S_1S_5) set fruit in the field and thus was cross-compatible. Cultivar Achaak, relabelled as S_2S_{25} in this work, was probably mis-scored by Ortega (2002) as S_2S_{10} using stylar ribonuclease analysis. More information will be required to confirm our results: if the cross of Achaak by Ferrastar fails, both cultivars should have the same genotype. Moreover, Achaak has never been tested by PCR analysis, which should be carried out in a future investigation.

The information obtained as a result of combining different methods for the purpose of cultivar *S* genotyping in almond shows the usefulness of the combined analyses in resolving inconsistencies. S_5 is one of the most frequently found *S* alleles in almond cultivars. Stylar ribonuclease analysis was useful for detecting discrepancies in almond genotypes. PCR analysis was unable to reveal differences between the S_5 and S_{25} alleles when the conserved AS1II/AmyC5R primer pair was used, although it was useful for confirming that Ardechoise was not carrying the expected S_{10} allele. Sequencing analysis confirmed that the S_5 and S_{25} alleles were indeed different. Testcrosses were made to verify the genotypes of seven cultivars, and the outcome was useful for confirming the predicted genotypes and for ensuring that the S_5 and S_{25} alleles were functionally different, although sequencing did suggest that both alleles could be related.

Conflicting *S* genotypes in almond cultivars found by stylar ribonuclease analysis have been revised and clarified, as has been done in other *Rosaceous* species like apple (Van Nerum et al. 2001; Broothaerts 2003) and cherry (Wiersma et al. 2001; Sonneveld et al. 2003). The *S* locus in almond is highly polymorphic (Ishimizu et al. 1998), and more bands corresponding to new *S* alleles are likely to be detected as the range of cultivar analysis increases. The detection of *S* allele inconsistencies will be facilitated as the techniques become more powerful. The information provided by this study will be included in the European *Prunus* database. An accurate identification of *S* genotypes is essential for designing crosses and selecting seedlings in breeding programmes and for choosing combinations of cultivars for productive orchards (Batlle et al. 1997; López et al. 2001). Although the *S* RNase and the genetic analyses have proven to be useful in reducing the number of crosses needed to identify *S* genotypes or in establishing cross-compatibilities between cultivars, these molecular methods should be applied with the support of testcrosses to check *S* genotype inconsistencies.

Acknowledgements The authors are grateful to P. Martínez-Gómez (CEBAS, CSIC, Murcia) and T.M. Gradziel (University of California, Davis, USA) for their valuable comments and suggestions. We also thank J. Clavé, M.A. Romero, J. Santos and J. Vergés (IRTA-Mas Bové), D. Gregory and K. Malone (University of Adelaide, Australia) and members of the laboratory of Genètica Vegetal at IRTA-Cabrils for their help with the field and laboratory work. This research was conducted under two INIA (Spanish Ministry of Education and Science) Projects (SC97-049 and RTA01-081). M.L. gratefully acknowledges a grant from the INIA postgraduate programme.

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