

Identification of the S_3 self-incompatibility allele in almond by specific primers

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Abstract

To date, the identification of the S_3 self-incompatibility allele in almond using PCR primers has been problematic, either because of its weak amplification in some allelic combinations, or because of a lack of polymorphism in comparison with the self-compatibility (S_f) allele. This paper describes the use of three new primers, one 'forward' (S3F) and two 'reverse' (S3R1 and S3R2), specifically designed to amplify the S_3 allele. Primers S3F and S3R1 were designed from the sequence of the second intron of this allele, while S3R2 was designed from the sequence upstream of the RC4 conserved region. Four primer combinations (S3F/S3R2, S3F/ConR, ConF/S3R1 and ConF/S3R2) satisfactorily amplified the S_3 allele. The use of consensus primers from the S -RNase intron sequences allows specific identification of the S -alleles, and indicates the expected size of the amplified fragment.

Additional key words: PCR, *Prunus amygdalus*, S -allele, selection by molecular markers.

Resumen

Identificación del alelo S_3 de autoincompatibilidad en el almendro mediante cebadores específicos

La identificación del alelo S_3 de autoincompatibilidad en el almendro mediante los cebadores PCR existentes es problemática, ya sea por su débil amplificación en determinadas combinaciones alélicas o por la falta de polimorfismo con el alelo de autocompatibilidad (S_f). Por ello, se diseñaron tres nuevos cebadores, uno 'directo' (S3F) y dos 'inversos' (S3R1 y S3R2) para la amplificación específica del alelo S_3 . Los cebadores S3F y S3R1 se diseñaron a partir de la secuencia del segundo intrón de este alelo, mientras que el S3R2 se diseñó a partir de la secuencia previa a la región conservada RC4. Cuatro combinaciones de cebadores (S3F/S3R2, S3F/ConR, ConF/S3R1 y ConF/S3R2) mostraron una amplificación satisfactoria del alelo S_3 . El uso de cebadores complementarios a las secuencias de los intrones de las S -RNasas permite la identificación específica de los alelos S y conocer previamente el tamaño del fragmento PCR amplificado.

Palabras clave adicionales: alelos S , PCR, *Prunus amygdalus*, selección por marcadores moleculares

Introduction

Almond [*Prunus amygdalus* Batsch syn. *Prunus dulcis* (Mill.) D.A. Webb] shows a gametophytic system of self-incompatibility (Socias i Company *et al.*, 1976), controlled by a single multi-allelic S -locus. Self-incompatibility compels the almond to outcrossing

and reduces the level of inbreeding (Dodds *et al.*, 1996) through the expression of ribonucleases (McClure *et al.*, 1989) in the style that arrest the growth of incompatible pollen tubes. A direct relationship between the stylar RNases produced and the S -alleles has been reported in this species (Bošković *et al.*, 1997; Tao *et al.*, 1997). Self-compatibility in almond occurs because of an S_f allele (Socias i Company and Felipe, 1988) that seems to function as a stylar part-mutant since no stylar S -RNase has been linked to the S_f allele (Bošković *et al.*, 1999).

The S -genotypes of some almond cultivars and selections have recently been examined by electrophoretic

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separation of the stylar RNases expressed by the different *S*-alleles (Bošković *et al.*, 1997, 1999). Those detected so far range from *S*₁ to *S*₂₃ (Bošković *et al.*, 2003); the potential number of *S*-alleles remains unknown.

S-genotype identification in the Rosaceae has advanced significantly through PCR amplification of DNA fragments coding for the different *S*-RNases. The DNA sequences from the *S*-RNase genes in the Rosaceae share five conserved domains (C1, C2, C3, RC4 and C5) and one hypervariable region (RHV) located between domains C2 and C3 (Ma and Oliveira, 2002). The identification of the *S*-genotypes and the study of the polymorphism and evolution of *S*-alleles has been undertaken in several species using primers based on these conserved domains, usually C1 and C5 (Richman *et al.*, 1997; Ishimizu *et al.*, 1998; Tamura *et al.*, 2000; Channuntapipat *et al.*, 2001).

The cloning of the most common *S*-alleles in Californian almond cultivars, *S*^b/*S*₁, *S*^c/*S*₇, *S*^d/*S*₈ (Ushijima *et al.*, 1998) and *S*^a/*S*₅ (Tamura *et al.*, 2000) represents a great step forward in the PCR identification of almond *S*-genotypes. The availability of primers for the C1 and C5 conserved regions allows these *S*-RNase genes to be amplified (Channuntapipat *et al.*, 2001; Ma and Oliveira, 2001a,b; Tamura *et al.*, 2000). Thus, using primers AS111 and AmyC5R (Tamura *et al.*, 2000), Martínez-Gómez *et al.* (2003) reported the amplification of fragments from the *S*₆, *S*₉, *S*₁₀ and *S*₁₈ alleles, while Ortega and Dicenta (2003) reported the same for *S*₁₁ and *S*₁₂. Channuntapipat *et al.* (2001) designed two other primers from the conserved

regions C1 and C5, known as ConF and ConR, which amplify *S*^b/*S*₁, *S*^c/*S*₇, *S*^d/*S*₈ and *S*_f fragments. At the same time, Ma and Oliveira (2001a) cloned the *S*₁ and *S*₃ alleles from the cultivar ‘Ferragnès’ and designed several primers, some of them degenerate, for the amplification of the *S*-alleles. Several of these primers (Table 1) share nucleotide sections, such as primers 1 and 6 (Ma and Oliveira, 2001a) with ConF (Channuntapipat *et al.*, 2001), and 2 and 7 (Ma and Oliveira, 2001a) with ConR (Channuntapipat *et al.*, 2001). Consequently, the proposed primer combinations 1/2, 6/7 and ConF/ConR, amplify bands of the same size when used with the same DNA sample. When primers from conserved regions are used in almond, however, a lack of amplification of some alleles is seen when the *S*₁ or *S*₇ alleles are present (Tamura *et al.*, 2000; Channuntapipat *et al.*, 2001). When these are absent amplification is quite normal.

Channuntapipat *et al.* (2003) proposed the use of specific primers for the alleles *S*₁, *S*₂, *S*₅, *S*₇, *S*₈, *S*₉, *S*₁₀, *S*₂₃ and *S*_f, designed from their intron sequences, as the most suitable tools for identifying *S*-genotypes, characterising cultivars, designing crosses, and selecting self-compatible seedlings in breeding programmes. However, no specific primers have been reported for the amplification of *S*₃, an allele present in many cultivars of European origin, some of which are commonly used as parents in European and Australian breeding programmes. The aim of the present work was to develop a strategy for the identification of *S*₃ using conserved and specific primers.

Table 1. Already-existing primers designed from the conserved C1 and C5 domains of the stylar *S*-RNase gene of almond

Primer	Sequence 5' → 3'	Primer combination	Product size (kbp)	Object allele	Reference
Primer 1	CARTTYGTBCARCARTGGCC	1 / 2	1.1	<i>S</i> ₁	Ma and Oliveira (2001a)
Primer 2	TACCACTTCATGTAACAACCTG	1 / 3	0.7		
Primer 3	GACATCCAAGCAATATAGAGAC	4 / 5	1.6	<i>S</i> ₃	
Primer 4	TCTAAGTATGGSKATKTTGAA	6 / 7	1.2		
Primer 5	AATTTTAYKGAAACRAGATG	8 / 2	1.2	<i>S</i> _f	
Primer 6	ACCAACTGCAGAGTTCGAACG				
Primer 7	TACCACTTCATGTAACAACCTGAG	ConF/ConR	1.1	<i>S</i> ^b / <i>S</i> ₁	
Primer 8	ACCACCTGCAGATTTAGCGGC				
ConF	GTGCAACAATGGCCACCGAC	ConF/ConR	1.2	<i>S</i> _f	Channuntapipat <i>et al.</i> (2001)
ConR	TACCACTTCATGTAACAACCTGAG				

Material and Methods

Plant material

Seedlings of four progenies obtained from the crosses 'Tuono' (S_1S_f) × 'Ferragnès' and 'Ferralise' (both S_1S_3) and their reciprocals were studied: 'Tuono' × 'Ferragnès' n=37 plants, 'Tuono' × 'Ferralise' n=22, 'Ferragnès' × 'Tuono' n=39, 'Ferralise' × 'Tuono' n=91. For those progenies in which 'Tuono' was the mother plant, the expected genotypes of the seedlings were S_1S_3 and S_3S_f . For progenies in which 'Tuono' was the pollen donor, the expected genotypes were S_1S_f and S_3S_f , all of which are self-compatible. The progeny material belongs to the almond breeding programme of the *Unidad de Fruticultura, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA)*, whose aim is to obtain new self-compatible and late blooming cultivars.

PCR primers

In preliminary studies, several primers already described for *S* allele identification in almond (Channuntapipat *et al.*, 2001; Ma and Oliveira, 2001a) were tested for the amplification of the S_1 , S_3 and S_f alleles in the seedlings (Table 1).

Three new specific primers, one forward (S3F) and two reverse (S3R1 and S3R2), were designed (Table 2) based on the S_3 sequence. The S3F and the S3R1 primers were obtained from the second intron sequence of the S_3 gene (Fig. 1). The S3R2 primer was designed from the exon sequence located next to the conserved region RC4. Different primer combinations involving the previously described conserved primers and the new specific primers were tested for their capacity to identify the *S*-genotypes of the seedlings.

PCR analysis

For *S*-allele identification by PCR analysis, genomic DNA was extracted from leaves of each seedling using a protocol based on that of Gepts and Clegg (1989). About 40 mg were ground to a fine powder in liquid nitrogen using a 1.5 ml tube as a mortar and a plastic pestle fitted to an electric drill. After the addition of 0.4 ml extraction buffer (50 mM Tris-HCl, 0.7 M NaCl, 10 mM EDTA, 1% SDS, 5% PVPP, 2% β -mercaptoethanol), the mixture was homogenised and incubated at 65°C for 20 min before mixing with an equal volume of chloroform-isoamyl alcohol (24:1). After centrifugation at 14,000 rpm for 20 min, the upper aqueous phase was separated and mixed with a 2/3 volume of cold isopropanol (−20°C). DNA strands were pooled, cleaned with washing buffer (76% ethanol containing 10 mM NH_4Ac) and dissolved in 0.2 ml of TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). The quantity and quality of the DNA were assessed using a Gene Quant II spectrophotometer (Pharmacia Biotech).

Fifty nanograms of genomic DNA were amplified in a final PCR reaction volume of 25 μl containing 10 mM Tris-Cl (pH 8.2), 50 mM KCl, 100 μM of each dNTPs, 1.9 mM MgCl_2 , 0.125 μM of each primer, and one unit of *Taq* DNA polymerase. The PCR programme to test the new primer combinations consisted of an initial denaturation time of 4 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 57°C and 2 min at 72°C, with a final extension step of 10 min at 72°C. For the other primer combinations, the PCR programmes were those described by Ma and Oliveira (2001a) or Channuntapipat (2001).

PCR products were separated on 2% (w v⁻¹) agarose gels in a buffer solution (40 mM Tris Base, 40 mM acetic

Table 2. Primer combinations for the specific identification of S_3 and S_f

Primer	Sequence 5' → 3'	Combination	Product size (bp)	Target allele	Reference
S3F	CTTCTGCGCTTACGAGAGGTT	S3F / S3R1	611	S_3	This work
S3R1	AAAACGTAAGGGATAGTTTCT	S3F / S3R2	790		
S3R2	TGTGATTTCCACATGTCT				
SfF	GTGCCCTATCTAATTTGTGAC	SfF / SfR	449	S_f	Channuntapipat <i>et al.</i> (2003)
SfR	GACATTTTTTTAGAAAGAGTG				

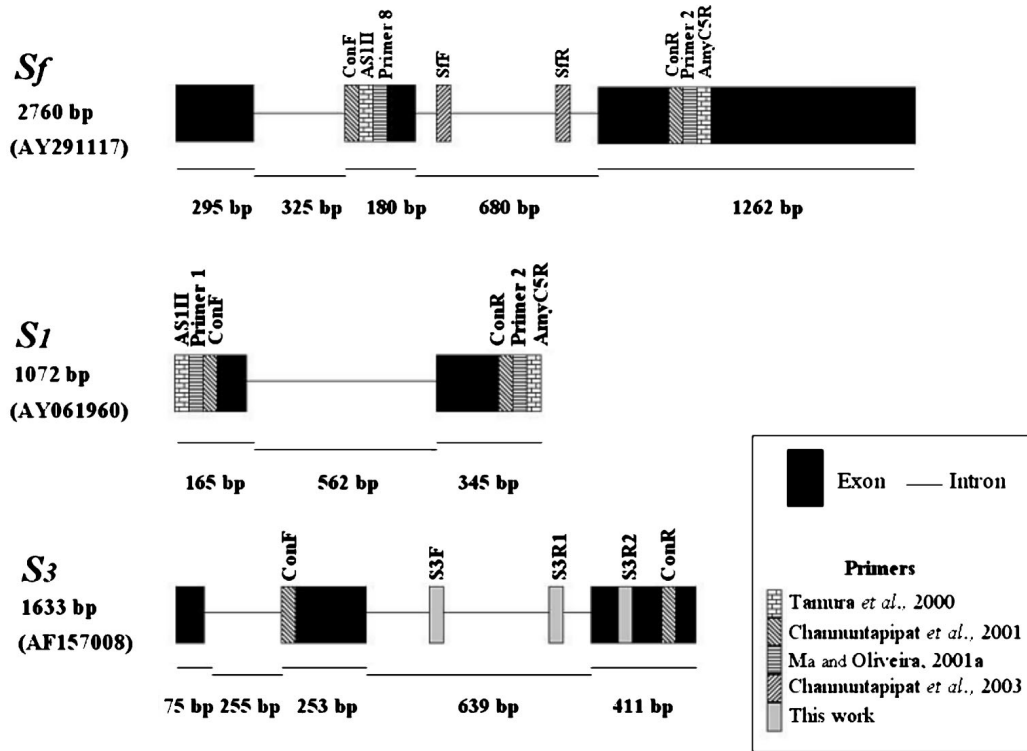


Figure 1. Structure of genomic DNA exons and introns of the *S_f*, *S₁* and *S₃* alleles and their primer annealing sites.

acid, 1 mM of Na₂EDTA) at 80 mA for 3h. To aid in *S* allele identification, the outer lanes contained 100 ng of a 123 bp DNA ladder. After electrophoresis, gels were stained with ethidium bromide (1µg ml⁻¹) and digital images captured with the help of a Gel Doc 2000 UV transilluminator (Bio-Rad). The pattern of amplification products of each seedling was compared with that of the parent cultivars.

Results

The primer combination 4/5 (Table 1) did not adequately amplify the *S₃* allele in our laboratory conditions. Primer combination 6/7 (Table 1) was not tested for *S₃* fragment amplification since these primers share a nucleotide section with the ConF/ConR primers. In fact, primer 7 and primer ConR are exactly the same, and therefore amplify bands of the same size when used with the same DNA sample.

The consensus ConF and ConR primers, developed to amplify other alleles, did amplify the *S₃* allele (Figs. 2 and 4.A-B). However, the fragment obtained

was 1196 bp long and therefore very similar in size to the fragment produced by the *S_f* allele (1205 bp). This very small difference — just 9 bp — hinders discrimination between these two alleles, and, since they are found together in many of the genotypes studied, this combination was deemed unsatisfactory.

When specific identification of the *S₃* allele was attempted using five primer combinations, S3F/S3R1, S3F/S3R2, S3F/ConR, ConF/S3R1 and ConF/S3R2, the fragments amplified were 611, 790, 950, 832 and 1036 bp long respectively (Fig. 3). These combinations produced the expected amplifications of the *S₃* fragments, except for S3F/S3R1, which may require further optimisation of the PCR conditions to be successful. Combinations S3F/S3R2 and S3F/ConR are particularly recommended given the quality of result they provide (Figs. 3 and 4.C).

The identification of the self-compatibility allele (*S_f*) was checked using the SfF/SfR primer combination (Fig. 4.D). The full correspondence obtained with the results of stylar *S*-RNase analysis (data not shown) shows the suitability of this primer pair for detecting the self-compatible genotypes.

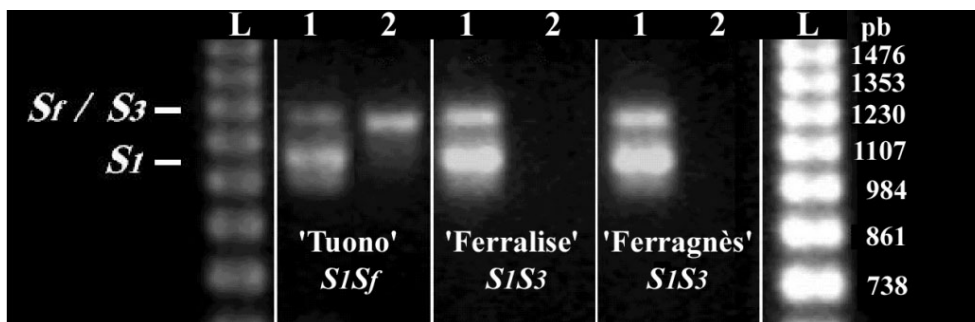


Figure 2. Similar PCR product sizes were obtained with the conserved primers used: (L) 123bp DNA ladder, (1) ConF/ConR (Channuntapipat *et al.*, 2001) (2) 2/8 (Ma and Oliveira, 2001a).

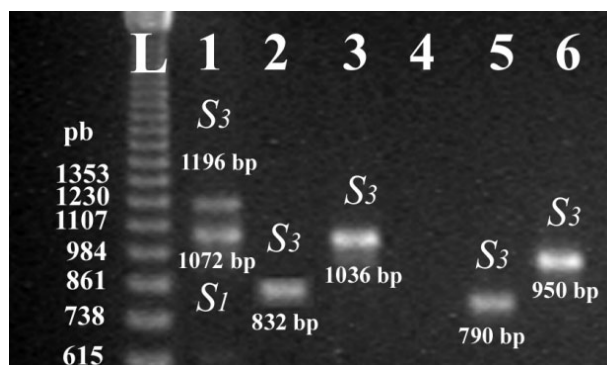


Figure 3. Primer combinations tested for specific S_3 allele identification in 'Ferralise' tracks; L= 123bp DNA Ladder, 1 = ConF/ConR, 2 = ConF/S3R1, 3 = ConF/S3R2, 4 = S3F/S3R1, 5 = S3F/S3R2 and 6 = S3F/ConR.

Discussion

Theoretically, the use of the different degenerate and regular primers developed by Ma and Oliveira (2001a) should allow the amplification of the S_I and S_3 self-incompatibility alleles and the self-compatibility allele S_f in the present progenies with just three primer combinations - 1/3, 4/5 and 8/2 (Table 1). However, only the 1/3 primer combination proved suitable for S_I allele identification under the present PCR conditions.

Primers designed from the S -RNase gene conserved regions easily amplify fragments from different S -alleles (Channuntapipat *et al.*, 2001; Martínez-Gómez *et al.*, 2003; Ortega and Dicenta, 2003, Sanchez-Pérez *et al.*, 2004). In the present work, the ConF/ConR combination of Channuntapipat *et al.* (2001) also amplified the S_3 allele, but the sequences were not fully reliable given the small differences between the AF157008 S_3 sequence (from 'Ferragnès') and the more recently described S_3

sequences AF510417 (also from 'Ferragnès') and AF490505 (from 'Ai'). Another important drawback in the use of these conserved primers is that the S_f amplified product is only 9 bp longer than the newly detected S_3 allele fragment; this hinders band differentiation. Further, the ConF/ConR combination (Channuntapipat *et al.*, 2001) resulted in the masking or the weak amplification of any alleles present with the S_I allele (Figs. 4A and B).

Primer combination 8/2 (Ma and Oliveira, 2001a) is not recommended for the detection of the self-compatibility allele (S_f) since the amplified fragment is 1184 bp long, quite similar to that produced by the ConF/ConR combination (1205 bp). Further, these primers may not show specificity for the S_f allele since primer 8 lies next to the C1 domain, and primer 2 and ConR were designed from the same C5 domain. ConR has only three more bases than primer 2, so they are effectively the same primer.

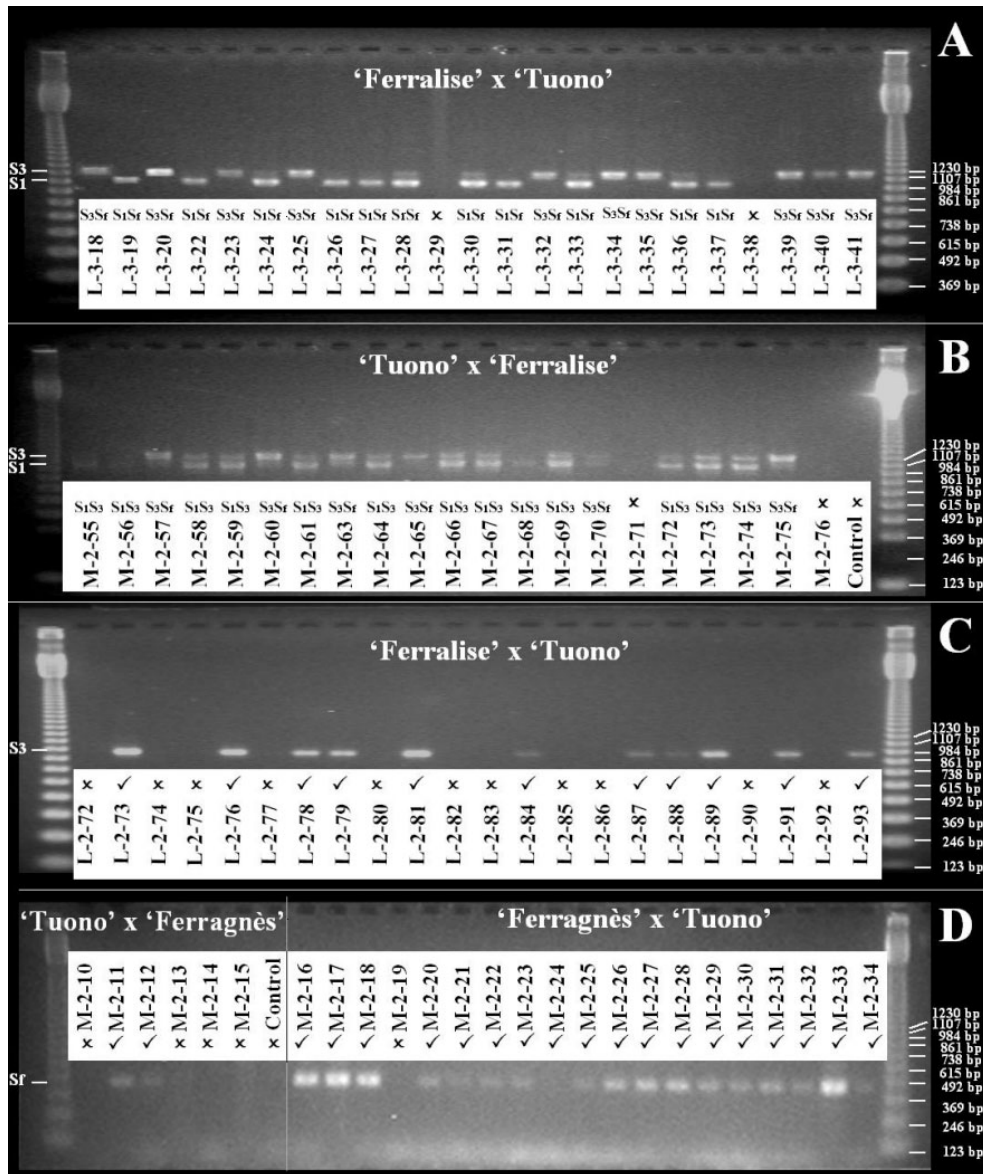


Figure 4. *S*-genotype identification by PCR: (A) 23 seedlings from ‘Ferralise’ × ‘Tuono’ with ConF/ConR, (B) 21 seedlings from ‘Tuono’ × ‘Ferralise’ with ConF/ConR, (C) 22 seedlings from ‘Ferralise’ × ‘Tuono’ with S3F/ConR *S*₃-specific primers and (D) 6 seedlings from ‘Tuono’ × ‘Ferragnès’ and 19 seedlings from ‘Ferragnès’ × ‘Tuono’ with SfF/SfR *S*₇-specific primers (Channuntapipat *et al.*, 2001).

Although these two primers work well with ‘Genco’ genomic DNA, they frequently do not work under the present laboratory conditions with ‘Tuono’ and its offspring. This makes their use problematic in almond breeding programmes since ‘Tuono’ is very widely used as a self-compatibility donor (Socias i Company, 2002).

The ConF/ConR combination satisfactorily amplified both the *S₇* and *S₃* fragments in plants with the *S₇S₃* genotype. However, in *S_iS_j* genotypes, the amplification of the *S_i* fragment usually masked *S_j* amplification. In addition, the amplification of the fragment of the *S₃* allele in the *S₃S_j* genotypes masked or silenced the amplification of the self-compatibility

allele (S_f); two very close bands were sometimes seen since the molecular weight of the two fragments is very similar. The present results show that the S_1S_f and S_1S_3 genotypes cannot be differentiated with the ConF/ConR primers. These two genotypes would not be expected to be found together in the present families, but in plants with these genotypes in which 'Tuono' is the mother plant, S_1S_f and S_fS_f could appear if accidental self-pollination occurred prior to 'Tuono' emasculation.

The specific primer combination SfF/SfR (Channuntapipat *et al.*, 2003), designed from the S_f intron sequences, satisfactorily detected the self-compatibility allele (S_f) in many of the seedlings studied. A band corresponding to a fragment of 490 bp size was amplified in the self-compatible genotypes. Thus, S_f -allele specific identification is solved by using the SfF/SfR combination, while the present work describes an efficient way to specifically identify the S_3 allele.

The results show the ease with which primers can be designed for the specific identification of the different self-incompatibility alleles using their intron DNA sequences. Since the size of the fragment to be amplified is known, this immediately allows the correct behaviour of the designed primers to be verified. In the case of the S_3 allele, four of the five proposed combinations (S3F/S3R2, S3F/ConR, ConF/S3R1 and ConF/S3R2) provided satisfactory amplifications, thus allowing the size of the marker fragment to be selected according to the allele combination. Two primer combinations, S3F/S3R2 and S3F/ConR, were selected for the identification of the S genotypes because of their excellent amplification of the expected fragments.

The present results, plus those already reported, show that the use of PCR for S -genotype identification in almond germplasm is slowly progressing, but that more information on the behaviour of the primers in different genotypes is required if efficiency is to increase. S -genotype determination by PCR in breeding progenies is most favourable when the S -alleles of the parents are known and specific primers for them exist - but currently this is not normally the case. Further, conserved primers may amplify fragments of different S -alleles but reveal no polymorphism, making their differentiation difficult or even impossible (as originally seen for the S_3 and S_f alleles). This problem is solved by using specific primers designed from intron

sequences, such as SfF/SfR, to detect the S_f allele, and those proposed in the present work (particularly combinations S3F/S3R2 and S3F/ConR) for the specific identification of S_3 .

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