

## Molecular and physiological identification of new *S*-alleles associated with self-(in)compatibility in local Spanish almond cultivars

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### ABSTRACT

The *S*-allele characterisation of ‘Alzina’ and ‘Garondès’, two local almond cultivars from the island of Majorca, by a multidimensional approach has allowed the confirmation of the presence of the *S<sub>J</sub>*-allele and the identification of a new allele not previously described in almond, *S<sub>36</sub>*. When these cultivars were phenotypically evaluated, both showed a self-incompatible phenotype and were cross-incompatible, as assessed by artificial self- and cross-pollinations and fruit sets after field pollinations, confirming that their *S<sub>J</sub>*-allele is in its active form, *S<sub>fa</sub>*. Thus a new CGI group in almond is proposed and named XXVIII. These results confirm the wide diversity of *S*-alleles in almond both at genotypic and phenotypic levels, as well as their similarity with the *S*-alleles from other close *Prunus* species. This similarity suggests the possibility of allele introgression between species or allele identity by descent from a common ancestor.

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### 1. Introduction

Most almond [*Prunus amygdalus* Batsch syn. *Prunus dulcis* (Mill.) D.A. Webb] cultivars are, with few exceptions, self-incompatible (Socias i Company, 1990). Gametophytic self-incompatibility (GSI) is a widespread mechanism in flowering plants that prevents self-fertilization and promotes outcrossing (de Nettancourt, 2001). The GSI mechanism, which is found in the Solanaceae, Rosaceae and Scrophulariaceae has an *S*-RNase as the pistil *S*-component and an F-box protein as the pollen *S*-component (Kao and Tsukamoto, 2004). In an incompatible situation, the pistil RNases degrade the pollen RNA, thereby preventing pollen tube growth.

Previously to the introduction of new cultivars in the years 1970s, traditional almond growing in Spain was based in two main cultivars, ‘Desmayo Langueta’ and ‘Marcona’, but with a large number of local cultivars normally grown only in a reduced region and rarely expanding outside their original area (Felipe, 2000). The wealth of local cultivars had been due to the initial almond propagation by seeds in the past, ensuing a close relationship among many of them and in the high frequency of a reduced number of *S*-alleles, as it has been described in populations showing a close relationship among their individuals (Kester et al.,

1994; Lansari and Lakhal, 2001). As a consequence, cases of cross-incompatibility have been found in several local cultivars (Felipe, 2000; Martínez-García et al., 2009), resulting in reduced production. Therefore, the knowledge of the *S*-genotype in almond cultivars is useful for orchard design by growers to ensure cross-pollination and reach a commercial crop level, as well as for parental choice in breeding programmes.

The *S* locus shows a high diversity in almond and more than 37 alleles have been identified (Kester et al., 1994; López et al., 2004; Ortega et al., 2006; Halász et al., 2008; Kodad et al., 2008a,b). The first attempt to establish cross-incompatibility groups (CIG) was achieved by Kester et al. (1994) in Californian cultivars by test crosses in the field, allowing the identification of six CIGs with allele assignment to 32 cultivars. Ortega et al. (2006) updated the table to 18 CIGs including 71 cultivars. More recently, five new alleles were identified (Kodad et al., 2008b) and 16 alleles were assigned to 29 local Spanish cultivars (Kodad et al., 2008a). Thus, the table given by Ortega et al. (2006) needed to be updated up to 25 CIGs with 108 cultivars (Kodad and Socias i Company, 2009).

*S*-genotype determination was initially carried out in field test crosses, although this method is particularly difficult because the results may vary depending on the climatic conditions at blooming. Molecular advances in the study of SI in Rosaceae (McClure et al., 1989) led to the use of non-equilibrium pH gradient electrofocusing (NEpHGE) for *S*-allele identification in almond (Bošković et al., 2003). However, this technique is not very sensitive and does not distinguish alleles with the same isoelectric point (PI) (Bošković et al., 2003), although it is useful to confirm the

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RNase activity of some *S*-alleles (Fernández i Martí et al., 2009; Kodad et al., 2009). PCR-based strategies have been developed for identification of *S*-RNase alleles using genomic DNA (Ushijima et al., 1998; Tamura et al., 2000), resulting in the confirmation of the identity of many *S*-alleles and the identification of new ones (Channuntapipat et al., 2003; Ortega et al., 2006; Kodad et al., 2008a). However, the primers designed from conserved regions (Channuntapipat et al., 2001; Tamura et al., 2000) do not always distinguish alleles with a similar number of nucleotides (López et al., 2004; Ortega et al., 2006; Kodad et al., 2008a). As a consequence, the identification of *S*-alleles in cultivars genotyped for the first time should be carried out by cloning and sequencing the fragments amplified in each allele as well as by test crosses (Kodad et al., 2008b). Thus, this full approach has been undertaken in the present work for the characterisation of new *S*-alleles in two local almond cultivars from the island of Majorca (Spain).

## 2. Materials and methods

### 2.1. Plant material

In addition to the two cultivars studied, 'Garondès' and 'Alzina', two other Spanish cultivars, 'Bulbuenta' ( $S_1S_5$ ) (Kodad et al., 2008a) and 'Desmayo Langueta' ( $S_1S_{10}$ ) (Ortega et al., 2006) were included. All plant samples were obtained from the Spanish almond germplasm collection located at the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) in Zaragoza, maintained as living plants grafted on the almond  $\times$  peach hybrid clonal rootstock INRA GF-677 using the standard management practices (Espiau et al., 2002).

### 2.2. DNA extraction and sequencing of *S*-RNase and *SFB* *S*-haplotype

Genomic DNA was extracted from leaves following the CTAB extraction method based on Doyle and Doyle (1987). For PCR amplification, DNA solution was diluted to 20 ng/ $\mu$ l in water.

The consensus primers AS1II (forward) and AmyC5R (reverse), designed from conserved coding regions flanking the second intron of almond *S*-RNases, were used in the present study according to Tamura et al. (2000). In addition, specific primers for the identification of the *S<sub>f</sub>*-alleles were used (Channuntapipat et al., 2003). Prior to cloning, the band size corresponding to the target alleles was purified using the Wizard Plus Miniprep DNA Purification System (Promega, Madrid, Spain) and quantified on 1.5% agarose gel using standard 1 kb DNA ladder (Invitrogen, Madrid, Spain). The purified PCR products were cloned into the vector pCR2.1 using the TA Cloning Kit (Invitrogen). The presence of the insert in plasmid DNA was confirmed by restriction enzyme digestion with EcoRI. Plasmids were isolated using the QIA prep Spin Miniprep Kit (Qiagen, Hilden, Germany). For each allele, at least three plasmids from different PCR were sequenced from both ends.

### 2.3. Pollination tests

To assess the cross-(in)compatibility between 'Alzina' and 'Garondès', flowers at stage D (Felipe, 1977) were collected from each selection and taken in plastic bags at the laboratory and prepared as described by Kodad and Socias i Company (2006). Samples of 10 pistils were collected from the trays four days after pollination and autoclaved in a 5% solution of  $\text{Na}_2\text{SO}_3$  for 12 min at 1.2 kg  $\text{cm}^{-2}$ . The samples were maintained at 2–4 °C until observation. The pistils were prepared dissecting their outer part and leaving only the transmitting tissue through which pollen tubes grow. This growth was assessed by observation in a Leitz Ortholux II microscope with UV illumination of a mercury lamp Osram HBO

200 W/4, by fluorescence of the callose deposits of the pollen tubes by aniline blue staining after squashing the pistils.

For set evaluation, several branches around the trees were selected in the field for each cultivar and at least 100 flowers were emasculated per branch and cross-pollinated. Fruit set was recorded in June for all treatments.

## 3. Results and discussion

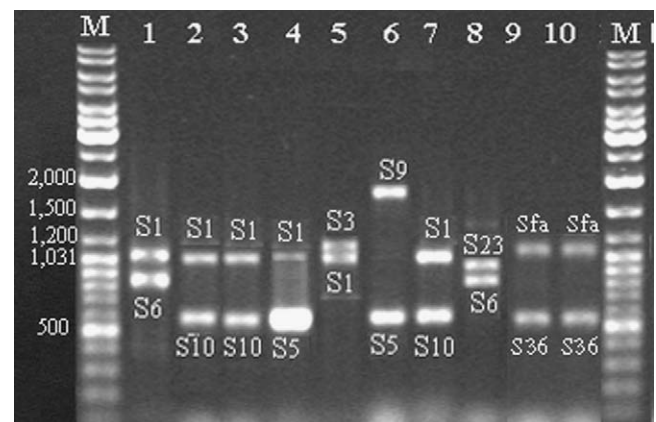
### 3.1. Confirmation of the identity of the *S<sub>f</sub>*-alleles

PCR amplification of the *S*-alleles using the consensus primers AS1II and AmyC5R, yielded two fragments in 'Alzina' and 'Garondès', one corresponding to the *S<sub>f</sub>* and the other presenting similar sizes than  $S_5$  and  $S_{10}$  (Fig. 1). The presence of the *S<sub>f</sub>*-allele was confirmed using the *S<sub>f</sub>*-specific primers designed from the second intron of the *S<sub>f</sub>*-allele of 'Tuono' (Channuntapipat et al., 2003). The sequencing of the *S<sub>f</sub>*-allele amplified from 'Alzina' and 'Garondès' using the consensus primers PaConsI-F and EM\_PC5-consRD confirmed its identity with the *S<sub>f</sub>*-allele (Fig. 2). The alignment of the amino acid sequence of the *S<sub>f</sub>* from 'Alzina' and 'Garondès' is identical to that sequenced from other SC almond cultivars, such as 'Cambra' (Kodad et al., 2009) and to the active *S<sub>f</sub>*-allele in the SI 'Ponç' (Kodad et al., 2009) (Fig. 2). The results of pollen tube growth and fruit set after self-pollination point out that 'Alzina' and 'Garondès' are physiologically SI. The same results have been found in 'Ponç' and 'Vivot', harboring the active *S<sub>f</sub>*-allele (Fernández i Martí et al., 2009; Kodad et al., 2009). Both 'Ponç' and 'Vivot' have shown two different *S*-proteins with RNase activity, indicating the active function of their two *S*-alleles.

The partial sequences of *S<sub>fa</sub>* from 'Alzina' and 'Garondès' have been deposited in the database with accession numbers FJ887783 and FJ887784, respectively.

### 3.2. Identification and molecular characterisation of a new *S*-allele

The use of the consensus primers AS1II and AmyC5R do not always distinguish alleles with a similar number of nucleotides, such as  $S_5$  and  $S_{10}$  (López et al., 2004; Kodad et al., 2008a) and  $S_3$  and  $S_{25}$  (Kodad et al., 2008a), where the bands of the two alleles appeared very close under standard agarose gel electrophoresis. However, the application of the restriction digestion of PCR fragments with different enzymes allowed the distinction between  $S_5$  and  $S_{10}$ , allowing to assign  $S_{10}$  to 'Garondès' (Kodad et al., 2008a). When the same procedure was applied in the present study,  $S_{10}$



**Fig. 1.** *S*-genotype identification in 10 almond cultivars amplified with the primer pair AS1II and AmyC5R. (1) 'Abizanda', (2) 'Desmayo Langueta', (3) 'Coop', (4) 'Bulbuenta', (5) 'Ferragnès', (6) 'Primorskij', (7) 'Zahaf', (8) 'Ramillete', (9) 'Alzina', (10) 'Garondès' and (M) 1 kb ladder (Fermentas).

S <sub>f</sub> a Alzina	VQQWPF	PTICRFSGK-PSNNRRPLPI	FTIHGIWPS	NYSNPRMRSNCTGS	QFK-KILSPRLR	SKLERAWPDVESGNDTKFWED	EWNKHGKCS	EQTLNQM
S <sub>f</sub> Cambra	VQQWPF	PTICRFSGK-PSNNRRPLPI	FTIHGIWPS	NYSNPRMRSNCTGS	QFK-KILSPRLR	SKLERAWPDVESGNDTKFWED	EWNKHGKCS	EQTLNQM
S <sub>36</sub> Alzina	VQQWPF	PTICRVR IKKPCS NRPRLQY	FTIHGLWPG	NYSNPTKPSNCTGI	KFDDRKYVPHMR	SKLKLISWPDVESGNDINFWER	EWNKHGTC	ERILNQF
S <sub>5</sub> P. webbii	VQQWPF	PTICRVR IKKPCS NRPRLQY	FTIHGLWPG	NYSNPTKPSNCTGI	KFDDRKYVPHMR	SKLKLISWPDVESGNDINFWER	EWNKHGTC	ERILNQF
S <sub>a</sub> Mission	VQQWPF	PTICAVSKQPGSQN--PPSI	FTIHGLWPS	NYSKKGAVNCTRT	RFN-NSLAPKLE	AKLKLISWPNVFNAYTEFWER	EWNKHGTC	EQTLNQF
S <sub>10</sub> D.largueta	VQQWPF	PTICAVRNMPCYQN--PPSI	FTIHGLWPS	NYSKYSWVNCPTI	LFN-NSLPRIE	AKLKLISWPNVFNAYTEFWER	EWNKHGTC	EHTLQK
		C1	C2		RHV		C3	
S <sub>f</sub> a Alzina	QYFERSHQWNSF	NIINILEKASIVP	NATQIWTYSDILS	PIKAATQR	IPLLRCKGNPQRQAKSQ	EKNRGSQ	PKSQAT	TQ
S <sub>f</sub> Cambra	QYFERSHQWNSF	NIINILEKASIVP	NATQIWTYSDILS	PIKAATQR	IPLLRCKGNPQRQAKSQ	EKNRGSQ	PKSQAT	TQ
S <sub>36</sub> Alzina	QYFERSYEMWRSH	NIITEILKNASIVP	HPTQIWTYSDIVS	PIKAATKR	PLLRCKYDRN	-----	-----	TQ
S <sub>5</sub> P. webbii	QYFERSYEMWRSH	NIITEILKNASIVP	HPTQIWTYSDIVS	PIKAATKR	PLLRCKYDRN	-----	-----	TQ
S <sub>a</sub> Mission	EYFQRSHDIWNAV	NIINFKKANILP	NG-AIWNYSYDIVS	PIKTVIRKMPALRCK	PKDPT	KEKHNK	-----	IS
S <sub>10</sub> D.largueta	QYFRSHDIWNAV	NIINILEKASIVP	NG-AKWYSDIVS	PIKIVIRKMPALRCK	PKDPT	LKPNNS	-----	MS
		RC4						C5

**Fig. 2.** Multiple alignment of the deduced amino acid sequence of the *S*-alleles included in the studied cultivars. The conserved regions (C1–C5) and the hypervariable region (RHV) described in Rosaceae (Ushijima et al., 1998) are boxed. EMBL/GenBank accession numbers are as follows: *S*<sub>6</sub> of 'Alzina' (FJ877783); *S*<sub>7</sub> of 'Cambra' (EU684318); *S*<sub>36</sub> of 'Alzina' (FJ876154); *S*<sub>5</sub> of *Prunus webbii* (DQ993664); *S*<sub>a</sub> of 'Mission' (AF148465); and *S*<sub>10</sub> of Desmayo Largueta (AM232659).

was also assigned to 'Alzina' (data not shown). Surprisingly, the deduced amino acid of the genomic DNA sequence from the C1 to C5 regions of the supposed *S*<sub>10</sub> allele in 'Alzina' and 'Garondès' (Fig. 1), was different from that of the *S*<sub>10</sub> allele of 'Desmayo Largueta' (AM231659) (Ortega et al., 2006), and to *S*<sub>a</sub> allele from 'Mission' (AF148465) (Tamura et al., 2000), equivalent to *S*<sub>5</sub> in European nomenclature (López et al., 2004). Thus, the allele identified in 'Alzina' and 'Garondès' (Kodad et al., 2008a) is considered a new allele and called *S*<sub>36</sub>. The partial sequences of *S*<sub>36</sub> from 'Alzina' and 'Garondès' have been deposited in the database with accession numbers FJ876154 and FJ876155, respectively.

The length of the DNA sequences from C1 to C5 of *S*<sub>5</sub>, *S*<sub>10</sub>, and *S*<sub>36</sub> was 584, 580 and 583 bp (Fig. 1). The length of the second exon of *S*<sub>36</sub> was 165 bp, thus nine nucleotides longer than those of *S*<sub>5</sub> and *S*<sub>10</sub> (156 bp) (data not shown). The length of the third exon of *S*<sub>5</sub> and *S*<sub>10</sub> was 22 nucleotides longer than that of *S*<sub>36</sub> (322 bp). The length of the second intron of *S*<sub>36</sub> was determined from the DNA sequence and compared to *S*<sub>5</sub> and *S*<sub>10</sub>. The intron of *S*<sub>36</sub> (95 bp) was 10 nucleotides longer than that of *S*<sub>a</sub> (85 bp) and 15 than that of *S*<sub>10</sub> (80 bp). Moreover, many indels and nucleotide differences were observed. The existence of such differences agrees with the hypothesis that new *S*-alleles may be generated by the accumulation of point mutations (Després et al., 1994). Ortega et al. (2006) suggested that the *S*-allele diversity is not only attributable to point mutations, but also to intragenic recombination near the intron, since Gilbert et al. (1997) pointed out that introns may be 'hot spots' for meiotic crossing-over.

The alignment of the deduced amino acids allowed comparison of the *S*<sub>36</sub>-allele with the alleles sequenced in almond and deposited in genebank databases, showing that *S*<sub>36</sub> from 'Alzina' and 'Garondès' is similar to, but different from, all other *S*-alleles identified in almond (data not shown). The deduced amino acid sequence of *S*<sub>36</sub> is 52.5% and 56.2% similar to that of *S*<sub>5</sub> and *S*<sub>10</sub>, respectively.

The identity and the similarity of the *S*<sub>36</sub>-allele with other *Prunus S*-RNases, showed that this allele was identical to *S*<sub>10</sub> (DQ993664) (Fig. 2) from *P. webbii* (Spach) Vierh. originating from Spain (Sánchez and Oliveira, 2005). Kodad et al. (2008b) reported that the *S*<sub>1</sub>, *S*<sub>33</sub> and *S*<sub>7</sub>-alleles in almond to be identical to *S*<sub>6</sub>, *S*<sub>8</sub> and *S*<sub>7</sub> identified in different ecotypes of *P. webbii* (Sánchez and Oliveira, 2005; Bošković et al., 2007). All these results support the hypothesis of the possibility of the introgression of these alleles by hybridisation from *P. webbii* into almond (Socias i Company, 2004) or that these alleles could be inherited from a common ancestor (Kodad et al., 2008b).

### 3.3. Cross-incompatibility of 'Alzina' and 'Garondès'

When 'Alzina' and 'Garondès' were cross-pollinated, pollen tubes were arrested in the middle third part of the style, indicating that these cultivars are cross-incompatible. Thus, a new CIG

including these two cultivars is proposed and named CIG XXVIII and added to those proposed by Kodad and Socias i Company (2009).

### 3.4. Conclusion

All approaches taken for determining the compatibility relationships in 'Alzina' and 'Garondès', such as pollen tube growth, fruit set after self-pollination, PCR analysis and sequencing of the *S*-RNase, confirmed their self-incompatibility. These results stress that the combination of both the molecular (PCR and sequencing analysis) and the physiological approaches is required to fully evaluate self-(in)compatibility in almond as well as the identity of new alleles.

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