The Expression of Self-compatibility in Almond May Not Only Be Due to the Presence of the S_f Allele

Ossama Kodad and Rafel Socias i Company¹

Unidad de Fruticultura, Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA),
Av. Montañana 930, 50059, Zaragoza, Spain

Ana Sánchez

Instituto de Tecnologia Química e Biológica (ITQB), Quinta do Marquês, 2784-505 Oeiras, Portugal

M. Margarida Oliveira

Instituto de Tecnologia Química e Biológica (ITQB), Instituto de Biologia Experimental e
Tecnológica (IBET), Quinta do Marquês, 2784-505 Oeiras, Portugal, and Universidade de Lisboa,
Faculdade de Ciências, Dep. de Biologia Vegetal, Campo Grande, 1749-016 Lisbon, Portugal

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ABSTRACT. The pistil (S -RNase) and the pollen [S -haplotype-specific F-box protein (SFB)] components of the S_f allele, presumably conferring self-compatibility in almond (*Prunus amygdalus* Batsch [syn. *P. dulcis* (Mill.) D.A. Webb]), were identified and sequenced in ‘Ponç’, a local Spanish almond cultivar, confirming their identity with the published sequences of these components. Despite the presence of the S_f allele, the ‘Ponç’ phenotype was self-incompatible as confirmed by different pollination tests, including self pollen tube growth, fruit set after self-pollination, and fruit set in bagged branches. However, the pistil and the pollen of ‘Ponç’ were fully viable when pollinated by a cross-compatible pollen or used on a cross-compatible pistil. The fact that ‘Ponç’ presents two different S -proteins with RNase activity may indicate an active function of its S_f -RNase, whereas in the self-compatible almond cultivars thus far studied, the S_f -RNase has been inactive. This activation indicates that the presence of the S_f allele may not be the exclusive source of self-compatibility in almond, and other factors may also be involved in the expression of almond self-compatibility.

Self-incompatibility (SI) is the ability of a fertile hermaphrodite flowering plant to prevent self-fertilization by discriminating between self and nonself pollen. In almond, as well as in other *Prunus* L. species, the SI system is of the gametophytic type (Socias i Company et al., 1976) and is controlled by a single S locus with multiple alleles (Crane and Lawrence, 1929). To ensure that flowers may be efficiently pollinated to reach an economically acceptable fruit set (Kester and Griggs, 1959), insect-dependent cross-pollination of intercompatible and simultaneously blooming cultivars is required. As an alternative to SI cultivars, self-compatible (SC) ones may be used to expand monovarietal orchards (Socias i Company, 1990).

To breed SC cultivars, it is important to understand the molecular mechanisms underlying this behavior. The S locus produces in the pistil a basic glycoprotein with ribonuclease (S -RNase) activity (McClure et al., 1989). This protein is taken up by the growing pollen tubes, causing their subsequent arrest in the style and thus preventing self-fertilization by its own pollen (Kao and Tsukamoto, 2004). The expression of SC in almond is attributed to the presence of the S_f allele, whose expression is

dominant over the other alleles of the S series (Socias i Company, 1984). Bošković and Tobutt (1996) reported that in cherry (*Prunus avium* L.), the S alleles code for stylar ribonucleases that can be detected by electrophoretic separation of stylar proteins and subsequent staining for activity. In almond, Bošković et al. (1999, 2003) applied the analysis of stylar S -RNases by nonequilibrium pH gradient electrofocusing (NePHGE) to detect SC seedlings in almond progenies from crosses in which one parent was SC. In these analyses, SC cultivars only showed one band with RNase activity. Additional studies carried out at the genetic level allowed the partial sequence of the S_f allele gene associated with S -RNase of SC in almond to be obtained (Channuntapipat et al., 2001; Ma and Oliveira, 2001).

The origin and the mechanism of almond SC are still unknown. Two hypotheses have been put forward to explain how SC appeared. The first hypothesis suggests a natural mutation in the SI system (Crossa-Raynaud and Grasselly, 1985), and the second suggests a gene transfer through spontaneous interspecific hybridization between *Prunus amygdalus* and *P. webbii* (Vierh.) Spach (Socias i Company, 2004). Independent of its origin, it is accepted that the loss of activity of an S -RNase in the style is the possible reason for SC in almond (Bošković et al., 1999). Supporting the previous suggestions from Bošković et al. (1999), Hanada et al. (2009) have reported that the possible origin of SC in almond may be due to the lack of or to the very low level of the transcription of the S -RNase in the pistil. However, Bošković et al. (2007) have suggested another possible origin for ‘Tuono’ SC—a mutation in the C2 region from histidine to arginine. Both hypotheses

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¹Corresponding author. E-mail: rsocias@aragon.es.

could explain the absence of RNase activity repeatedly observed in SC almond genotypes (Alonso and Socias i Company 2005; Bošković et al., 1999).

In other studies, Ushijima et al. (2003) sequenced, for the first time, the pollen *S* haplotype termed F-Box (SFB), finding that this could be a good candidate for the pollen *S* product because it was confirmed to be specifically expressed in the pollen tube and to be physically linked to the *S*-RNase gene (Entani et al., 2003; Ikeda et al., 2005). SFB features such as pollen-specific expression, tight linkage with the *S*-RNase gene, a high level of allelic polymorphism, and the presence of regions under positive selection are consistent with this being the pollen determinant in *Prunus* (Entani et al., 2003; Ikeda et al., 2004, 2005; Yamane et al., 2003). Hanada et al. (2009) have sequenced the SFB_F in SC 'Lauranne' and found the same sequence as did Bošković et al. (2007) in SC 'Tuono' and in SI 'Cinquanta Vignali' and 'Fra Giulio Grande'.

Recently, Kodad et al. (2008) identified the *S* genotype of 39 cultivars in the almond germplasm bank of Zaragoza, Spain, using the PCR approach, combining different primers. Several Spanish local cultivars from the island of Majorca, including Ponç, were found to possess the consensus *S_F* allele as sequenced by Channuntapipat et al. (2001), considered to confer the SC trait in almond. This cultivar presents high productivity, good nut quality, medium blooming time, and good behavior under drought conditions (Rubí, 1980). The confirmation of SC in 'Ponç' would also open new horizons in almond breeding programs as an alternative source of SC other than 'Tuono' and closely related cultivars from the Italian region of Puglia (Socias i Company, 2002). This alternative would avoid the problems related to inbreeding in the expression and transmission of SC in almond, which presumably results from lethal and deleterious genes that also affect some horticultural traits, including vigor, bud density, and plant size (Alonso and Socias i Company, 2007).

The aim of this work was to confirm the molecular identity of the *S_F*-RNase and SFB_F of 'Ponç' by genomic DNA sequencing and by comparison with the *S* genotype of other SC cultivars. This study was complemented by assessing pollen tube growth and fruit set, after controlled artificial self-pollination and bagging branches at bloom, as an indicator of phenotypic expression of SC/SI.

Materials and Methods

PLANT MATERIAL. Nine almond genotypes were studied. Four of these, 'Ponç', 'Desmayo Largueta' [*S_FS₁₀* (Ortega et al., 2006)], 'Marcona' [*S₁₁S₁₂* (Bošković et al., 1999)], and 'Bertina' [*S₆S₁₁* (Bošković et al., 1999)] are local Spanish cultivars, and three, 'Soleta' (*S_FS₂₃*), 'Cambra' (*S_FS₃*), and selection G-2-22 [*S_FS₆* (Kodad and Socias i Company, 2008; Kodad et al., 2008)], are releases from the Centro de Investigación y Tecnología Agroalimentaria de Aragón (CITA) breeding program. 'Tuono' [*S_FS₇* (Cossa-Raynaud and Grasselly, 1985)] is the most used SC cultivar in breeding programs, and 'Ferragnès' [*S_FS₃* (Cossa-Raynaud and Grasselly, 1985)] is a release from the Institut National de la Recherche Agronomique, France (INRA) breeding program. All plant samples were obtained from the Spanish almond germplasm collection located at CITA, maintained as living plants grafted on the almond × peach [*Prunus persica* (L.) Batsch] hybrid clonal rootstock INRA GF-677 using the standard management practices (Espiau et al., 2002).

DNA EXTRACTION. Genomic DNA was extracted from leaves following the CTAB extraction method based on Doyle and Doyle (1987). For PCR amplification, DNA was diluted to 20 ng·μL⁻¹ in water.

***S*-RNASE AND SFB ALLELES PCR AMPLIFICATION.** To confirm the identity of the *S* alleles of these cultivars, the *S* alleles were amplified according to Tamura et al. (2000), using the primers AS1II and AmyC5R designed for the C1 and C5 conserved regions of almond *S*-RNase alleles. The regions between the signal peptide and the conserved region C5 of the *S_F* allele of 'Cambra' and the putative *S_F* allele of 'Ponç' were amplified according to Ortega et al. (2006) using the forward primer PaConsI-F (Sonneveld et al., 2003) and the reverse primer EM_PC5consRD (Sutherland et al., 2004). PCR reactions were performed in a volume of 30 μL containing 1× PCR buffer (Promega, Madrid, Spain), 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 0.5 μM of each primer, one unit of Taq DNA polymerase, and 75 ng of genomic DNA. The PCR program for *S*-RNase allele identification consisted of an initial denaturation of 2 min at 94 °C, followed by 34 cycles of 1 min at 95 °C, 2 min at 50 °C, and 4 min at 72 °C followed by a final extension of 10 min at 72 °C. A fragment of the SFB locus was PCR-amplified with primers SFB-C1F and SFB-FB3 (Yamane et al., 2003), SFB-C2F (Ikeda et al., 2004), and F-BOX3A' (Vaughan et al., 2006). About 50 ng of genomic DNA was used for PCR amplification in 50 μL of reaction mixture containing 1× PCR buffer, 1.5 mM of MgCl₂, 200 μM each of dNTPs, 400 μM of each primer, and one unit of Taq DNA polymerase. For the SFB haplotype amplification, the PCR reactions were run with a program of 35 cycles at 94 °C for 1 min, 54 °C for 1 min, and 72 °C for 1 min 30 s, with initial denaturing at 94 °C for 3 min and a final extension of 72 °C for 10 min.

The PCR products were separated on 1% agarose gels containing 1× TAE buffer and were stained with ethidium bromide (0.4 μg·mL⁻¹). DNA bands were visualized under ultraviolet light and the images were registered using Gel Doc 2000 (Bio-Rad, Hercules, CA) with Quantity One software (version 4.0.1; Bio-Rad). GeneRuler™ DNA Ladder Mix (Fermentas, Porto, Portugal) was used as the molecular size standard.

CLONING AND gDNA SEQUENCING. For the SFB haplotype, the cloning of the target sequences was done directly from the PCR product after checking the presence of the amplification on agarose gel. For the *S*-alleles, before cloning, the bands corresponding to the target *S* alleles were purified using the Wizard Plus Miniprep DNA Purification System (Promega) and were quantified on a 1.5% agarose gel using a standard 1-kb DNA ladder (Invitrogen, Madrid, Spain). The purified PCR products were cloned into the vector pCR2.1 using a TA Cloning Kit (Invitrogen). Insertion was confirmed by restriction enzyme digestion with *Eco*RI. Plasmids were isolated using the QIA prep Spin Miniprep Kit (Qiagen, Hilden, Germany). For each allele, at least three plasmids from different PCR reactions were sequenced from both ends.

STYLAR *S*-RNASE ANALYSIS. *S*-genotype of 'Ponç' was also determined by separation of stylar RNases linked to SI alleles by NEpHGE as described by Bošković and Tobutt (1996), using as reference the other cultivars included in this study.

POLLINATION TESTS. Physiological SI in 'Ponç' and 'Cambra' was studied with three different methods: pollen tube growth, fruit set after artificial hand pollination, and fruit set after bagging. During two consecutive years (2006–07), 50 flowers

at stage D (Felipe, 1977) were collected from trees growing in the field and taken in plastic bags to the laboratory, emasculated, and placed in trays with tap water. Their pistils were self- or cross-pollinated with 'Marcona' pollen as a control. Pollen was collected and its viability was checked before pollination (Kodad and Socias i Company, 2006). After pollination, trays were kept at room temperature and samples of 10 pistils were collected every 24 h after pollination, completing 120 h since pollination. The pistils were autoclaved in a 5% solution of Na₂SO₃ for 12 min at 1.2 kg·cm⁻². Self- and cross-compatibility was assessed by pollen tube growth after observation in a Leitz Ortholux II microscope (Leitz, Wetzlar, Germany) with ultra-violet illumination of a mercury lamp (Osram HBO 200 W/4; Torrejón de Arsoz, Madrid, Spain) by fluorescence of the callose deposits of the pollen tubes by aniline blue staining after squashing.

Eight branches per cultivar were selected in the field and at least 100 flowers were emasculated per branch and were self-pollinated. In other branches, the flowers were cross-pollinated with 'Marcona' and 'Desmayo Langueta' pollen. Another group of branches were bagged to evaluate the level of self-fertilization of 'Ponç'. Fruit set was recorded in June for all treatments.

Results

'PONÇ' S-RNASE ALLELE IDENTITY. The screening of the S-RNase allele diversity in the almond germplasm bank of Zaragoza using allele-specific and conserved primers has allowed the identification of the *S_f* allele in 'Ponç' (Kodad et al., 2008). This allele, using the AS1II and AmyC5R primers, corresponds to a band of size ≈1200 bp. The deduced amino acid sequence of the *S_f*-allele in 'Ponç' is identical to that of the first *S_f* allele (AY291117) amplified in almond from two SC genotypes, 'Lauranne' and Institut de Recerca i Tecnologia Agroalimentària (IRTA) selection 12-2 (Channuntapipat et al., 2001). However, it is only 98% identical to the *S_f* allele sequenced in 'Tuono' (AF157009) by Ma and Oliveira (2001) and 99.3% to that by Bošković et al. (2007; AM690356), who gave different sequences for the same allele. The other allele with a band of size ≈1700 bp is identical to *S₂₇* (AM231675; Ortega et al., 2006).

Using the forward primer PaConsI-F and the reverse primer EM_PC5consRD, a band of 1600 bp was amplified in 'Ponç'

and 'Cambra'. The sequencing of this *S_f* showed the typical feature of the *Prunus* T2-type RNase with five conserved domains (C1, C2, C3, RC4, and C5) and one hypervariable region (Fig. 1). The position of the two introns was also identical to that of other almond S-RNases reported by Ortega et al. (2006). The comparison of the sequences as the *S_f* allele of 'Ponç' at the g-DNA and at the deduced amino acid sequence levels showed the same sequence and position of the signal peptide and the first intron of the *S_f* allele identified in 'Cambra', a SC cultivar obtained from the cross of 'Tuono' × 'Ferragnès' (Socias i Company and Felipe, 1999). Thus, the two alleles are identical and no alteration was observed in the primary structure of the encoded almond *S_f*-RNase.

The application of NEpHGE in 'Ponç' revealed two different S-RNase bands instead of one, as expected in SC genotypes (Bošković et al., 1999), corresponding to *S₂₇* and *S_f* (Fig. 2). The presence of two bands suggests that the two S-RNases of 'Ponç' are functional, including its *S_f*-RNase.

SFB IDENTITY IN 'PONÇ'. When the SFB-C1F and SFB-FB3 primers were tested in 'Cambra' (*S_fS₃*) and 'Ferragnès' (*S_fS₃*), DNA sequencing revealed two different kinds of clones; however, just one kind of clone was found in 'Ponç'. The deduced amino acid sequence from the partial genomic DNA of the SFB₁ and SFB₃ identified in 'Ferragnès', SFB₁₁ and SFB₁₂ in 'Marcona', SFB₃ and SFB_f in 'Cambra', and SFB_f in 'Ponç' contained a partial sequence of the F-Box motif determined in other SFB S-haplotypes (Ushijima et al., 2003), and two variable regions V1 and V2 (Ikeda et al., 2004).

For 'Ponç' and 'Cambra', the use of SFB-C2F and F-BOX3A' primers allowed the sequencing of the hypervariable regions HVa and HVb (Ikeda et al., 2004), located downstream to the V2 region (Fig. 3). The deduced amino acid of the SFB_f sequence identified in 'Ponç' was identical to that sequenced in 'Tuono' (AM711126; Bošković et al., 2007) and in 'Lauranne' (AB361036; Hanada et al., 2009).

The partial sequences of SFB₃, SFB₁₂, and SFB_f have been deposited in the database with accession numbers EU293149, EU310402, and EU310402, respectively.

POLLINATION TESTS. After confirming the identity of the *S_f*-allele RNase and of the SFB S-haplotype of 'Ponç' with those of other SC cultivars, pollination tests were conducted to confirm 'Ponç' SC. Pollen viability of 'Ponç' was tested before pollination, showing a high percentage of germination in vitro

Fig. 1. Multiple alignment of the deduced amino acid sequence of the almond *S_f* allele identified in the present study and those deposited in the database. The conserved regions (C1–C5) and the hypervariable region (RHV) described in Rosaceae (Ushijima et al., 1998) are boxed. Conserved cysteine and histidine residues are shadowed. European Molecular Biology Laboratory Nucleotide Sequence database (EMBL/GenBank) accession numbers are as follows: *S_f* of 'Tuono' a (AM690356; Bošković et al., 2007); *S_f* of 'Tuono' b (AF157009; Ma and Oliveira, 2001); *S_f* of 'Cambra' (EU684318); *S_f* of IRTA selection 12-2 (AY291117; Channuntapipat et al., 2001); *S_f* of 'Ponç' (EU293146); and *S₃₀* of 'Fra Giulio Grande' (AM690361; Bošković et al., 2007).

Fig. 2. Zymogram of almond *S* alleles after NEpHGE showing two bands for 'Ponç' compared to a single band for the self-compatible 'Tuono' and selection G-2-22.

(≈92%). Pollen germination was also high in self-pollinated pistils in the laboratory, with similar numbers of pollen tubes in the upper third of the style compared with cross-pollinated pistils, with an average of 47 pollen tubes. Pollen tube growth was arrested in the middle third of the style in the self-pollinated pistils, and no pollen tubes were observed at the style base or in the ovary. Conversely, in the cross-pollinated pistils with pollen of 'Marcona' and 'Desmayo Langueta', more than three pollen tubes were observed at the style base. These results were consistent during the 2 years of study. In the field tests, no fruit set was obtained after hand self-pollination, but normal sets, according to the criteria of Kester and Griggs (1959), were obtained after cross-pollination with 'Marcona' and 'Desmayo Langueta' pollen, as well as after selfing 'Cambra' (Table 1). Furthermore, no fruit set was obtained in

bagged branches of 'Ponç' during the 2 years of study. The field pollinations by hand or in bagged branches were unaffected by adverse weather conditions in both years, as confirmed by the normal sets in the compatible crosses.

Discussion

S-RNASE AND SFB IDENTITY AND SELF-(IN)COMPATIBILITY EXPRESSION IN 'PONÇ'. The cloning and sequencing of the *S_f*-RNase allele in 'Ponç' (EU310402) showed that it was found to be identical to that of 'Cambra' (EU684318). As well, the deduced amino acid sequence (C1-C5) of the 'Ponç' *S_f*-RNase allele was identical to that determined here in 'Cambra' and to that of 'Lauranne' and the IRTA selection 12-2 (AY291117; Channuntapipat et al., 2001), reported to be derived from selfing 'Lauranne' (Bošković et al., 1999). Hanada et al. (2009) have also identified the partial sequence of the *S_f*-RNase allele in 'Lauranne' and found it to be identical to the AY291117 sequence. However, all of these sequences are different from that of 'Tuono' published by Ma and Oliveira (2001; AF157009) or by Bošković et al. (2007; AM690356). The former (AF157009) has valine instead of isoleucine and histidine instead of arginine in the C2 region (Fig. 1). The latter has histidine instead of arginine (Fig. 1). Taking into account that 'Cambra' and 'Lauranne' are both seedlings from 'Tuono', these cultivars must have inherited the identical *S_f*-RNase allele from 'Tuono', as well as the IRTA selection 12-2 from 'Lauranne'.

Separately, Bošković et al. (2007) have identified in the SI 'Fra Giulio Grande', also from the same Italian region of Apulia as 'Tuono', an *S*-RNase identical to our *S_f*-RNase of 'Ponç' and 'Cambra' and to *S_f*-RNase of 'Lauranne' and IRTA 12-2 (Channuntapipat et al., 2001). However, Bošković et al. (2007) reported for 'Tuono' a different sequence (AM690356), with a substitution of histidine by arginine in the C2 region from the consensus *S_f* sequence (Fig. 1). Thus, they suggested a new

SBF30_F.G.Grande	MAF	TLRKKEIMIDILVRLPAKSLVRLCTCKSWSDLIGSSSFVSTHL	HRNVRKHAHVYLLCLHHPNF	ERKDDPDDPYVEQE	FQWSLFSKETLEE
SBff_Tuono	MAF	TLRKKEIMIDILVRLPAKSLVRLCTCKSWDLIGSSSFVSTHL	HRNVRKHAHVYLLCLHHPNF	ERKDDPDDPYVEQE	FQWSLFSKETLEE
SBff_Lauranne	MAF	TLRKKEIMIDILVRLPAKSLVRLCTCKSWDLIGSSSFVSTHL	HRNVRKHAHVYLLCLHHPNF	ERKDDPDDPYVEQE	FQWSLFSKETLEE
SBff_Ponç	---	-----LVRFLCTCKSWSDLIGSSSFVSTHL	HRNVRKHAHVYLLCLHHPNF	ERKDDPDDPYVEQE	FQWSLFSKETLEE
F-box motif			V1		
SBF30_F.G.Grande	CSKLSHPGSGSTEHYVIYGSSNGLVCISDEILNFDSP	IIHNWNPVSKKFRTPPVSTINIMKFSHVALQGFH	PGVNDYKAVRMMRTNKNALAVEVYSLRT		
SBff_Tuono	CSKLSHPGSGSTEHYVIYGSSNGLVCISDEILNFDSP	IIHNWNPVSKKFRTPPVSTINIMKFSHVALQGFH	PGVNDYKAVRMMRTNKNALAVEVYSLRT		
SBff_Lauranne	CSKLSHPGSGSTEHYVIYGSSNGLVCISDEILNFDSP	IIHNWNPVSKKFRTPPVSTINIMKFSHVALQGFH	PGVNDYKAVRMMRTNKNALAVEVYSLRT		
SBff_Ponç	CSKLSHPGSGSTEHYVIYGSSNGLVCISDEILNFDSP	IIHNWNPVSKKFRTPPVSTINIMKFSHVALQGFH	PGVNDYKAVRMMRTNKNALAVEVYSLRT		
SBF30_F.G.Grande	DSWRMVEAIPPLKCTWQHHKGIFFNGVAYHII	IEKGPISFVSIFDSGSEEFEEFIAPDTICTSWDLCIDVYKEQIC	CLLKFYSCEEEIMDKI	DLW	
SBff_Tuono	DSWRMVEAIPPLKCTWQHHKGIFFNGVAYHII	IEKGPISFVSIFDSGSEEFEEFIAPDTICTSWDLCIDVYKEQIC	CLLKFYSCEEEIMDKI	DLW	
SBff_Lauranne	DSWRMVEAIPPLKCTWQHHKGIFFNGVAYHII	IEKGPISFVSIFDSGSEEFEEFIAPDTICTSWDLCIDVYKEQIC	CLLKFYSCEEEIMDKI	DLW	
SBff_Ponç	DSWRMVEAIPPLKCTWQHHKGIFFNGVAYHII	IEKGPISFVSIFDSGSEEFEEFIAPDTICTSWDLCIDVYKEQIC	CLLKFYSCEEEIMDKI	DLW	
			V2		
SBF30_F.G.Grande	VLQEKRWKQLCPFV	YPFNYYYGT	IGISIDNKLL	MLKRDDIRGQPDHL	LCDYDSKPTQQVLETGIKLATMKYGEIEFLFSLSY
SBff_Tuono	VLQEKRWKQLCPFV	YPFNYYYGT	IGISIDNKLL	MLKRDDIRGQPDHL	LCDYDSKPTQQVLETGIKLATMKYGEIEFLFSLSY
SBff_Lauranne	VLQEKRWKQLCPFV	YPFNYYYGT	IGISIDNKLL	MLKRDDIRGQPDHL	LCDYDSKPTQQVLETGIKLATMKYGEIEFLFSLSY
SBff_Ponç	VLQEKRWKQLCPFV	YPFNYYYGT	IGISIDNKLL	MLKRDDIRGQPDHL	LCDYDSKPTQQVLETGIKLATMKYGEIEFLFSLSY
HVa		HVb			

Fig. 3. Alignment of the deduced almond SFB_f amino acid sequences. The F-box motif, the variable regions V1 and V2, and the hypervariable regions HVa and HVb are boxed (Ikeda et al., 2004). European Molecular Biology Laboratory Nucleotide Sequence database (EMBL/GenBank) accession numbers are as follows: SFB30 of 'Fra Giulio Grande' (AM711127; Bošković et al., 2007); SFBf of 'Tuono' (AM711126; Bošković et al., 2007); SFBf of 'Lauranne' (AB361036); and SFBf of 'Ponç' (EU310402).

Table 1. Fruit set after artificial hand-pollination of almond cultivars in the field (100–150 flowers per treatment).

Pistil parent (and <i>S</i> genotype)	Pollen parent				
	Ponç	Cambra	Desmayo Largueta	Marcona	
	Fruit set (%)				
Ponç (<i>S_{fa}S₂₇</i>)	0	25.7	28.2	19.7	
Cambra (<i>S₃S_f</i>)	25.4	24.3	26.3	23.7	
Desmayo Largueta (<i>S₁S₁₀</i>)	20.2	27.3	0	23.5	
Marcona (<i>S₁₁S₁₂</i>)	29.7	33.1	32.2	0	

allele, *S₃₀*, (AM690361) present in ‘Fra Giulio Grande’, but not differing from the consensus *S_f* sequence, concluding that this *S₃₀* is the wild-type allele from which *S_f* was a stylar mutation called *S₃₀*[°] conferring SC in almond. This terminology is confusing because they attribute a new number (*S₃₀*) for an existing allele (*S_f*) and establish a new allele (*S₃₀*[°]) as a result of a possible missequencing. Consequently, the *S₃₀*-RNase allele identified by Bošković et al. (2007) and the *S_f*-RNase allele of ‘Ponç’ correspond to the *S_f*-RNase allele already sequenced in SC almond cultivars and selections (Channuntapipat et al., 2001).

The pollination tests showed that ‘Ponç’ is physiologically SI. However, although the growth of the self-pollen was arrested in ‘Ponç’ pistils, the good growth of a cross-compatible pollen showed that these pistils are able to sustain the tube growth of foreign pollen. In addition, the good germination of ‘Ponç’ pollen indicates its high viability, as well as the fruit set of ‘Marcona’ after hand pollination by ‘Ponç’ pollen. There were no available cultivars sharing the same *S* genotype as ‘Ponç’, therefore the functionality and ability of the pollen harboring the same *S*-genotype than ‘Ponç’ in reaching the style base could not be tested.

The ribonuclease activity of the *S*-RNases is shown to be essential for the SI reaction (Huang et al., 1994; Sassa et al., 1997). In almond, SC has been associated with the absence of a stylar RNase band (Bošković et al., 1999), as well as in Japanese pear (*Pyrus serotina* Rehd.) (Sassa et al., 1992). The fact that RNase activity is required for the function of the *S*-RNases, and that *S*-RNases are taken up by self- and nonself-pollen tubes, suggests that the *S_f*-RNase of ‘Ponç’ is functional and degrades the RNA of self-pollen. Bošković et al. (2007) have also identified three Italian SI almond cultivars, ‘Cinquanta Vignali’, ‘Fra Giulio Grande’, and ‘Santoro’, with the *S*-RNase allele identical to that found in ‘Ponç’ that showed ribonuclease activity. The ribonuclease activity of the *S_f*-RNase allele of ‘Ponç’ explains the results of pollen tube growth and fruit set when ‘Ponç’ was self-pollinated.

SC in ‘Cambra’, IRTA 12–2, and ‘Lauranne’ could be the result of a mutation or alteration in the coding region of the *S_f*-RNase gene, as mentioned in other species. In *Lycopersicon peruvianum* (L.) Mill., some spontaneous SC accessions have an amino acid substitution at one of the essential histidines in the catalytic domain of the *S*-RNase that leads to a complete loss of enzymatic activity (Kowiyama et al., 1994; Royo et al., 1994). In *Petunia inflata* R. Fries, the mutant *S₃* allele mutagenized by replacing the codon for His-93 with a codon

for asparagine, producing a mutant protein that does not exhibit any detectable ribonuclease activity (Huang et al., 1994). However, in the present study, no mutation of histidine to other amino acids was detected in the *S_f* allele in SC almond cultivars.

Recently, Tao et al. (2007) reported an inactive *S_{2m}*-RNase in several cultivars of peach and attributed the reduction of RNase stability to the replacement of a structurally important cysteine residue in the C5 region by tyrosine. In the present study, we have not completely sequenced the *S_f*-RNase clone, but the complete deduced amino acid sequence of *S_f*-RNase of IRTA selection 12–2 (AY291117) contains a cysteine residue.

The SC observed in some cultivars of several *Prunus* species has been reported to be due to a defective function of the pollen, as a result of a mutation, deletion, or insertion in the coding region of the SFB *S*-haplotype, such as in *P. avium* and *P. mume* Sieb. et Zucc. (Ushijima et al., 2004), *P. armeniaca* L. (Vilanova et al., 2006), and *P. persica* (Tao et al., 2007). However, the identity of the partial sequence of ‘Ponç’ SFB_f and that of ‘Lauranne’ (AB361036; Hanada et al., 2009) and ‘Tuono’ (AM711126; Bošković et al., 2007) indicates that there is no alteration in any of the two components of the *S_f* allele in ‘Ponç’, either in the pistil or the pollen parts. Moreover, the identity of SFB_f from ‘Lauranne’ and ‘Tuono’, and of SFB₃₀ (AM711127) from ‘Fra Giulio Grande’, a SI Italian cultivar (Bošković et al., 2007), indicates that the origin of SC in almond is not related to any alteration or mutation in the SFB gene in almond.

These results confirm that ‘Ponç’ and ‘Cambra’ in the present study, as well as ‘Lauranne’ (Hanada et al., 2009), share the same SFB_f allele but present a different phenotypic expression. As far as we know, this is the first time that an almond cultivar has been identified as possessing the *S_f* allele, associated with SC expression, but being phenotypically SI. Alonso and Socias i Company (2005) reported that some inbred SC genotypes, having ‘Tuono’ as a donor for the SC trait, in spite of the presence of the *S_f* allele, showed a SI phenotype, indicated by a very low number of pollen tubes at the base of low number of pistils. However, this was the result of the inbreeding depression observed in that population, whereas in the present work, all self-pollinated pistils showed the arrest of the pollen tubes in the middle part of the style, which is a different phenomenon. This *S_f* allele, showing ribonuclease activity and being expressed in ‘Ponç’, may be called *S_{fa}* (active *S_f*-allele).

HYPOTHESIS ON THE ORIGIN OF SELF-COMPATIBILITY IN ALMOND. It has been suggested that SC in almond could be explained by quantifying the transcript expression of the *S*-RNases in the style. Watari et al. (2007) reported that the low transcriptional level of the *Se*-RNase resulted in an insufficient *Se*-RNase accumulation in the pistils, thus conferring SC in Japanese plum (*Prunus salicina* Lindl.). Qin et al. (2006) have estimated the threshold amount of *S₁₂*-RNase required for pollen rejection in *Solanum chacoense* Bitt. and reported that an insufficient accumulation confers SC. In almond, SC has already been attributed to the lack of ribonuclease activity of the *S_f* allele in the pistil (Bošković et al., 1999). However, it is not known thus far whether the change affecting *S_f*-RNase expression in almond is at the transcriptional or translational level.

The absence of any alteration in the coding region of the *S_f* allele in the SI and SC cultivars indicates that the appearance of SC in almond could be due to a change outside the coding region or to the presence of a modifier gene. Yamane et al.

(2003) reported that the inactivation of the S_{6m} -RNase in *Prunus cerasus* L. is due to an insert of 2600 bp located upstream of the S -RNase coding sequence. Further analysis should be undertaken to ascertain if there is any insertion in the S_f allele from SC and SI almond cultivars that could explain the inactivation of the S_f allele in SC almond cultivars. In *Prunus pseudocerasus* Lindl., it has been suggested that SC could be caused by mutations in other genes critical for the SI reaction (Huang et al., 2008).

In conclusion, we have isolated and sequenced, for the first time, the coding sequence for S_f -RNase and partial SFB_f from two cultivars originated from two different geographic regions and showing a different phenotypic expression. The present results show that the coding region of the S_f gene may not be the exclusive origin of SC in almond. Further experiments involving the complete sequence of the S_f -RNase gene in SC and SI cultivars to confirm its identity and the expression analysis of the S_{fa} -RNase in 'Ponç' is required to determine the characteristics of the transcribed proteins of the expressed S_{fa} allele in almond.

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