Molecular characterization of five new *S* alleles associated with self-incompatibility in local Spanish almond cultivars

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Abstract. Almond is a highly heterozygous species with a high number of *S*-alleles controlling its gametophytic self-incompatibility system (GSI). In this work we have analysed Spanish local almond cultivars for *S*-RNase allele diversity. By cloning and sequencing five new *S*-RNase alleles were identified: S_{31} (804 bp) in 'Pou de Felanitx' and 'Totsol', S_{32} (855 bp) in 'Taiatona', S_{33} (1165 bp) in 'Pou d'Establiments' and 'Muel', S_{34} (1663 bp) in 'Pané-Barquets', and S_{35} (1658 bp) in 'Planeta de les Garrigues'. The high number of new alleles identified reveals the wide diversity of almond germplasm still existing and requiring characterisation, and points to the possibility of new findings by a wider study focusing on other provenances.

Keywords. *Prunus amygdalus* – *S*-RNases – Molecular characterisation – Breeding programme.

Caractérisation moléculaire de cinq nouveaux allèles-S liés à l'autoincompatibilité chez des cultivars locaux espagnols d'amandier

Résumé. *L'amandier est une espèce hautement hétérozygote avec un nombre très élevé d'allèles-S qui contrôlent son système d'autocompatibilité gamétophytique (GSI). Dans le présent travail on a analysé des cultivars locaux espagnols pour étudier la diversité des allèles de la S-RNase. Par clonage et séquençage, cinq nouveaux allèles ont été identifiés :* S_{31} *(804 bp) chez 'Pou de Felanitx' et 'Totsol',* S_{32} *(855 bp) chez 'Taiatona',* S_{33} *(1165 bp) chez 'Pou d'Establiments' et 'Muel',* S_{34} *(1663 bp) chez 'Pané-Barquets', et* S_{35} *(1658 bp) chez 'Planeta de les Garrigues'. Le nombre élevé de nouveaux allèles identifiés révèle la grande diversité encore existante chez le germoplasme de l'amandier et ayant besoin d'être caractérisée, montrant la possibilité d'entreprendre de nouveaux travaux plus approfondis avec des génotypes d'autres provenances.*

Mots-clés. Prunus amygdalus – S-*RNases* – *Caractérisation moléculaire* – *Programme d'amélioration.*

I – Introduction

In almond (*Prunus amygdalus* Batsch) fertilization is essential for fruit production because the commercial part of the almond fruit is a seed. However, most almond cultivars are, with few exceptions, self-incompatible (Socias i Company 1990). The determination of the *S*-genotype in almond cultivars is useful for orchard design by growers to ensure cross-pollination and a commercial crop level, as well as for parental choice for breeding programs. PCR-based strategies have been developed for identification of *S*-RNase alleles using genomic DNA (Tamura *et al*., 2000; Ushijima *et al*., 1998), resulting in the confirmation of the identity of many *S* alleles and the identification of new ones (López *et al*., 2006).

So far, 35 *S*-incompatibility alleles, in addition to the S_f self-compatibility allele, have been identified in almond using different molecular analysis, ribonucleases, PCR, and sequencing analysis. More than 154 almond cultivars have been genotyped (López *et al.*, 2006) and 19 cross-incompatible groups have been established (Ortega *et al.*, 2006). The use of consensus (Tamura *et al*., 2000) and specific (Channuntapipat *et al*., 2003; Ma and Oliveira, 2002) primers has allowed to identify the *S*-allele composition of a set of local Spanish cultivars and new releases from the CITA breeding programme (Kodad *et al*., 2007), genotyping 39 almond cultivars and detecting the presence of 2 new *S* alleles. Based on these results, we have extended this study to other cultivars, detecting three other potentially new almond *S*-RNases. In the present study was to characterize these five new *S* alleles reported.

II – Material and methods

1. Plant material

Seven local Spanish cultivars from various regions (Table 1) were surveyed. All plant samples were obtained from the Spanish almond germplasm collection located at the CITA, Zaragoza, maintained as living plants grafted on the almond × peach hybrid clonal rootstock INRA GF-677, using the standard management practices (Espiau *et al*., 2002).

Table 1. Almond local cultivars surveyed and *S* genotypes determined

Cultivar	Origin	*S* genotype†
'Pou de Felanitx'	Majorca Id., Balearic Ids.	$\boldsymbol{S_{31}}\,S_?$
'Totsol'	Majorca Id., Balearic Ids.	$S_8\boldsymbol{S_{31}}$
'Taiatona'	Majorca Id., Balearic Ids.	$\boldsymbol{S_{32}}\,S_?$
'Pou d'Establiments'	Majorca Id., Balearic Ids.	$S_{12}\boldsymbol{S_{33}}$
'Muel'	NE Spain, Zaragoza	$S_{22}\boldsymbol{S_{33}}$
'Pané-Barquets'	NE Spain, Lérida	$S_1\boldsymbol{S_{34}}$
'Planeta de les Garrigues'	NE Spain, Lérida	$S_{22}\boldsymbol{S_{35}}$

†New alleles are highlighted in Bold.

2. DNA Extraction and PCR amplification

Genomic DNA was extracted from leaves following the CTAB extraction method based on Doyle and Doyle (1987). The PCR to identify the *S* alleles of the different cultivars was carried out using universal primers [AS1II(F) and AmyC5(R)] according to Sánchez-Pérez *et al*. (2004) and specific primers according to Channunpupinat *et al.* (2003).

3. Cloning and gDNA sequencing

The putative new alleles were cloned from different cultivars (Table 1). PCR products were cloned into the vector pCR2.1 using the TA Cloning Kit (Invitrogen). The coding sequence from C1 to C5 of the cloned *S*-allele, were translated and the deduced amino acid sequences were aligned by the ClustalX method (Thompson *et al*., 1997) using MegAlign Software (DNASTAR, Madison, WI, USA).

III – Results and discussion

PCR amplification of the *S* alleles of the local cultivars (Fig. 1), using the consensus primers AS1II and AmyC5R, yielded two fragments of various sizes, except in the case of 'Totsol', and 'Taiatona', where a single allele was observed. Using the allele-specific primers available for almond it was possible to ascribe S_1 allele to 'Pané-Barquets' and S_8 to 'Totsol'. Additionally, in 'Pou d'Establiments', and 'Muel' and 'Planeta de les Garrigues' bands, with ≈1600 bp and ≈1450 bp appear to correspond to the S_{12} and S_{22}, respectively. Genomic DNA sequences from C1-C5 regions were obtained for each of the target alleles and deposited in the EMBL Nucleotide Sequences Database.

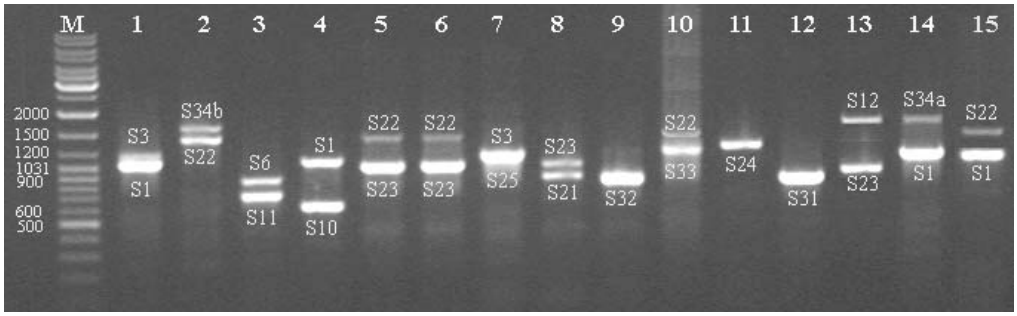

Fig. 1. PCR products of 15 almond cultivars, obtained with the primers AS1II and AmyC5R. Assessment of *S*-alleles was accomplished after band isolation, cloning and sequencing. Lanes: 1- 'Ferragnès'. 2- 'Planeta de les Garrigues'. 3- 'Bertina'. 4- 'Desmayo Largueta'. 5- 'Verdreta'. 6- 'Pau'. 7- 'Redonda de Palma'. 8- 'Tendra Amarga'. 9-'Taiatona'. 10- 'Muel'. 11- 'Mollar'. 12- 'Pou de Felanitx'. 13- 'Pou d'Establiments'. 14- 'Pané-Barquets'. 15- 'Tejada-2'. M: 1kb Ladder (Fermentas).

The sequences of the genes amplified in 'Pou de Felanitx' and 'Totsol' were identical, corresponding to a new allele identified in this study and named S_{31}. The bands with an approximate size of 850 bp sequenced from 'Taiatona' correspond to a new allele and named S_{32}. The sequencing of the fragments of approximately 1140 bp amplified in 'Muel' and 'Pou d'Establiments' has shown that there are different alleles with similar sizes. However, the band amplified in 'Pou d'Establiments' and 'Muel' is of 1165 bp and represents a new allele named S_{33}. The bands of 1660 bp amplified in 'Pané-Barquets' and 'Planeta de les Garrigues' were found to be 99.4% similar, and 98.9% similar to S_h (AF267510) (Ma and Oliveira, 2002). However, the length of the S_h allele was only 1389 bp long. The alignment of these three alleles (not shown) revealed a 251 bp insertion in the second intron of 'Pané-Barquets' and 'Planeta de les Garigues', at the position 400 from the dinucleotide splice donor. These alleles may thus be considered different from S_h.

The alignment of the deduced amino acids (Fig. 2) allowed the characterisation of the *S*-alleles of these cultivars based on the percentage of similarity between amino acid sequences. The deduced amino acids of the alleles of 1660 bp sequenced from 'Planeta de les Garrigues' and 'Pané-Barquets' are 99.4% identical, and each 98.4% and 99% identical to S_h respectively. Furthermore, the second intron of the allele sequenced from 'Pané-Barquets' was 5 bp longer than the second intron of 'Planeta de les Garrigues', and many indels and nucleotide differences were observed (not shown). Based on these results we can assume that these sequences correspond to two different new alleles. Thus, the allele sequenced from 'Pané-Barquets' was named S_{34} and that from 'Planeta de les Garrigues' S_{35}. The differences between S_{34}/S_{35} could be explained by point mutations and deletions occurring in the two sequences (not shown). Wünsch and Hormaza (2004) suggested that if two sequences have a similar intron length and

were exceptionally similar both at nucleotide and amino-acid levels, the two alleles could have derived from a common ancestral allele or one could have evolved from the other after several mutations. However, the high similarity or identity of two alleles does not mean that they have the same functionality.

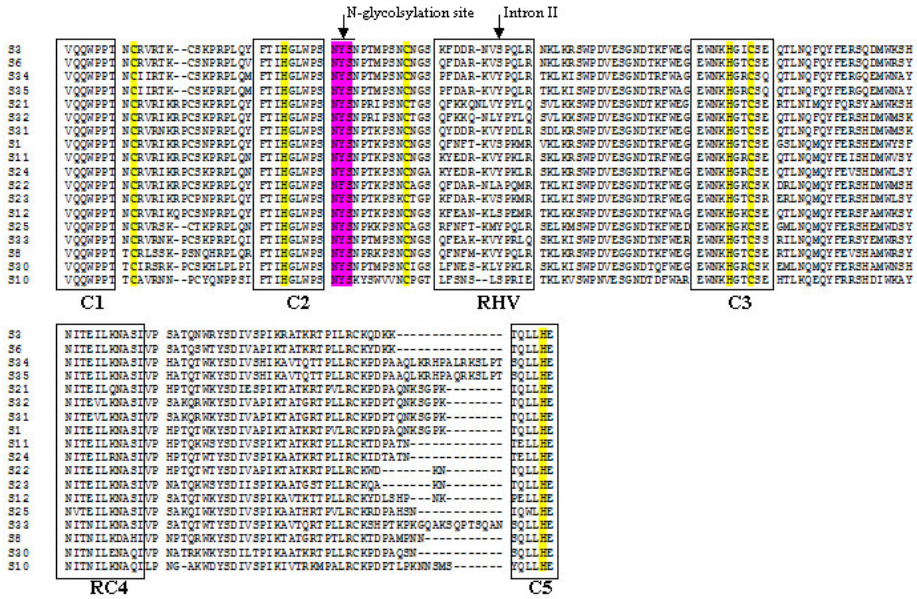

Fig. 2. Multiple alignment of the deduced amino acid sequence of the *S* alleles identified in the studied cultivars. The conserved regions (C1-C5) and the hypervariable region (RHV) described in Rosaceae (Ujishima *et al*., 1998) are boxed, and the positions of the second intron and the N-glycosilation site are indicated. Conserved cysteine and histidine residues are highlighted in yellow.

Two *S*-alleles with different specificity but with the same RHV have been recently reported in European pear (Zisovich *et al.*, 2004), suggesting that the RHV region by itself does not determine specific pollen rejection. The same could have occurred in almond with S_{34} and S_{35}. Fruit set evaluation after test cross pollinations in the field between the genotypes having common *S* alleles, as well as S_{34} and S_{35}, should be undertaken to confirm that these alleles are functionally identical.

A precise identification of the *S* genotype is essential in a breeding programme and for selection of cultivar combinations for productive orchards where self-compatible cultivars have not been introduced. The identification of five new almond *S*-RNases in 7 local cultivars not previously characterised raises the expectation of finding more alleles in almond, by enlarging the range of plants analysed.

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References

Channuntapipat C., Wirthensohn M., Ramesh S.A., Batlle I., Arús P., Sedgley M. and Collins G., 2003. Identification of incompatibility genotypes using specific primers based on the introns of the *S*-alleles. In: *Plant Breed*., 122, p. 164-168.

Doyle J.J. and Doyle J.L., 1987. A rapid DNA isolation procedure for small quantities of fresh tissue. In: *Phytochem. Bull*., 19, p. 11-15.

Espiau M.T., Ansón J.M. and Socias i Company R., 2002. The almond germplasm bank of Zaragoza. In: *Acta Hort*., 591, p. 275-278.

Kodad O., Sánchez A., Oliveira M. and Socias i Company R., 2007. *S*-allele genotyping of local Spanish almond cultivars. In: *XII Eucarpia Symposium on Fruit Breeding and Genetics*, Zaragoza (Spain), 16-20 September 2007.

López M., Vargas F.J. and Battle I., 2006. Self-(in)compatibility almond genotypes: A review. In: *Euphytica*, 150, p. 1-16.

Ma R.-C. and Oliveira M.M., 2002. Evolutionary analysis of *S*-RNase genes from Rosaceae species. In: *Mol. Gen. Genet*., 267, p. 71-78.

Ortega E., Bošković R., Sargent D.J. and Tobutt K.R., 2006. Analysis of *S*-RNase alleles of almond (*Prunus dulcis*): Characterization of new sequences, resolution of synonyms and evidence of intragenic recommendation. In: *Mol. Gen. Genet*., 276, p. 413-426.

Sánchez-Pérez R., Dicenta F. and Martínez-Gómez P., 2004. Identification of *S*-alleles in almond using multiplex PCR. In: *Euphytica*, 138, p. 263-269.

Socias i Company R., 1990. Breeding self-compatible almonds. In: *Plant Breed. Rev*., 8, p. 313-338.

Tamura M., Ushijima K., Sassa H., Hirano H., Tao R., Gradziel T.M. and Dandekar A.M., 2000. Identification of self-incompatibility genotypes of almond by allele-specific PCR analysis. In: *Theor. Appl. Genet.*, 101, p. 344-349.

Thompson J.D., Gibson T.J., Plewniak F., Jeanmougin F. and Higgins D.G., 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. In: *Nucleic Acids Res*., 25, p. 4876-4882.

Ushijima K., Sassa H., Tao R., Yamane H., Dandekar A.M., Gradziel T.M. and Hirano H., 1998. Cloning and characterization of cDNAs encoding *S*-RNases from almond (*Prunus dulcis*): primary structural features and sequence diversity of *S*-RNases in Rosaceae. In: *Mol. Gen. Genet*., 260, p. 261-280.

Wünsch A. and Hormaza J.I., 2004. *S*-allele identification by PCR analysis in sweet cherry cultivars. In: *Plant Breed*., 123, p. 327-331.

Zisovich A.H., Stern R.A., Shafir S. and Goldway M., 2004. Identification of seven *S*-alleles from the European pear (*Pyrus communis*) and the determination of compatibility among cultivars. In: *J. Hort. Sci. Biotechnol.*, 79, p. 101-106.