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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_f-allele and the identification of a new allele not previously described in almond, S_{36}. 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_f-allele is in its active form, S_{fa}. 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.

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 Tsukamato, 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 Largueta' 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 assignation 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, 'Bulbuente' (S_1S_5) (Kodad et al., 2008a) and 'Desmayo Largueta' (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 × 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/μl 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_f-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 Na_2SO_3 for 12 min at 1.2 kg 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 trough 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_f-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_f and the other presenting similar sizes than S_5 and S_{10} (Fig. 1). The presence of the S_f-allele was confirmed using the S_f-specific primers designed from the second intron of the S_f-allele of 'Tuono' (Channuntapipat et al., 2003). The sequencing of the S_f-allele amplified from 'Alzina' and 'Garondès' using the consensus primers PaConsI-F and EM_PC5-consRD confirmed its identity with the S_f-allele (Fig. 2). The alignment of the amino acid sequence of the S_f 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_f-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_f-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_{fa} 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}

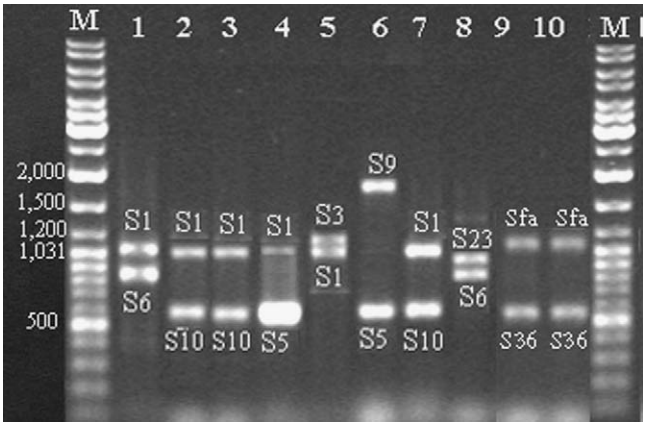

Fig. 1. *S*-genotype identification in 10 almond cultivars amplified with the primer pair AS1II and AmyC5R. (1) 'Abizanda', (2) 'Desmayo Largueta', (3) 'Coop', (4) 'Bulbuente', (5) 'Ferragnès', (6) 'Primorskij', (7) 'Zahaf', (8) 'Ramillete', (9) 'Alzina', (10) 'Garondès' and (M) 1 kb ladder (Fermentas).