***S*-RNase allele identification and incompatibility group assignment in apricot cultivars**

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Abstract

Apricot (*Prunus armeniaca*) is a member of the Rosaceae originated in China. Most of the European apricot cultivars have been traditionally considered self-compatible (SC) although *S*-RNase-based-Gametophytic Self-Incompatibility (GSI), a common incompatibility mechanism found in the Rosaceae and genetically determined by a locus (*S*) with multiple alleles, is common in the species. This locus encodes an allele-specific *S*-RNase, expressed in the style, which inhibits the growth of pollen tubes with the same *S* alleles. In the last years, an important renewal of plant material is taking place worldwide, with the introduction of new cultivars from different breeding programs. The use of self-incompatible (SI) parental genotypes has resulted in an increasing number of SI new cultivars with unknown pollination requirements. In order to establish the incompatibility relationships among apricot cultivars, in this work we perform a *S*-RNase allele identification in a group of 48 cultivars from different breeding programs. The *S*-alleles of each cultivar were determined by PCR amplification of the *S*-RNase gene. The results allowed allocating the cultivars in their corresponding incompatibility groups, a highly valuable tool for fruit growers to design apricot orchards, and for breeders to choose parental genotypes in breeding programs.

Keywords: *Prunus armeniaca*, self-incompatibility, *S*-genotype, *S*-alleles

INTRODUCTION

Apricot (*Prunus armeniaca* L.) was originated in Central Asia where it has been cultivated for millennia and from where it spread worldwide. Apricot cultivars can be classified in six main ecogeographical groups (Layne et al., 1996). The majority of apricot cultivars originated in Middle-Asian and Iranian-Caucasian groups are self-incompatible (SI), however, most of the European apricot cultivars have been traditionally considered self-compatible (SC) (Hormaza et al., 2007).

In the last years, the introduction of new cultivars from different breeding programs has provided an important renewal of plant material (Zhebentyayeva et al., 2012). Although most traditional apricot cultivars are self-compatible (Burgos et al., 1997), the use of self-incompatible parental genotypes in breeding programs (Hormaza et al., 2007) has resulted in the recent release of new self-incompatible cultivars, which need to be grown together with cross-compatible cultivars to ensure an appropriate yield. However, the pollination requirements of many of these new cultivars are unknown.

Self-incompatibility in Rosaceae is based on cell-cell recognition that is determined genetically by a Gametophytic Self-Incompatibility System (GSI) that acts through the inhibition of the pollen tube growth in the style. This mechanism is controlled by a multiallelic locus named *S*, encoding two linked genes that determine the pistil and pollen genotype (Charlesworth et al., 2005). A ribonuclease (*S*-RNase) determines the allele specificity of the

style (Tao et al., 1997) and an F-box protein (SFB) determines pollen allele specificity (Ushijima et al., 2003).

The identification of the *S-RNase* gene in apricot (Romero et al., 2004; Sutherland et al., 2004) allowed developing an *S*-allele genotyping PCR strategy, similar to those developed for cherry and almond (Sutherland et al., 2004). In the first PCR study of *S*-allele genotyping in apricot reported, seven self-incompatibility alleles (S_1 to S_7) and one allele for self-compatibility (S_c) were identified (Vilanova et al., 2005). Afterwards nine additional *S*-alleles (S_8 - S_{16}) were identified by Halász et al. (2005). These studies allowed the determination of different apricot *S*-genotypes from different countries (Halász et al., 2010; Kodad et al., 2013; Lora et al., 2017). Most of these *S*-alleles have been characterized using detection of *S*-locus genes by PCR fragment amplification and some of them have also been sequenced. The PCR-analysis can show little differences in the fragment size of some *S*-alleles, even if was carried out on an automatic sequencer (Lora et al., 2017).

In this work, we determine the *S*-allele identification of 48 apricot cultivars. The genetic identification of some *S*-alleles that could not be distinguished in previous works has been solved by a sequencing approach. Each cultivar has been assigned to its corresponding incompatibility group.

MATERIALS AND METHODS

Adult trees of 48 apricot cultivars from different collections and orchards of Aragón (Spain) were used, including traditional and new cultivars from different breeding programs (Table 1).

Genomic DNA was extracted from young leaves and used for *S*-allele genotyping following the protocol described by Hormaza (2002) and using the DNeasy Plant Mini Kit (Qiagen). For the PCR analyses, the primer pair SRc-F and SRc-R (Vilanova et al., 2005) was used to amplify the first intron of the apricot *S-RNase* in a PCR reaction of 15 μ L, containing 10x NH4 Reaction Buffer, 25 mM Cl_2Mg , 2.5 mM of each dNTP, 10 μ M of each primer, 100 ng of genomic DNA and 0.5 U of BioTaq™ DNA polymerase (Bioline, London, UK). The temperature profile used had an initial step of 3 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 3 min at 72°C, and a final step of 5 min at 72°C. For the amplification of the second intron, the primers Pru-C2 and Pru-C4R were used as recommended in Vilanova et al. (2005), but with the addition of 10 cycles and 55°C of annealing temperature as indicated in Sonneveld et al. (2003).

The PCR fragments were isolated using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel). Cloning was performed using CloneJET PCR Cloning Kit (Thermo) and by electroporation in *E. coli* Single-Use JM109 Competent Cells (Promega). The search for similarities was performed in NCBI blast (<http://www.ncbi.nlm.nih.gov/BLAST>, version 2.2.10).

RESULTS AND DISCUSSION

The *S*-alleles were characterized by using the primers SRc-F/SRc-R that allowed amplifying the first intron (Vilanova et al., 2005) and identifying eight alleles. Two of them, S_1 and S_7 , showed a similar fragment size of 400 bp and, in those cases, the primers Pru-C2/Pru-C4R could distinguish both alleles, showing a fragment size of 2260 bp for the S_1 allele or 900 bp for S_7 according to Vilanova et al. (2005). Primers Pru-C2/Pru-C4R were initially designed to amplify the second intron in *Prunus avium* (Tao et al., 1999).

In a previous study we identified the *S*-alleles of 40 apricot cultivars, some of them showing a 420 bp or 430 bp fragment that was assigned as S_x and S_y , respectively (Lora et al., 2017). These band sizes are close to the S_6 allele, which has been reported as 424 bp (Kodad et al., 2013) or 423 bp (Halasz et al., 2010). To elucidate if S_x (420 bp) and S_y (430 bp) correspond to S_6 or are different alleles, both fragments were cloned and sequenced, resulting

in a sequence of 414 bp for S_x and 421 bp for S_y . These results showed differences of several bp from the PCR fragment analysed by an automatic sequencer and the real sequence. Differences in fragment size were also found in the S_2 (334 bp herein, 327 bp in Vilanova et al., 2005; 332 bp in Kodad et al., 2013) and S_c (358 bp herein; 353 bp in Vilanova et al., 2005; 355 bp in Kodad et al., 2013) alleles.

The alignment of the S_y allele in the sequence database showed a 99% identity (100% cover) with an unpublished allele, S_{52} (KF951503.2). Since the S_6 allele has not been previously sequenced, allele S_{52} could indeed correspond to the S_6 allele. The S_6 allele could also be identified with the primers Pru-C2/Pru-C4 showing a PCR-fragment of around 1400 bp that includes the second intron (Vilanova et al., 2005). Thus, we amplified it using these primers. The PCR results showed a fragment of 1400 bp strongly suggesting that S_y , S_6 and S_{52} are the same allele. Moreover, the primers Pru-C2/Pru-C4 enclose a sequence of 1386 bp in the allele S_{52} . Thus, in this work, allele S_6 was assigned to S_y .

The sequence of the S_x allele showed sequence similarity to S -alleles, but not S -alleles of *Prunus armeniaca*. The second intron of this allele was amplified with the primers Pru-C2/Pru-C4, showing a PCR-fragment of around 700 bp. Its cloning, sequencing and alignment revealed a 99% identity (100% cover) with the allele S_9 (AY853594, Feng et al., 2006) and, consequently, the allele S_9 was assigned to the allele S_x . These results have enabled the identification of 12 cultivars that were previously identified with the allele S_x or S_y (Lora et al., 2017).

In this work, the S -*RNase* genotype has been analysed in a group of 48 apricot cultivars (Table 1). The Sc allele was identified in 13 cultivars, which were considered self-compatible (Vilanova et al., 2006). The cultivars 'Katy', 'Lorna' and 'Palsteyn' were also assigned to the self-compatible group, because they have been described as self-compatible although they did not exhibit the Sc allele (Zuriaga et al., 2013; Herrera et al., 2017; Lora et al., 2017). Although two alleles were identified in most cultivars, a unique allele was identified in 13 cultivars. Nineteen cultivars were assigned to seven incompatibility groups. Results herein showed differences in the S -*RNase* genotype of 'Palsteyn' (S_1S_2) and 'Lorna' (S_1S_2) reported in previous studies as S_1Sc (Raz et al., 2009) and S_2 (Donoso et al., 2009).

These results are valuable for the selection of parental genotypes in apricot breeding programs and for an appropriate distribution of pollenizer cultivars in commercial orchards. Moreover, these results underline the need for a sequencing approach for accurate S -allele identification that unifies PCR fragment size reports.

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Table 1. Incompatibility groups and *S*-genotype of 48 apricot cultivars.

I.G.	<i>S</i> -genotype	Cultivars analysed in this study
I	S_1S_2	Goldrich, Hargrand
III	S_2S_6	ASF0401, Bergarouge, Moniqui
VIII	S_6S_9	ASF0402, ASF0405, Orangered ¹ , Stark Early Orange, Wonder Cot
XVIII	S_2S_3	Mayacot, Sun Glo
XIX	S_2S_9	Goldstrike, Magic Cot
XX	S_3S_9	Durobar, Flodea, Henderson, Tsunami
XXI	S_7S_9	Goldbar
Self-compatible cultivars	ASF0404 (S_6), Bergecot (S_2S_6), Canino (S_2S_6), Charisma (S_7S_6), Faralia (S_6S_6), Flopria (S_9S_6), Kioto (S_6), Mitger (S_6), Paviot (S_2S_6), Pricia (S_3S_6), Soledane (S_6), Tadeo (S_6), Tom Cot (S_9S_6), Katy (S_1S_2), Lorna ¹ (S_1S_2), Palsteyn ¹ (S_1S_2)	
Unclassified	Aurora (S_9), Big Red (S_1), Early Queen (S_2), Golden Sweet (S_3), Harcot (S_4), JNP (S_9), Lilly Cot (S_3), Muñoz (S_2), Pandora (S_2), Perle Cot (S_9), Pinkcot (S_9), Veecot (S_2), Westley (S_2)	

¹Cultivars in which the *S*-RNase genotype reported herein differs from that reported in other studies.