Pollination requirements of new apricot (*Prunus armeniaca* L.) cultivars

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Abstract

Apricot (*Prunus armeniaca* L.) exhibits a gametophytic self-incompatibility system (GSI), the pollen-pistil recognition system present in the *Rosaceae* that prevents the self-fertilization. This mechanism is genetically controlled by a multiallelic locus (*S*), containing at least two linked genes that determine the pistil and pollen *S*-genotypes. Most of the traditional European apricot cultivars are self-compatible. However, the number of new commercial cultivars from different genetic backgrounds has increased in the last years. Most of these cultivars have been selected in several breeding programs for their tolerance to Sharka disease, caused by Plum Pox Virus, using self-incompatible cultivars selected in North America as parents. The pollination requirements of many of these new cultivars are unknown, and this can limit yield in the new orchards depending on the cultivars planted. In this work, the pollination requirements of 50 accessions have been determined, including 4 landraces, 36 commercial cultivars and 10 recent unregistered releases from different breeding programs. Self-(in)compatibility was analyzed by self-pollination experiments, followed by the observation of the pollen tube behavior through the pistil using fluorescence microscopy. The *S*-genotype was determined by PCR amplification of regions of the *S-RNase* and *SFB* genes and the self-incompatible cultivars were allocated to their corresponding incompatibility groups. The knowledge of pollination requirements is useful for apricot growers to select adequate pollinizers in commercial orchards, and for breeders to choose parental genotypes in breeding programs.

Keywords: *Prunus armeniaca*, self-incompatibility, *S*-genotype, pollen tube, fluorescence microscopy

INTRODUCTION

Gametophytic self-incompatibility (GSI) is a pollen-pistil recognition system to prevent self-fertilization and promote out-crossing present in the *Rosaceae* and other plant families (Silva and Goring, 2001). In GSI, self-incompatibility is genetically controlled by a multiallelic locus named *S* that contains two linked genes that determine the pistil and pollen *S*-genotypes. The *S-RNase* gene encodes pistil-expressed glycoproteins with ribonuclease activity that reject the pollen tube when the *S*-haplotype of the pollen matches either of the two *S*-alleles of the pistil (Tao et al., 1997). On the other hand, an F-box protein (SFB) is specifically expressed in the pollen grain and determines pollen allele specificity (Ushijima et al., 2003).

Apricot is a temperate fruit tree species that originated in Central Asia (Groppi et al., 2021). The majority of apricot cultivars belonging to the Middle-Asian and Iranian-Caucasian groups are self-incompatible (SI), but most of the European apricot cultivars have been traditionally considered self-compatible (SC) (Burgos et al., 1997; Hormaza et al., 2007). However, in the last decades many widespread commercial cultivars have been described as self-incompatible (Burgos et al., 1997; Milatovic et al., 2013b; Herrera et al., 2018a, b). With the purpose of introducing a source of resistance to Sharka disease in the new apricot releases, some incompatible cultivars that were developed in North America have been used as parents in several breeding programs (Zhebentyayeva et al., 2012; Krška, 2018). As a

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consequence, the pollination requirements of many new commercial cultivars from different genetic backgrounds are unknown, and this can limit yield in the new orchards depending on the cultivars planted (Hormaza et al., 2007).

Different approaches have been developed to determine self-(in)compatibility in apricot (Herrera et al., 2020). Fruit set recording after controlled pollinations in orchard conditions has been used to establish the pollination requirements in different cultivars (Burgos et al., 1993; Rodrigo et al., 2009). However, self- and cross-(in)compatibility can be evaluated faster and more reliably by the observation of pollen tube growth through the style in hand-pollinated flowers in laboratory conditions rather than by monitoring hand-pollinated flowers in the field until harvest. Pollinations in laboratory conditions avoid adverse environmental conditions and allows the analysis of a higher number of cultivars (Rodrigo and Herrero, 1996; Guerra and Rodrigo, 2015; Herrera et al., 2021).

Indeed, a molecular approach based on the study of the genetic determinants of self-incompatibility has enabled the characterization of the allelic composition of the *S* locus in apricot. To date, 33 *S*-alleles, including one allele for self-compatibility (*Sc*), have been identified (Halasz et al., 2005; Vilanova et al., 2005; Zhang et al., 2008; Muñoz-Sanz et al., 2017; Murathan et al., 2017). *S*-genotyping has allowed classifying the cultivars in their corresponding incompatibility groups according to their compatibility relationships. Up to now, 26 incompatibility groups have been described (Szabó and Nyéki, 1991; Egea and Burgos, 1996; Halasz et al., 2010; Lachkar et al., 2013; Herrera et al., 2018a, b).

In this work, we have determined the pollination requirements of 50 apricot accessions, including traditional and new cultivars released from different several breeding programs.

MATERIALS AND METHODS

Flower and leaf samples of 50 apricot accessions from different collections and orchards of Aragón (Spain) were used, including 4 landraces, 36 commercial cultivars registered in the Community Plant Variety Office (CVPO, 2022) and 10 recent unregistered releases from two breeding programs.

To explore self-(in)compatibility, self-pollinations were carried out in semi-in vivo culture of flowers in the laboratory, and subsequent observation of pollen tube growth under the fluorescence microscope (Herrera et al., 2020). As control, a set of flowers from each accession was cross-pollinated with pollen from 'Canino' or 'Katy', which are considered as universal pollinizers for apricot (Zuriaga et al., 2013), in order to ensure a cross-compatible reaction. In each accession and pollination treatment, 30 flowers at the balloon stage were emasculated and the pistils were maintained on wet florist foam. Pollen grains were sieved through a fine mesh from dehisced anthers previously placed on a piece of paper for 24 h at room temperature. Hand-pollinations were carried out with the help of a paintbrush for each self- pollination 24 h after emasculation. After 72 h, pistils were fixed in ethanol (95%)/acetic acid (3:1, v/v) for 24 h, and conserved at 4°C in 75% ethanol (Williams et al., 1999). In order to evaluate pollen viability, after each self- or cross-pollination pollen was scattered on a solidified pollen germination medium (Hormaza et al., 1996) and pollen germination was observed under the microscope 24 h later. Pollen grains were considered viable when the length of the growing pollen tube was higher than the pollen grain diameter. To determine self-(in)compatibility, pistils were stained with 0.1% (v/v) aniline blue in 0.1 N K_3PO_4 and the pollen tubes were observed under a fluorescence microscope (Linskens and Esser, 1957). Cultivars were considered as self-compatible when more than 70% of the pistils displayed at least one pollen tube reaching the base of the style. Self-incompatibility was considered in self-pollinated flowers when pollen tube growth was arrested along the style in more than 70% of the pistils observed.

Genomic DNA was extracted following the protocol described by Hormaza (2002) using the DNeasy Plant Mini Kit (Qiagen). For PCR analyses, the fluorescently labeled forward primer SRc-F with the reverse primer SRc-R (Vilanova et al., 2005) was used to amplify the first intron of the apricot *S-RNase* in a PCR reaction of 15 μL, containing 10x NH_4 Reaction Buffer (Bioline, London, UK), 25 mM Mg Cl_2, 2.5 mM of each dNTP, 10 μM 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 95°C, 35 cycles of 30 s at 95°C, 45 s at 54°C and 1 min 15 s at 72°C, and a final step of 10 min at 72°C. The sizes of the products obtained by PCR were analyzed in a CEQ™ 8000 capillary electrophoresis DNA analysis system (Beckman Coulter, Fullerton, CA, USA) and compared and classified according to the nomenclature established by Vilanova et al. (2005).

The second intron of the *S-RNase* was also analyzed using different primer combinations because similar fragment first-intron amplified sizes have been reported in two pairs of alleles (S_1 and S_7, S_6 and S_9) (Vilanova et al., 2005; Herrera et al., 2018a, b). To differentiate the S_6 and S_9 alleles the Pru-C2 and Pru-C4R primers were used (Tao et al., 1999) with the addition of 10 cycles and 55°C of annealing temperature (Sonneveld et al., 2003). SHLM1-2 and SHLM 3-4 specific primers were used to identify the S_1 and S_7 alleles, respectively (Herrera et al., 2018b). Amplified fragments of the second intron were separated on 1% (w/v) agarose gels and DNA bands were visualized using the nucleic acid stain SYBR Green (Thermo Scientific, St Leon-Rot, Germany).

The S_c and S_8 alleles exhibit an identical fragment length of 355 bp from the first intron when amplified using the SRc-(F/R) primers (Feng et al., 2006; Halasz et al., 2007). Thus, the *SFB* specific primers AprFBC8-F and AprFBC8-R were used to distinguish both alleles. The identification was carried out according to the PCR conditions of Halasz et al. (2010).

To compare the character of self-(in)compatibility between the group of self-compatible and the self-incompatible accessions in cross- and self-pollinations, the Mood's median test (Mangiafico, 2016) was performed using the function 'mood.medtest' from package 'RVAideMemoire' v. 0.9-81 (Hervé, 2021) using the R programming environment v. 4.1.0 (R Core Team, 2022).

RESULTS AND DISCUSSION

Thirty-three accessions were considered self-compatible (Table 1) since pollen tubes grew through the style and at least one pollen tube reached the base of the style in more than 70% of the pistils observed. Twelve accessions were reported as self-compatible for the first time. On the other hand, the pollen tubes stopped in the style without reaching the ovary in the pistils of 17 accessions that, consequently, were considered self-incompatible, nine of them for the first time (Table 1). Significant differences (p<0.01) were observed between self-compatible and self-incompatible accessions in the percentage of pistils with pollen tubes at the base of the style. However, no significant differences were found in the cross-pollinations between both groups (Table 1). Our results confirmed the self-incompatibility of 23 cultivars previously determined by the evaluation of the percentage of fruit set after self-pollinations in the field (Egea and Burgos, 1996; Burgos et al., 1997) or by the observation of pollen tube growth in pistils after self- and cross-pollinations (Egea and Burgos, 1996; Milatovic et al., 2013a,b; Herrera et al., 2018a). In addition, pollen germination in vitro showed that all the accessions analyzed had viable pollen.

The *S*-genotype was characterized in 29 accessions, but only one allele could be identified in 21 accessions (Tables 2 and 3). This could be due either to homozygosis in the case of the S_c allele in self-compatible accessions or to amplification problems in the other genotypes. Thus, the identification of additional *S-RNase* alleles in these genotypes requires additional work focused on characterizing the *S*-locus. The S_2 and S_3 alleles were detected by using the SRc-F/SRc-R primers that allowed amplifying the first *S-RNase* intron (Vilanova et al., 2005). In 20 accessions, the S_6 and S_9 alleles showed a similar fragment size for the first intron (Table 2), but they could be distinguished by using the Pru-C2/Pru-C4R primers, showing a fragment size of 1386 bp for S_6 (Vilanova et al., 2005; Herrera et al., 2018a) and 700 bp for S_9 (Feng et al., 2006; Herrera et al., 2018a). The specific primer pair SHLM1-SHLM2 allowed distinguishing the S_1 allele in 13 accessions. However, the S_7 allele, amplified with the primers SHLM3-SHLM4, was not found in any accession.

Table 1. Classification of 50 apricot cultivars into self- or self-incompatible cultivar groups.

	Pistils (%) with pollen tubes at the base of the style	
	Cross-pollination	**Self-pollination**
Self-compatible cultivars		
AC2	100	100
A01[a]	100	93
A02[a]	100	100
A03[a]	100	100
A04[a]	90	100
Apriqueen	100	91
Aprix 20	100	73
Aprix 33	100	100
Aprix 9	90	86
B03[a]	100	100
B04[a]	87.5	80
Berdejo	100	93
Faralia	100	100
Farbaly	100	92
Farbela	100	100
Farclo	100	100
Fardao	100	100
Farfia	100	100
Farius	100	100
Farlis	100	100
IBCOT 13-12[a]	100	100
IPS16121[a]	80	100
IPS20390[a]	90	76
IPS21345[a]	100	90
Kioto[a]	50	78.5
Mediva	100	89
Peñaflor	100	83.3
Pepito del Rubio	80	100
Playa Cot	100	100
Rubilis[a]	100	100
Sandy Cot	66.7	100
Swired	87.5	100
Tadeo	100	100
Self-incompatible cultivars		
A05[a]	100	0
A06[a]	75	0
Almater	100	5
Aurora	74	0
B01[a]	100	0
B02[a]	100	0
Flash Cot	66	0
Hargrand	88	14
Goldrich	93	0
IBCOT 18-2[a]	100	0
IBCOT 29-5[a]	70	0
JNP	87	5
Medaga	100	0
Ninja[a]	100	0
Robada	100	0
Samourai[a]	100	0
Tsunami[a]	100	10
Chi-squared	5.12	22.81
df	1	1
p-value	ns	1,78E-03

ns: non-significant values at p<0.01 significance level.

[a] Self-(in)compatibility reported herein for the first time.

Table 2. *S*-genotyping of apricot cultivars with four primer pairs used in this work.

Cultivar	SRc-(F/R) (bp)	PruC2-PruC4R (bp)	SHLM1-SHLM2 (bp)	AprFBC8-(F/R) (bp)	*S*-genotype
A01	334/408		650		S_1S_2
A02	355/420	700		500	S_9S_c
A03	355/420	700		500	S_9S_c
A04	334/355			500	S_2S_c
A05	334/420	700			S_2S_9
A06	420	700/1386			S_6S_9
AC2	355/420	700		500	S_9S_c
Almater	408/420	700	650		S_1S_9
Apriqueen	355			500	S_c
Aprix 20	355/420	1386		500	S_6S_c
Aprix 33	355			500	S_c
Aprix 9	355/420	1386		500	S_6S_c
Aurora	408/420	700	650		S_1S_9
B01	334/420	700			S_2S_9
B02	408/420	700	650		S_1S_9
B03	408		650		S_1
B04	408/420	700	650		S_1S_9
Berdejo	334/355			500	S_2S_c
Faralia	355/420	1386		500	S_6S_c
Farbaly	355			500	S_c
Farbela	355			500	S_c
Farclo	355			500	S_c
Fardao	355			500	S_c
Farfia	355			500	S_c
Farius	355			500	S_c
Farlis	355/420	1386		500	S_6S_c
Flash Cot	420	700/1386			S_6S_9
Goldrich	334/408		650		S_1S_2
Hargrand	334/408		650		S_1S_2
IBCOT 13-12	355			500	S_c
IBCOT 18-2	408		650		S_1
IBCOT 29-5	334				S_2
IPS16121	355			500	S_c
IPS20390	408		650		S_1
IPS21345	355			500	S_c
JNP	408/420	700	650		S_1S_9
Kioto	355			500	S_c
Medaga	408/420	700	650		S_1S_9
Mediva	334/355			500	S_2S_c
Ninja	420	700/1386			S_6S_9
Peñaflor	334/355			500	S_2S_c
Pepito del Rubio	334/355			500	S_2S_c
Playa Cot	355			500	S_c
Robada	355/420	1386		150	S_6S_8
Rubilis	408		650		S_1
Samourai	274				S_3
Sandy Cot	334/355			500	S_2S_c
Swired	355			500	S_c
Tadeo	355			500	S_c
Tsunami	274/420	700			S_3S_9

Table 3. Incompatibility group (I.G) and S-genotype of 50 apricot cultivars analyzed in this study and 26 reference apricot cultivars.

I.G.	S-genotype	Cultivars
I	S_1S_2	Goldrich[a], Hargrand[a], AC1[b]
II	S_8S_9	Perle Cot[b], Pinkcot[b]
III	S_2S_6	ASF0401[b], Bergarouge[b], Moniqui[b]
V	S_2S_8	Holly Cot[b], Sweet Cot[b]
VIII	S_6S_9	A06[c], Flash Cot[c], Ninja[c], ASF0402[b], Feria Cot[b], Orangered[b], Stark Early Orange[b], Sunny Cot[b], Wonder Cot[b]
XVIII	S_1S_3	Cooper Cot[b]
XIX	S_2S_3	Mayacot[b], Sun Glo[b]
XX	S_2S_9	A05[c], B01[c], Goldstrike[b], Magic Cot[b]
XXI	S_3S_8	Lilly Cot[b], Spring Blush[b]
XXII	S_3S_9	Tsunami[c], Almadulce[b], Flodea[b], Henderson[b]
XXIII	S_7S_9	Goldbar[b]
XXIV	S_1S_6	Primaya[b]
XXV	S_1S_9	Almater[c], Aurora[c], JNP[c], Medaga[c], B02[c]
XXVI	S_6S_8	Robada[c]
Self-compatible cultivars	A01 (S_1S_2)[c], A02 (S_9S_c)[c], A03 (S_9S_c)[c], A04 (S_2 S_c)[c], AC2 (S_9S_c)[d], Apriqueen (S_c)[d], Aprix 20 (S_6S_c)[d], Aprix 33 (S_c)[d], Aprix 9 (S_6S_c)[d], B03 (S_1)[c], B04 (S_1 S_9)[c], Berdejo (S_2S_c)[d], Faralia (S_6S_c)[d], Farbaly (S_c)[d], Farbela (S_c)[d], Farclo (S_c)[d] Fardao (S_c)[d], Farfia (S_c)[d], Farius (S_c)[d], Farlis (S_6S_c)[d], IBCOT 13-12 (S_c)[c], IPS16121 (S_c)[c], IPS20390 (S_1)[c], IPS21345 (S_c)[c], Kioto (S_c)[c], Mediva (S_2S_c)[d], Peñaflor (S_2S_c)[d], Pepito del Rubio (S_2S_c)[d], Playa Cot (S_c)[d], Rubilis (S_1)[c], Sandy Cot (S_2S_c)[d], Swired (S_c)[d], Tadeo (S_c)[d]	
Unclassified cultivars	IBCOT 18-2 (S_1)[c], IBCOT 29-5 (S_2)[c], Samourai (S_3)[c]	

[a] Our results confirmed the S-genotype proposed by Egea and Burgos (1996).

[b] Reference apricot cultivars published in Herrera et al. (2018a).

[c] Cultivar reported herein for the first time.

[d] Self-compatibility previously reported in Herrera et al. (2018a) confirmed with primers AprFBC8-F/AprFBC8-R.

The S_c allele was identified in 28 self-compatible accessions, confirming that this S-allele is associated to self-compatibility (Vilanova et al., 2006). The cultivars 'A01', 'B03', 'B04', 'IPS20390', and 'Rubilis' behaved as self-compatible but they did not exhibit the S_c allele. The cultivars 'Canino' or 'Katy' have been reported to exhibit the S_1- or S_2-haplotypes and overcome self-incompatibility barriers (Vilanova et al., 2006; Zuriaga et al., 2013). Some works have reported the existence of an additional mutation in the M-locus and not linked to the S-locus, which causes a loss of pollen S-activity (Vilanova et al., 2006; Zuriaga et al., 2012, 2013; Muñoz-Sanz et al., 2017). Thus, further analysis could reveal if self-compatibility found in those five cultivars is also related to the M-locus. The results of the PCR-based method confirmed the self-compatibility of 33 cultivars determined by the controlled pollinations including 21 cultivars previously published in Herrera et al. (2018a). On the other hand, the 14 self-incompatible accessions were assigned to 6 incompatibility groups (Table 3). Cultivars with the same S-alleles were assigned to the same incompatibility group and are therefore inter-incompatible. However, cultivars of different groups, carrying at least one different S-allele, could be considered as inter-compatible and may be used as pollinizers if they overlap in flowering times.

Knowing the pollination requirements of cultivars is important for apricot growers to select adequate pollinizers in commercial orchards, and for breeders to choose parental genotypes in breeding programs.

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