Scientia Horticulturae 185 (2015) 29–33

Contents lists available at ScienceDirect

Scientia Horticulturae

journal homepage: www.elsevier.com/locate/scihorti

Unilateral recognition of the S_f allele in almond

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ARTICLE INFO

Article history:
Received 21 November 2014
Received in revised form 15 January 2015
Accepted 17 January 2015
Available online 6 February 2015

Keywords:
Prunus amygdalus Batsch
Self-compatibility
Breeding
S_f allele
Allele expression
Allele recognition

ABSTRACT

The presence of two phenotypic expressions of the S_f allele in almond has raised the question of their possible interaction. As a consequence, several seedlings with homozygous S_fS_f genotype were obtained in order to combine the two forms of the S_f allele in the same genotype: the active form (S_{fa}) inducing self-incompatibility, and the inactive form (S_{fi}) inducing self-compatibility. The seedling genotype was determined by PCR amplification of genomic DNA with universal and specific primers and the phenotype by pollen tube growth. The results showed full self-incompatibility of the $S_{fi}S_{fa}$ genotypes as a result of the recognition of any kind of S_f pollen (S_{fi} or S_{fa}) by the style, where S_f-RNase is produced due to the presence of the S_{fa} allele. These results confirm the allelism of the S_f allele with the series of *S* alleles of self-incompatibility and that a mutation in the stylar part of the S_{fa} haplotype has led to the self-compatibility of the S_{fi} form. The recognition of the S_{fi} pollen by the S_{fa} style confirms that the presence of the S_{fi} haplotype does not always ensure self-compatibility in almond, as also observed in other *Prunus* species. This fact must be taken into account when designing future crosses in *Prunus* breeding programmes.

1. Introduction

Self-compatibility (SC) has been considered a priority objective in almond [*Prunus amygdalus* Batsch. syn. *Prunus dulcis* (Mill.) D.A. Webb] breeding (Socias i Company, 1990). After confirming that SC was a transmissible trait (Socias i Company and Felipe, 1977) it was attributed to the presence of the S_f allele, allelic to the series of *S* alleles of self-incompatibility (SI) (Socias i Company, 1984), being inherited as a single Mendelian trait (Socias i Company and Felipe, 1988).

When molecular approaches were applied to the study of SC/SI in almond, it was firstly established that the *S* alleles code for stylar ribonucleases whose activity can be detected by separation of stylar proteins by non-equilibrium pH gradient electrofocusing and subsequent staining for activity, but no RNase activity was found for the S_f allele (Bošković et al., 1999), thus suggesting the role of the *S*-RNases in pollen recognition and rejection. Later, *S* alleles, including S_f, were identified by PCR analysis using conserved and allele-specific primers, obtaining the partial sequence of the S_f allele gene associated with S_f-RNase (Channuntapipat et al., 2001; Ma and Oliveira, 2001). Ushijima et al. (2003) subsequently sequenced the pollen *S* haplotype termed F-Box (*SFB*) finding that this could be a good candidate for the pollen *S* gene-product.

Once the S_f allele could be identified, the amino acid sequences of both the *S*-RNase and the *SFB* genes could be determined. Despite the different sequences reported for the S_f-RNase by different authors (Hanada et al., 2009; Socias i Company et al., 2010), the first sequence reported by Channuntapipat et al. (2001) is considered as correct and must be taken as the consensus one. Probably some miss-sequencings and misinterpretations must have occurred during allele analysis, leading Bošković et al. (2007) to incorrectly name a new allele, S_{30}, which is identical to S_f, but showing a different activity, since it results in SI. The two different expressions of the S_f-RNase gene require their differentiation, and the denomination S_{fa} has been suggested for the active S_f allele showing a SI expression (Kodad et al., 2009), whereas the denomination S_{fi} has been suggested for the inactive S_f allele showing a SC expression (Fernández i Martí et al., 2009). The two forms of the S_f allele were equally identified by specific primers and showed an identical allele sequence (Fernández i Martí et al., 2009; Kodad et al., 2009). This identity was not only restricted to the coding region (C1 to C5), as deduced from their sequences, but also to the alignment of their 5′-flanking regions as shown by the construction of a fosmid library (Fernández i Martí et al., 2010). The change of function of the two phenotypic expressions of the S_f allele has been recently attributed to an epigenetic mutation in the 5′ upstream region of the S_f-RNA haplotype (Fernández i Martí et al., 2014).

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http://dx.doi.org/10.1016/j.scienta.2015.01.016
0304-4238/

The double phenotypic expression of the same genotype has also been observed in other *Prunus* species, such as Japanese plum (Watari et al., 2007; Guerra et al., 2009) and sweet cherry (Wünsch et al., 2010), and may explain some cases of unilateral incompatibility observed in almond (Bošković et al., 2007; Fernández i Martí et al., 2009; Socias i Company et al., 2012). These cases of unilateral incompatibility only resulted in fruit set in one direction whereas in the other the pollination was incompatible. This phenomenon is not the same as observed in the almond 'Jeffries' (Kester et al., 1994), attributed to a dysfunctional *S*-allele haplotype in both pistil and pollen (Ushijima et al., 2001).

Along the crosses of several breeding programmes, homozygous S_fS_f seedlings have been obtained in order to ensure that self-pollination was fully SC and not only semi-compatible (Dicenta et al., 2002) and as an strategy to obtain only SC seedlings in breeding crosses (Ortega and Dicenta, 2003). However, due to the double expression of the S_f allele, two different kinds of S_fS_f homozygotes may be obtained depending on the parents involved in the cross: $S_{fi}S_{fi}$, where the two alleles are in their inactive form, and $S_{fi}S_{fa}$, where the activity of the two alleles is different. The possibility of homozygous $S_{fa}S_{fa}$ must be ruled out due to the impossibility of self-pollinating a SI S_xS_{fa} genotype.

The origin of the homozygous S_fS_f seedlings studied so far indicates that they are $S_{fi}S_{fi}$ homozygotes and that their behaviour is fully SC. Consequently, our objective was to study the possible interaction between the two phenotypic forms of the S_f allele when present in the same genotype.

2. Materials and methods

2.1. Plant material

Three almond cultivars with identical *S*-genotype (S_fS_{23}) were utilised as parents of the population included in this study. One was 'Vivot', a local SI cultivar from the island of Majorca (Spain), possessing the active form of the S_f allele, S_{fa} (Kodad et al., 2010). The other two cultivars are releases from the CITA almond breeding programme, selected because of their SC, 'Belona' and 'Soleta', possessing the inactive form of the S_f allele, S_{fi} (Socias i Company and Felipe, 2007). The two SC cultivars were used as female parents in crosses with pollen from 'Vivot' in order to obtain $S_{fi}S_{fa}$ hetero/homozygotes.

The crosses were made in the spring of 2009, and the fruit were collected in the following fall, the seeds were stratified and the germinated seedlings were placed in growing plots and later transferred to the open field for flowering.

2.2. S-genotyping

Genomic DNA was extracted from leaves following the CTAB extraction method based on Doyle and Doyle (1987). For PCR amplification, DNA stocks were diluted to 20 ng μl^{-1} 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_{23}- and S_f-alleles were used (Channuntapipat et al., 2003). All PCR reactions were performed in 20 μl containing 20 mM Tris–HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM each dNTP, 0.2 μM each primer, 1 Unit of *Taq* DNA polymerase (Invitrogen, Madrid, Spain), and 90 ng almond genomic DNA. The PCR programme included an initial denaturation at 95 °C for 3 min, followed by 34 cycles of 30 s at 95 °C, 45 s or 1 min at the annealing temperature (Table 1), and 1 or 2 min at 72 °C, with a final extension step of 10 min at 72 °C. PCR products were separated in 1.5% (w/v) agarose gels in 150 ml TBE buffer, stained with a solution of 0.4 μg ml^{-1} ethidium bromide, and visualised under UV light. Band scoring was carried out using a standard 1 kbp DNA ladder (Invitrogen). For each allele, at least three different PCR were amplified to be sure of the identity of the *S*-genotypes in the progeny.

2.3. Seedling phenotyping

The phenotype of the seedlings was determined as soon as they produced the first flowers by pollen tube growth, since this method has been shown to be an efficient method for SC evaluation (Socias i Company et al., 2014). Flowers at stage D (Felipe, 1977) were collected during two consecutive years from each seedling and taken in plastic bags at the laboratory. Twenty flowers per seedling were emasculated and placed in trays with tap water, with the peduncles passing through the holes of a plastic mesh floating on the water over several pieces of wood. Anthers were extracted from the same flowers and left to dry on paper trays for 48 h, after which the pistils were self-pollinated. After pollination the pistil trays were placed in constant temperature chambers at 22 °C and the pistils were collected from the trays 96 h after pollination to allow pollen tubes to reach the style base (Socias i Company et al., 1976). The pistils were 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.

For pollen tube growth observation the pistils were prepared according to the method of Kodad and Socias i Company (2006), dissecting the outer part of the pistils 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 200W/4, by fluorescence of the callose deposits of the pollen tubes by aniline blue staining after squashing the pistils (Linskens and Esser, 1957). The pistils were rated according to the furthest level where pollen tubes were observed as defined by Socias i Company et al. (2013). Finally, each genotype was classified according to the average rating of all the pistils observed, pooling the data of the two years of observation in order to obtain the SC classification for the genotype.

2.4. Statistical analysis

Allele transmission was tested for different hypothesis by the chi square (χ^2) test of goodness-of-fit using Yates (1934) correction for one degree of freedom.

Table 1
PCR primers designed from the second intron used for *S*-allele determination.

Primer	Target	Nucleotide sequence (5′ → 3′)	Product size (bp)	Annealing temp (°C)
S_{23}F	S_{23}	ATTGTCATCTGAAGACCATATAC	437	60
S_{23}R		TGAGACATCCAAGCAATATATAC		
S_fF	S_f	GTGCCCTATCTAATTTGTTGAC	459	60
S_fR		GACATTTTTTTAGAAAGAGTG		
AS1II (F)	–	TATTTTCAATTTGTGCAACAATGG	#	53
AmyC5(R)		CAAAATACCACTTCATGTAACAAC		

Variable, depending on the genotype.