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Self-compatibility Evaluation in Almond: Strategies, Achievements, and Failures

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Abstract. **Several approaches have been used in evaluating self-compatibility in almond. These include fruit set after self-pollination and bagging, pollen tube growth, and the more recent S_f allele identification by molecular markers and gene sequencing. However, none of these methods have given fully reliable results because they all show advantages and limitations. Pollen contamination may distort pollination results as well as inaccuracies during fruit setting operations. Factors other than self-compatibility such as inbreeding may affect fruit set and pollen tube growth. Detection of *S* alleles by RNase activity and polymerase chain reaction analysis by consensus primers has not always been conclusive. The differential phenotypic expression of the S_{fa} and the S_{fi} alleles has revealed that the presence of the S_f allele is not the only requirement for self-compatibility expression in almond. As a consequence, the coding region of the S_f allele may not be the sole factor involved in that expression, which may be caused by modifier genes outside this region. Missequencing of alleles has also created confusion for allele identification. Thus, self-compatibility evaluation in almond must involve a better knowledge of the plant material as a whole, and not only of its genotype. All factors involved in setting a commercial crop in conditions of solid plantings of a single cultivar must be put together to evaluate almond self-compatibility. This approach is fundamental for the understanding of self-compatibility in almond and for the evaluation of the new selections in a breeding program.**

Although self-compatibility was discovered in almond as early as 1945 (Almeida, 1945), no attention was paid to the issue until the 1970s. The importance of self-compatibility in almond-growing and in breeding for new self-compatible cultivars was then fully understood (Socias i Company, 1978). The first attempts for self-compatibility identification involved fruit set evaluation after artificial self-pollinations (Almeida, 1945). This approach is based on the horticultural importance of almond self-compatibility, that is, to obtain commercial yields after an acceptable fruit set (Socias i Company et al., 2009).

Several approaches, each one showing advantages and limitations, have been used to assess the level of self-compatibility in almond. Effective self-compatibility implies, first of all, pollen tube growth after self-pollination similar to that after cross-pollination with cross-compatible pollen. Second, this good pollen tube growth after self-pollination should result in similar fruit sets, which may not always be the case. Third, these fruit sets must reach the level of a commercial crop. From a horticultural point of view, there is a fourth requirement, because these fruit sets must be obtained by autogamy, and that is the ability of a genetically self-compatible cultivar to pollinate itself in the absence of insects (Weinbaum, 1985). Additionally, a good cultivar must always be productive with a crop of good kernel quality.

Identification of *S* alleles was first attempted to establish cross-incompatibility groups by test pollination crosses (Kester et al., 1994). However, this approach could not allow the identification of the S_f allele. Only after Bošković et al. (1999) found no RNase activity for the S_f allele could an efficient identification of this allele be initiated.

More recently, once the genetic structure of the S_f allele was further understood, the detection of self-compatibility was also undertaken by molecular markers. Gametophytic self-compatibility such as that found in almond is controlled by a single polymorphic locus containing at least two tightly linked genes, one specifically expressed in the pistil and the other in the pollen (Kao and Tsukamoto, 2004). The pistil component of this gene codes for an *S*-RNase responsible for the pollen tube growth inhibition in the styles (Bošković et al., 1997). The candidate gene for the *S* pollen component (SFB) has been identified by Ushijima et al. (2003) showing a tight linkage with the *S*-RNase gene (Ikeda et al., 2005). Undoubtedly, the knowledge of the molecular basis for self-compatibility in the rosaceous fruit species has advanced significantly in recent years (Tao and Iezzoni, 2010; Yamane and Tao, 2009).

However, this information is only genetic and not horticultural. The final evaluation of self-compatibility of a cultivar or selection is its productivity under field conditions. This implies solid blocks of one clone isolated from any other almond clone and even in the absence of pollinating insects. Thus, our objective was to review the different physiological and genetic aspects of almond self-compatibility. This approach is required to better understand how these aspects are evaluated and how the results of their evaluation may be applied efficiently in a breeding program. This wider approach has become more necessary, especially after stating that some confusing results have been reported recently. These results refer to the S_f allele identification by molecular markers and gene sequencing as well as to the presence of modifier genes affecting the expression of self-compatibility in almond.

Fruit Set

The first studies of almond pollination were based on fruit set, concluding that the cultivars studied were self-incompatible (Tufts, 1919). The same approach was later adopted when testing the almond cultivars across the different growing regions of the world (reviewed by Socias i Company, 1990). Consequently, the first results on almond self-compatibility were obtained by Almeida (1945, 1949) by evaluating fruit sets after artificial self-pollinations. However, fruit set evaluation in the field is subject to many environmental hazards despite being the most natural approach to the real self-compatibility level of any genotype. As a consequence, fruit sets show a very high variability between years (Socias i Company et al., 2005). Fruit set levels, however, are not only related to the genetic self-compatibility of the selection, but also to other genetic conditions, including inbreeding depression (Alonso and Socias i Company, 2005a). The differences between the results of different years point to unspecified environmental conditions affecting fruit set, stressing the need for self-compatibility evaluation in more than 1 year (Socias i Company et al., 2004).

The environmental conditions not only affect natural fruit set in the field, but also the operations of emasculation and pollination. These operations are carried out for comparing self- and cross-pollination in the open air. Temperatures are usually very low at almond blooming time and, if winds are blowing, much attention must be paid to conduct these operations. Thus, fruit set determination in the field is mostly restricted to the final steps of self-compatibility evaluation in elite selections.

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Although the level of fruit set has been emphasized during the evaluation process in almond breeding (Oukabli et al., 2000; Socias i Company and Felipe, 1987, 2007; Torre Grossa et al., 1994), it must be obtained by autogamy. Previous studies have included bagging of branches (Grasselly and Olivier, 1984) or even enclosing whole trees in cages with or without honeybees (Godini et al., 1994; Socias i Company and Felipe, 1992). Autogamy, however, only received attention later (Dicenta et al., 2001; Godini et al., 1992; Kodad and Socias i Company, 2008; Socias i Company and Felipe, 1992; Socias i Company et al., 2004, 2005; Vargas et al., 1998). This aspect is particularly important because only natural autogamy can allow solid plantings of a single cultivar isolated from any other almond orchard and in the absence of pollinating insects. Flower morphology, in particular the relative positions of the stigma and anthers, is of great importance for natural autogamy (Bernad and Socias i Company, 1995; Godini et al., 1994; Kodad and Socias i Company, 2008; Socias i Company et al., 2004).

Special attention must be paid to pollen management and branch/tree isolation during fruit set studies, because very often pollen contamination may take place, thus distorting fruit set results. Some mistakes could have been made in pollen management in the many studies devoted to evaluating fruit set in almond (reviewed in Socias i Company, 1990). However, their final conclusion that fruit set is the ultimate and conclusive measure of self-compatibility expression in almond remains convincing.

Pollen Tube Growth

Pollen tube growth is a clear indication of the compatibility of any pollination. As a consequence, it has been repeatedly used to determine compatibility since the first evaluation of self-compatibility in almond genotypes (Socias i Company et al., 1976). The flowers examined for assessing pollen tube growth can be kept in different environments as well as on the original branches or separated from them, giving the same unequivocal results (Socias i Company, 2001).

The studies conducted in the field show the most reliable response because they reflect the natural conditions of the pollination. However, these studies are subject to unpredictable weather conditions such as frosts. Frosts may destroy the pistils, but this is not the case of the pollen tubes, that only suffer growth arrest at low temperatures, including frosts (Socias i Company, 1982).

The problems encountered in field work for pollen tube growth studies are the same as for fruit set evaluation. The weather contingencies may be avoided by taking whole branches to the laboratory or greenhouse and emasculating and pollinating them. Another possibility is to take only single flower buds at Stage D (Felipe, 1977) and place them on trays (Kodad and Socias i Company, 2006), hence saving space. In addition, the trays with the pollinated flowers can be kept in chambers to control the temperature. Higher temperatures than usual increase the speed of compatible pollen tube growth but aggravate the symptoms of pollen incompatibility (Socias i Company et al., 1976).

Pollen tube growth studies have often been associated with fruit setting after artificial pollinations, giving similar results (Ben Njima and Socias i Company, 1995; Kodad and Socias i Company, 2006; Socias i Company and Felipe, 1987). However, some confusion has arisen with studies based only on pollen tube growth such as in 'Moncayo' (Kodad et al., 2008) and 'AS-1'. This is a local Spanish selection mistakenly described as self-compatible by Herrero and Felipe (1975) but shown to be clearly self-incompatible (Kodad et al., 2009b). As well as for fruit set results, inbreeding depression may affect the expression of self-compatibility by pollen tube growth (Alonso and Socias i Company, 2005a).

RNase Activity

Bošković and Tobutt (1996) reported that the *S* alleles code for stylar ribonucleases in cherry (*P. avium* L.). These RNases can be detected by separation of stylar proteins by non-equilibrium pH gradient electrofocusing (NePHGE) and subsequent staining for activity. The same approach was later applied to almond *S* alleles and Bošković et al. (1999) found no RNase activity for the S_f allele. Consequently, they concluded that genotypes showing only one band for RNase activity were self-compatible. However, the presence of one band is not enough to assess the presence of the S_f allele. The absence of RNase activity may not only be the result of the lack of transcription of the *S*-RNase in the pistil, but also the very low level of this transcription. This low transcription level, reported in Japanese plum (*Prunus salicina* Lindl.) by Watari et al. (2007), has not been clearly noticed so far in almond (Hanada et al., 2009). Inbreeding may also produce an incompatible expression of self-compatible genotypes with a single RNase band (Alonso and Socias i Company, 2005a).

Some problems, however, have arisen when RNase detection has been applied to different genotypes. Two different RNase bands may coincide after electrophoresis separation, thus giving a wrong "one-band" result when a real superposition of two bands is occurring. Consequently, this technique is only fully reliable for seedling identification when the genotypes of the two parents are previously known (Bošković et al., 2003).

Allele Identification

The more recent advances in genetic analysis at the gene level have allowed a closer approach to the S_f allele in almond both of the stylar and the pollen components. First, *S* alleles, including S_f, were identified by polymerase chain reaction (PCR) analysis using conserved and allele-specific primers (Channuntapipat et al., 2001; Ma and Oliveira, 2001). Later, the partial sequence of the S_f allele gene associated with S_f-RNase was obtained (Channuntapipat et al., 2001; Ma and Oliveira, 2001). Finally, Ushijima et al. (2003) sequenced the pollen SFB finding that this could be a good candidate for the pollen *S* product. This candidacy is based on its specific expression in the pollen tube, to be physically linked to the *S*-RNase gene, to show allele sequence diversity, and dysfunction in the self-compatible *S* haplotype (Entani et al., 2003; Tao and Iezzoni, 2010; Ushijima et al., 2003; Yamane et al., 2003). This was identified in self-incompatible almond genotypes, but later the self-compatible SFB_f was sequenced by Bošković et al. (2007) and Hanada et al. (2009).

Various consensus primer sets have been designed to determine *S* genotypes in almond. They were designed from conserved regions of *S* genes to amplify across the second intron (Channuntapipat et al., 2003; Tamura et al., 2000), the first intron (Ortega et al., 2005), or both (Sutherland et al., 2004). However, PCR primers designed from conserved regions do not always distinguish between alleles with a similar number of nucleotides (López et al., 2004). This fact must be taken carefully into account, as it also occurs with RNase identification. As a result, this technique is only fully reliable for seedling genotyping when the genotypes of the two parents are previously known. In addition, the detection of some alleles is masked by the presence of another allele, thus giving a wrong single band. This confusion was first detected by Channuntapipat et al. (2003) when the presence of either S_1 or S_7 masked the amplification of S_8 by PCR when using conserved primers. The same masking has also been observed with other alleles (Alonso and Socias i Company, 2005b; Fernández i Martí et al., 2009). As a consequence, other primer sets have been specifically designed to amplify some *S* genes, including S_f (Channuntapipat et al., 2001; Ma and Oliveira, 2001). PCR-based markers of almond *S* alleles have been used to facilitate the integration of self-compatible *S*-alleles from related species (Gradziel et al., 2001). Screening efficiency and flexibility have also been greatly improved with the development of successful multiplex PCR techniques by Sánchez Pérez et al. (2004). This technique prevents one allele being masked by the expression of another.

Allele Sequencing

Once the S_f allele could be identified, the amino acid sequences of both the RNase and the SFB genes could be determined. Since the beginning, several amino acid sequences for the S_f-RNase have been deposited in the databases by different authors. When these sequences have been compared, several differences could be observed between them. The diversity of the S_f-RNase sequences was closely examined by Hanada et al. (2009) to solve previous confusions as to their identity.

As a result of this examination, the sequences could be contrasted because most of them had been determined in 'Tuono' and genotypes derived from it, consequently for the same S_f-RNase. This identity allowed different sources of self-compatibility for the genotypes studied to be discarded. The first sequences reported by Channuntapipat et al. (2001) and Ma and Oliveira (2001) were already different. Further sequencings suggest that the sequence by Channuntapipat et al. (2001) was correct and must be taken as the consensus sequence.

Figure 1 shows the alignment of some of the sequences published for the S_f-RNase as well as some other *S*-RNases for comparison. This alignment mostly agrees with the results of Hanada et al. (2009) and Fernández i Martí et al. (2010a). The first is considered the consensus sequence and was amplified in 'Lauranne' and selection IRTA12-2 (Channuntapipat et al., 2001). These two self-compatible genotypes are derived from 'Tuono', but despite this origin, their sequence is different from several published sequences for the 'Tuono' S_f-RNase (Table 1). It is however identical to the S_f sequence of 'Cambra', another cultivar derived from 'Tuono', to the S_{fi} sequence of 'Blanquerna', a cultivar derived from 'Genco', not from 'Tuono', and to five *S* alleles reportedly conferring self-incompatibility in almond (Table 1).

These results show that some missequencings and misinterpretations must have occurred during allele analysis. Ma and Oliveira (2001) showed valine instead of isoleucine and histidine instead of arginine in the C2 region, probably as a result of a mistake in sequencing (Fig. 1). Bošković et al. (2007) had to recognize missequencing in a note added in proof, thus invalidating most of the reasoning of their conclusions. Their 'Tuono' S_f did not really show the supposed histidine substitution instead of arginine in its sequence. Barckley et al. (2006) gave an amino acid sequence for 'Tuono' S_f identical to S_1, probably as a result of missampling. Consequently, the 'Tuono' genotype studied by Barckley et al. (2006) in California must be the same as that used for the other analysis as opposed to their original suggestion.

These mistakes led Bošković et al. (2007) to incorrectly name a new allele, S_{30}, which is identical to S_f but showing a different activity (Kodad et al., 2009a). This new name may create new confusion in almond *S* allele research because the identity of the S_f allele must be preserved despite showing two different phenotypic expressions. As a consequence, the denomination S_{fa} has been suggested for the active S_f allele showing a self-incompatible expression (Kodad et al., 2009a). Similarly, the denomination S_{fi} has been suggested for the inactive S_f allele showing a self-compatible expression (Fernández i Martí et al., 2009). The two forms of the S_f allele are equally identified by specific primers and show an identical allele sequence (Fernández i Martí et al., 2009; Kodad et al., 2009a). This identity is not only restricted to the coding region (C1 to C5), as deduced from their sequences (Fig. 1), but also to the alignment of their 5′-flanking regions as shown by the construction of a fosmid library (Fernández i Martí et al., 2010a).

The SFB_f allele has not been so hard to identify. The sequences for 'Tuono' (AM711126) by Bošković et al. (2007) and for 'Lauranne' (AB361036) by Hanada et al. (2009) are identical. This sequence is also the same as for the self-incompatible SFB_f allele sequenced in 'Ponç' (EU310402) by Kodad et al. (2009a) and in 'Fra Giulio Grande' (AM711127) by Bošković et al. (2007). These coincidences indicate that the SFB_f allele as well as the S_f-RNase allele, show two different phenotypic expressions. In addition, both the pistil and the pollen components of the S_f allele show the same compatible or incompatible behavior simultaneously. These results suggest that the coding region of the S_f gene may not be the exclusive origin of self-compatibility in almond (Kodad et al., 2009a). Thus, some genetic modifications outside this coding region are affecting that expression (Fernández i Martí et al., 2009).

Modifier Genes

The presence of self-compatible genotypes without possessing the S_f allele (Fernández i Martí et al., 2009) is another reason for suggesting the presence of modifier genes outside the coding region. The presence of modifier genes substantiates the proposal by Socias i Company (1990) that almond is a self-incompatible species with a genetic background of pseudo-self-compatibility. The possibility of pseudo-self-compatibility in almond is based on the small self sets observed in some cultivars (reviewed by Socias i Company, 1990). Over this background, only one S_f allele could break the self-incompatibility system, but probably by interacting with this background of pseudo-self-compatibility. This interaction has been shown by the effect of two QTLs related to the expression of self-compatibility in genotypes not showing the presence of the S_f allele, thus theoretically self-incompatible (Fernández i Martí et al., 2010b). This new approach may shed new light on the origin,

Table 1. Similarity of different almond *S*-RNases with the consensus S_f-RNase.

Allele	Genotype	Database code	Coincidence with the S_f consensus allele (%)	Reference
S_f consensus	'Lauranne' and selection IRTA12-2	AY291117	100	Channuntapipat et al. (2001)
S_f	'Tuono' a	AF157009	98	Ma and Oliveira (2001)
S_f	'Tuono' b	DQ156217	64	Barckley et al. (2006)
S_f	'Tuono' c	AM690356	99.3	Bošković et al. (2007)
S_{fa}	'Ponç'	EU293146	100	Kodad et al. (2009a)
S_f	'Cambra'	EU684318	100	Kodad et al. (2009a)
S_{30}	'Fra Giulio Grande'	AM690361	100	Bošković et al. (2007)
S_{fi}	'Blanquerna'	AB467371	100	Fernández i Martí et al. (2010a)
S_{fa}	'Alzina'	FJ887784	100	Kodad et al. (2010)
S_{fa}	'Garondès'	FJ887783	100	Kodad et al. (2010)
S_{fa}	'Vivot'	AB467370	100	Fernández i Martí et al. (2010a)

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Sf  Tuono (a)   VQQWPPTTCRFSGK-PSNNRRPLPIFTIRGVWPSNYSNPRMRSNCTGSQFKKILS-PRLRSKLERAWPDVESGNDIKFWEDEWNKHGKCSEQTLNQMQYFERSHQMW
Sf  Tuono (b)   VQQWPPTNCRVRIKRPCSNPRPLQYFTIHGLWPSNYSNPTKPSNCNGSQFNFTKVSPKMRVKLKRSWPDVESGNDIRFWEGEWNKHGTCSEGSLNQMQYFERSHEMW
Sf  Tuono (c)   VQQWPPTTCRFSGK-PSNNRRPLPIFTIRGIWPSNYSNPRMRSNCTGSQFKKILS-PRLRSKLERAWPDVESGNDIKFWEDEWNKHGKCSEQTLNQMQYFERSHQMW
Sfa Ponç        VQQWPPTTCRFSGK-PSNNRRPLPIFTIHGIWPSNYSNPRMRSNCTGSQFKKILS-PRLRSKLERAWPDVESGNDIKFWEDEWNKHGKCSEQTLNQMQYFERSHQMW
Sf  Cambra      VQQWPPTTCRFSGK-PSNNRRPLPIFTIHGIWPSNYSNPRMRSNCTGSQFKKILS-PRLRSKLERAWPDVESGNDIKFWEDEWNKHGKCSEQTLNQMQYFERSHQMW
S30 F.G.Grande VQQWPPTTCRFSGK-PSNNRRPLPIFTIHGIWPSNYSNPRMRSNCTGSQFKKILS-PRLRSKLERAWPDVESGNDIKFWEDEWNKHGKCSEQTLNQMQYFERSHQMW
Sf  Lauranne    VQQWPPTTCRFSGK-PSNNRRPLPIFTIHGIWPSNYSNPRMRSNCTGSQFKKILS-PRLRSKLERAWPDVESGNDIKFWEDEWNKHGKCSEQTLNQMQYFERSHQMW
                 C1                C2                RHV               C3

Sf  Tuono (a)   SSFNITNILEKASIVPNAIQTWIYSDILSPIKAATQRIPLLRCKGNPQRQAKSQPKNRGKSQFKSQATTQLLHEVV
Sf  Tuono (b)   YSFNITEILKNASIVPHPTQTWKYSDIVAPIKTATKRTPVLRCKPDP------------AQNKSGPKTQLLHEVV
Sf  Tuono (c)   SSFNITNILEKASIVPNAIQTWIYSDILSPIKAATQRIPLLRCKGNPQRQAKSQPKNRGKSQFKSQATTQLLHEVV
Sfa Ponç        SSFNITNILEKASIVPNAIQTWIYSDILSPIKAATQRIPLLRCKGNPQRQAKSQPKNRGKSQFKSQATTQLLHEVV
Sf  Cambra      SSFNITNILEKASIVPNAIQTWIYSDILSPIKAATQRIPLLRCKGNPQRQAKSQPKNRGKSQFKSQATTQLLHEVV
S30 F.G.Grande SSFNITNILEKASIVPNAIQTWIYSDILSPIKAATQRIPLLRCKGNPQRQAKSQPKNRGKSQFKSQATTQLLHEVV
Sf  Lauranne    SSFNITNILEKASIVPNAIQTWIYSDILSPIKAATQRIPLLRCKGNPQRQAKSQPKNRGKSQFKSQATTQLLHEVV
                        RC4                                                        C5
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Fig. 1. Multiple alignment of the deduced amino acid sequence of different *S* almond alleles. Accession numbers and references are given in Table 1.

the evolution, and the expression of self-compatibility in almond.

Conclusion

The different approaches applied to identify self-compatibility in almond show different advantages and limitations. In particular, the missequencing of alleles has created confusion for allele identification. An active S_f allele has recently been identified, which does not confer self-compatibility despite its full identity with the inactive S_f allele, considered to date as the one that confers self-compatibility. This coincidence shows that the presence of the S_f allele is not the only requirement for self-compatibility expression in almond. Thus, the coding region of the S_f allele may not be the sole factor involved in that expression. Knowledge of the genotype only is not enough in almond self-compatibility research as confirmed by the effect of newly identified QTLs in genotypes lacking the S_f allele. Fruit set, however, must be considered as the main evaluation criterion for self-compatibility selection in almond (Kodad and Socias i Company 2008) independently of the genotype of any plant. The effect of the S_f allele and of the two QTLs recently identified (Fernández i Martí et al., 2010b) may explain the wide range of fruit sets observed in self-pollination studies. These genetic effects are independent of the changing year effect on these sets (Socias i Company et al., 2005). This implies a global study of the plant material for evaluating the real ability of any genotype to set fruit under autogamy conditions. Only this insurance may justify its further selection to be finally released as a registered cultivar.

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