

Discrimination and preliminary selection of self-compatible progenies among controlled crosses in almond using S_f specific primer

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Abstract

Self-incompatibility in almond and *Prunus* species is an important trait that prevents self-fertilization. Self-incompatibility in almond is controlled gametophytically by the multiallelic S-locus. Present study was done in order to identification and preliminary selection of self compatible progenies resulted from controlled crosses in almond using specific primer S_f/S_fR. Some important morphological traits of parental crosses were evaluated using almond descriptor. Also, progenies (F₁) of five crosses including; A (Tuono × 101 Genotype), B (Supernova × 101 Genotype), C (Genco × Shahrood 21), D (Tuono × Shahrood 12) and E (Tuono × shahrood 17) were tested using PCR in order to DNA amplification and self-compatibility evaluation. The result of PCR method found that using S_f/S_fR pair primer, self-compatible progenies showed 449 bp bands, while this band not observed in self-incompatible progenies. In addition, all self- incompatible progenies appeared no S₁ allele at any condition. According to Chi-square test, ratios of self-compatible to self-incompatible progenies were to Mendelian principles. It can be suggested that PCR method of this research has high potential in order to distinguish self-compatible and incompatible genotypes.

Keywords: Almond, PCR, S_f-allele, S₁-allele, Self-incompatibility, S_f/S_fR primer.

Introduction

Almond is one of the temperate zone fruit trees which is cultivated in different countries for its nutritional value. In addition, the almond is one of the oldest crops using by humans, but its exact environmental requirements have restricted its commercial production to specific areas of the world (Kester and Asay 1979). Self-incompatibility is one of the most important difficulties in almond production which reduces fruit set dramatically and makes orchards management difficult. Self-incompatibility is a widely spread and heritable reproductive phenomenon in flowering plants, that self-fertilization is prevented by rejection of pollen from the same plant. This is an evolutionary advantage due to its effectiveness in avoiding inbreeding and the encouragement of out crossing (Momenpour et al. 2011). *Prunus* species, such as almond are characterized by a gametophytic type of self-incompatibility. Which means there is no pollen germination on the stigma (Yamashita et al. 1987) or tube growth stops most often in the upper third of the style (Sanchez-perez et al. 2004).

Self-incompatibility in almond is gametophytic and controlled by a single S-locus with multiple co-dominant alleles (Socias I Company et al. 1976) This trait is expressed in the style by special glycoproteins (S-RNases) that arrest the growth of pollen tubes in self-incompatible cultivars (Socias I Company et al. 1976; Boskovic et al. 1997) Nowadays, using molecular methods is very important for identification of self-compatible and self-incompatible cultivars. In addition these methods are very reliable for determination of S-genotypes in almond. So far, it has been shown that there are about 35 self-incompatible alleles (S₁, S₂,...S₃₅) and one self-compatible allele (S_f). Only a limited number of the numerous almond cultivars grown worldwide are self-

compatible. The majority come from the Italian region of Apulia (Godini, 1979; Reina et al. 1986). Prominent among these are Tuono and Genco, Filipo Ceo (Grassley & Oliver 1976) Mazzetto — synonymous for Tuono — (Jaouani 1973) Exinograd (Cyprus Agricultural Research Institute 1973) Falsa Barese, Ferrante and Palatina (Godini, 1977) As these cultivars were shown to be capable of transmitting their self-compatibility to their offspring (Socias I Company & Felipe 1977) using them has proved to be the most effective method for obtaining new self-compatible cultivars. A knowledge of the inheritance of self-compatibility is an essential step in the attainment of such an objective (Grassley et al. 1981) found that almost all the descendants from the cross Ferragnes × Filipo Ceo were self-compatible and attributed this fact to the possibility that the self-compatible parents were homozygous (Grassley and Olivier, 1984), when crossing self-incompatible cultivars with Tuono, obtained the results that would be expected if this parent were heterozygous for self-compatibility. However, in some of the crosses with the female parent related to Cristomorto, the proportion of self-compatible individuals was higher than expected. Dicenta and Garcia (1993) studied the inheritance of self-compatibility in 742 almond seedlings, from 25 families, by fluorescence microscopy. The observed frequencies of self-compatible descendants were in accordance with the accepted theory concerning the gametophytic system of *Prunus*. The results confirmed the presence of a common allele in the cultivars Genco, Tuono and Ferragnes (Ortega and Dicenta 2003) So far, 35 S-incompatibility alleles, in addition to the S_f self-compatibility allele, have been identified in almond using different molecular analysis, ribonucleasis, S-allele PCR and sequencing analysis. More than 154

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almond cultivars have been genotyped (Lopez *et al.* 2006) and 19 cross-incompatible groups have been established (Ortega *et al.* 2006) The techniques used to identify genotypes self can be more pointed; using of paper bags and flowers cover the branches of -contain flower and finally examine the fruit set, evaluation of pollen tube growth in pistils using fluorescence microscopy, study of activity style ribonuclease in pistil and use of molecular methods including PCR (Lopez *et al.* 2004) PCR method to detect genotypes by researchers such as Chanuntapipat (2001 and 2003) Gomez (2003), Tamura and *et al* (2003) and Alonso (2005) have been carried out in most parts of the world has produced many self genotypes. Based on these results, main objective of this study was initial screening self-compatible

progenies by using PCR method that resulting of hybridization between native and foreign cultivars.

Materials and methods

Plant materials

Native self-incompatibility cultivars including Shahroud17, Shahroud21, Genotype101 and Shahroud12 as female parents in crosses with the varieties of foreign self-compatible, supernova, Genco and Touno as male parents went to the nursery section of horticulture Seed and Plant Improvement Institute, Karaj were prepared. From the seedling stage, leaf samples were taken. Offspring's (F₁) of five crosses including; A (Genotype 101 × Tuono), B (Genotype 101 × Supernova), C (Shahroud 17 × Tuono), D (Shahroud 21 × Genco) and E (Shahroud 12 × Tuono) were evaluated in this study that their information are reported in Table 1.

Table 1. Names of crossing compounds and some information of them.

Cross name	Female parent (♀)	Male parent (♂)	Number of evaluated progenies	Expected Genotypes
A (Gen. 101 × Tuono)	Genotype 101(S _x S _x)	Tuono(S ₁ S ₇)	18	S ₁ S _x S ₇ S _x
B (Gen. 101 × Supernova)	Genotype 101(S _x S _x)	Supernov a (S ₁ S ₇)	12	S ₁ S _x S ₇ S _x
C (Shahroud 17 × Tuono)	Shahroud17(S _x S _x)	Tuono (S ₁ S ₇)	16	S ₁ S _x S ₇ S _x
D (Shahroud 21 × Genco)	Shahroud21 (S _x S ₃)	Genco (S _x S ₇)	16	S ₁ S _x S ₇ S _x S ₁ S ₃ S ₇ S ₃
E (Shahroud 12 × Tuono)	Shahroud12(S _x S ₃)	Tuono(S ₁ S ₇)	12	S ₁ S _x S ₇ S _x S ₁ S ₃ S ₇ S ₃

Measurements of quantitative and qualitative traits were done in different and suitable ways for each them. Coding of some traits was done according to IBPGR and UPOV almonds descriptors (Gulcan, 1985) .

DNA extraction

Total DNA was extracted from young leaves collected in early spring, following the method described by Murray and Thompson (1980) modified and adapted to almond as follows with the difference that 2 µl of β-mercaptoethanol added immediately after application of extraction buffer. In the end, the purified total DNA was quantified by gel electrophoresis, and its quality verified by Nano Drop 1000 Spectrophotometer V3.7. DNA samples were

stored at - 20° C. Three independent extractions were performed for each sample.

PCR amplification

DNA sample with concentration of 10 ng per µl were prepared for PCR reaction. Amplification reactions were carried out in 25 µl volumes containing; 1x PCR buffer, 3mM MgCl₂, 0.4 mM dNTPs, 0.625 mM of each primer (forward and reverse) 1 unit of SmarTaq DNA polymerase (Cinnagen) and 10 ng of genomic DNA. The PCR reaction program consisted of three minutes at 95°C for denaturation primary, in 34 cycles with 30 seconds, temperature 95°C, 45 seconds at 53°C and then one minute at 72 °C and after cycles, 10 min at 72°C was performed. After PCR, the products at 4°C (refrigerator) were stored until electrophoresis.

Table 2. Profile of primers used in this study are shown in this table.

Marker (locus)	Sequence		Primer mix	Band size(bp)	Visible allele	T annealing	Reference
	5'	3'					
SFF	GTGCCCTATCTAATTTGTTGAC		SFF/ SFR	449	S ₇	60 °C	Chanuntapipat <i>et al.</i> (2003)
SFR	GACTTTTTTTTAGAAAGAGTG						

Electrophoresis

Amplified PCR products were separated using 2% agarose gel electrophoresis using 0.5X Tris-Boric acid-EDTA buffer. The molecular sizes of the amplification products were estimated using 100 bp DNA ladder plus (Fermentas). After electrophoresis, agarose gel electrophoresis (Biowittaker Maine, USA) stained with ethidium bromide (1 µg/ml) and visualized under UV light (Morgante et al. 2001).

Also, in order to significant test results from the progenies of different crosses and consistent with Mendelian ratio was used Chi-square test (χ^2).

Results

Results of some important traits of the progenies' parents were evaluated according to the almond descriptor (Gulcan, 1985) (Table 3). According to the results some traits such as flowering time, leaf area, harvest maturity, shell hardness, nut weight, double kernel percentage and kernel color had variation between parents. For example "Shahrood12" was extremely late flower cultivar without double kernel (Table 3). Also, "Shahrood17" and "Supernova" were late flower cultivars (Table 3). Based on these results, if late flowering time is the objective of breeding program, we can use of these cultivars in classical crosses. "Tuono" and "Supernova" cultivars were self-compatible and they are suitable for using in breeding programs in order to have self-fertility progenies.

For screen of self-compatible and self-incompatible progenies, 74 seedlings resulting of controlled hybridization were studied. Results of PCR analysis indicated that S_f allele band with 449 bp size was observed in self-compatible progenies. In five groups of crosses (A to E), the results were expected according to Chi-square test (Table 4). These results is consistent with Martin-Gomez et al. 2003., Channuntapipat et al. 2003., Alonso 2005 and Kamali et al. 2009. In A cross (Genotype 101 × Tuono) 18 descendants were evaluated that in 9 descendants S_f allele were found (Table 4). At B cross (Genotype 101 × Supernova) 12 offspring were evaluated that five of them have S_f allele and others were self-incompatible (Table 4) (Figure1). In the current study, 6 seedling of 16 descendants in hybrid C (Shahrood 17 × Tuono), 7 progenies of 16 descendants of hybrid D (Shahrood 21 × Genco) and 4 progenies of 12 descendants of hybrid E (Shahrood 12 × Tuono) were self-compatible and the rest of progenies were self-incompatible (Table 4) (figures1, 2, 3, 4 and 5). In following figures, "Tuono" and "A200" were control cultivars of self-compatible and self-incompatible, respectively. The pair primer ConF-ConR by channuntapipat et al. (2001) that previously was used in order to identifying S_1 , S_3 , S_f alleles, because similarity S_3 allele (1196 bp) and S_f allele (1205 bp) in size, occasionally, it may cause mistake in distinguish between S_3 and S_f alleles. Therefore, it is better, this pair primer put to use for distinguish S_1 and S_f alleles and for identification of S_3 allele, pair primer S_3F - S_3R_1 can be used. Thus by this primer, S_3 allele produces 611bp band.

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Table 3. Some important quantitative and qualitative traits of the parents of progenies that were evaluated according to the almond descriptor (Gulcan, 1985).

Row	Cultivar or genotype name	Origin	Blooming time	Tree vigor	Foliage density	Leaf area	Harvest maturity	Shell hardness	Nut weight	Nut wide	Nut length	Kernel percentage	Double Kernel	Kernel weight	Kernel color
		origin	code ¹	code ²	code ³	(mm ²)	code ⁴	odec ⁵	gr	cm	cm	(%)	(%)	gr	code ⁶
1	Ttuon	Italy	5	7	7	1151/01	3	3	3.96	3.08	3.38	40	12	1.23	3
2	Genco	Italy	6	3	5	1029/25	5	3	2.14	2.10	2.70	48	35	1.03	3
3	Genotype 101	Iran	5	7	7	652/41	5	1	1.20	1.90	2.96	38	10	0.74	3
4	Supernova	Italy	7	7	5	1091/83	5	3	4.06	2.63	3.47	38	8	1.25	1
5	Shahrood12	unknown	9	5	7	1512/32	5	3	4.21	2.74	3.97	32	0	1.96	5
6	Shahrood17	unknown	7	7	7	1207/47	7	5	2.00	1.97	2.63	40	2	0.75	5
7	Shahrood21	unknown	5	5	7	860/37	3	3	3.35	2.04	2.71	45	15	1.04	2

- 1= Extremely early, 2= Very Early, 3= Early, 4= Early / intermediate, 5= Intermediate, 6= Intermediate / late, 7= Late, 8= Very late, 9= Extremely late.
- 3= Weak, 5= Intermediate, 7= Strong.
- 3 = Low, 5= Intermediate, 7= Dense.
- 1= Extremely early, 3 =Early, 5= Medium, 7= Late, 9= Extremely late.
- 1= Extremely hard, 2 = Hard, 5= Intermediate (broken by hand with effort), 7= Soft, 9= Paper.
- 1= Extremely light, 3= Light, 5= Intermediate, 7= Dark, 9= Extremely dark.

Table 4. Analysis of self-compatible and self-incompatible progenies resulting from different crosses by PCR method using Chi-square test (χ^2).

Cross name	Number of evaluated progenies	Parents genotype (φ) \times (σ)	Expected Genotypes	Obtained Genotypes	χ^2
A (Gen. 101 \times Tuono)	18	($S_x S_x$) \times ($S_f S_f$)	$S_1 S_x$ $S_f S_x$	9 9	0.00 ^{ns}
B (Gen.101 \times supernova)	12	($S_x S_x$) \times ($S_f S_f$)	$S_1 S_x$ $S_f S_x$	7 5	0.166 ^{ns}
C (Shahrood 17 \times Tuono)	16	($S_x S_x$) \times ($S_f S_f$)	$S_1 S_x$ $S_f S_x$	10 6	0.500 ^{ns}
D (Shahrood 21 \times Genco)	16	($S_x S_x$) \times ($S_f S_f$)	$S_1 S_x$, $S_f S_x$ $S_1 S_3$, $S_f S_3$	9 7	0.125 ^{ns}
E (Shahrood 12 \times Tuono)	12	($S_x S_3$) \times ($S_f S_f$)	$S_1 S_x$, $S_f S_x$ $S_1 S_3$, $S_f S_3$	8 4	0.667 ^{ns}

* S_x : all parents and offspring that at least one allele of them is not S_f .

** ns: non-significant.



Fig 1. Existence of S_f allele with 449 bp band in some progenies of A (Genotype 101 \times Tuono) and B (Genotype 101 \times Supernova) crosses.

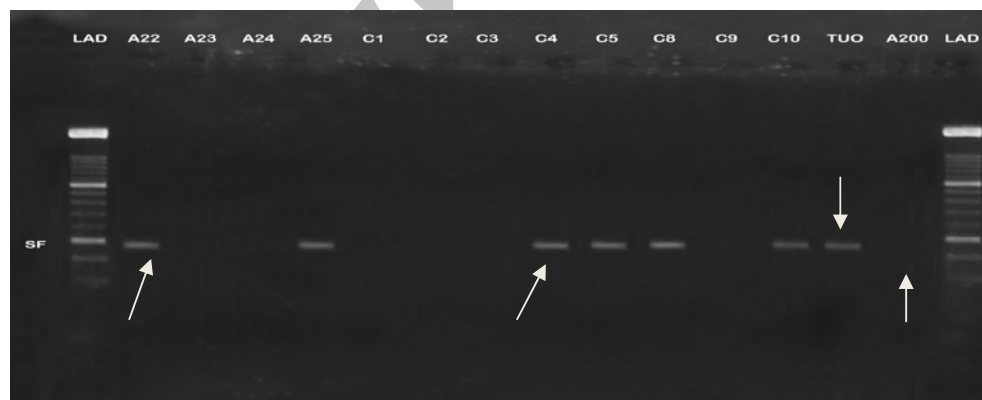


Fig 2. Existence of S_f allele with 449 bp band in some progenies of C (Shahrood 17 \times Tuono) cross.

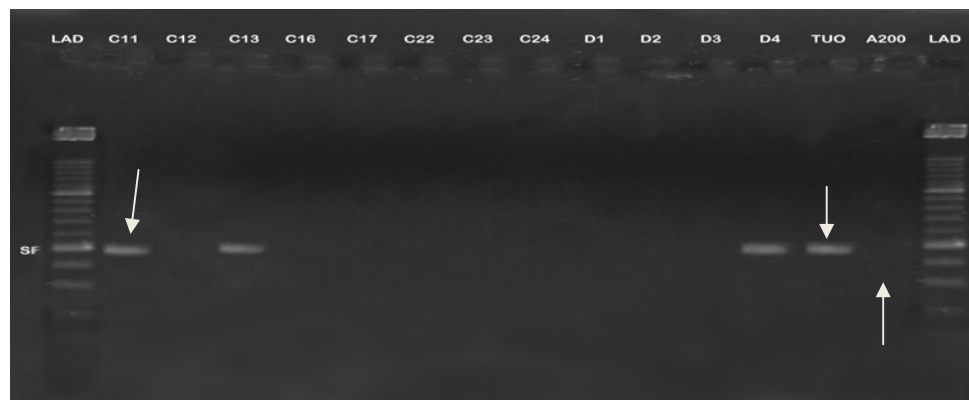


Fig 3. Existence of S_f (449 bp) allele in some progenies of C (Shahrood 17× Tuono) and D (Shahrood 21× Genco) crosses.

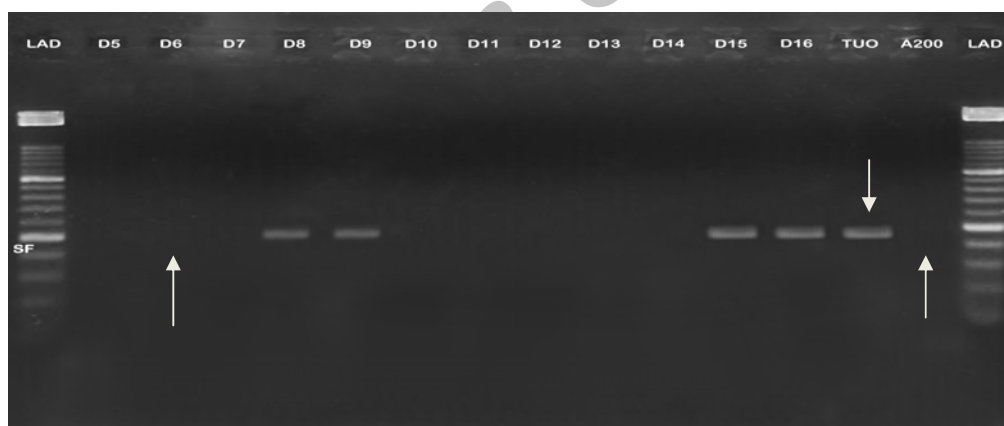


Fig 4. Existence of self-compatibility allele (S_f (449 bp)) in some progenies of D (Shahrood 21× Genco) cross..

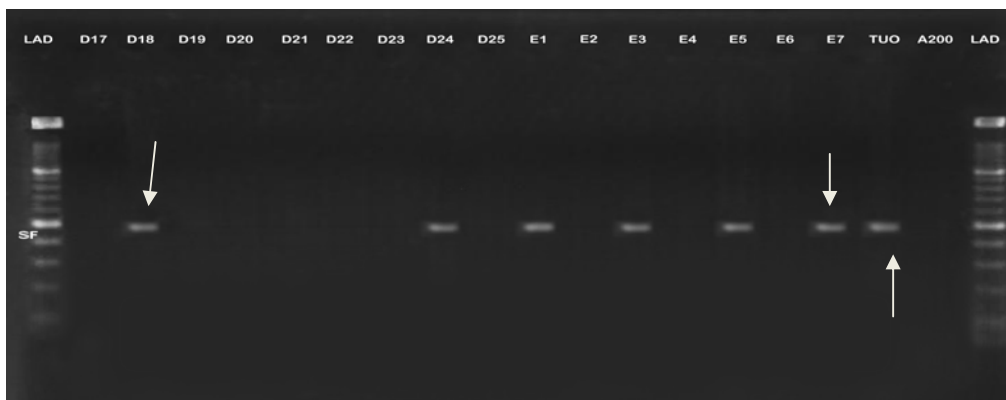


Fig 5. Existence of self-compatibility allele ($S_{f(449\text{ bp})}$) in some progenies of D and E crosses.



Fig 6. Existence of self-compatibility allele ($S_{f(449\text{ bp})}$) in some progenies of E (Shahrood 12 \times Tuono) crosses

Discussion

Since all the male parents (Touno, Supernova, Genco) with S genotype (S_1S_7) were self-compatible and female parents (Genotype 101, Shahrood17, Shahrood21 and Shahrood12) are all self-incompatibility (Kamali et al. 2009).

a) If in the female parent, there is S_1 allele, so all offspring will be the self-compatible, because pollen by S_1 allele of male parent is unable to growth in to the style tissue and the zygote will not formed and only pollen with S_7 alleles is able to penetrate in to the style and it can fertile ovule, so all offspring will has (S_7S_x , S_1S_7) S genotype (S_x = uncertain).

b) If there is no S_1 allele in the female parent genotype and female parent S genotype to consider S_xS_x , self-compatible and self-incompatibility genotypes of the offspring are expected with S_7S_x , S_1S_x , S_1S_x and S_7S_x forms. This case shows that 50% of progenies are self-compatible and 50% of progenies are also self-incompatible (Table 4). Progenies obtained from crosses madebetween foreign cultivars (Tuono, Supernova and Genco) had S genotype S_7S_1 as male parents, with internal cultivars (Shahrood17, Shahrood21, Genotype 101 and Shahrood12) as parents female show that, in all progenies of the

hybrids the number of offspring have been S_7 alleles and the results are visible by bands obtained the PCR (Table 4) (figures1 to 6).

In case of progenies obtained from A, B, C, D and E crosses can be said that according to all cultivars of native trade are self-incompatible and has not S_7 allele (Table 4). Besides all the male parents (Foreign cultivars) that are self-compatible and all of them have been S_7 allele (Kamali et al. 2009). So, if one alleles of female parents be S_7 allele is expected that S_1 allele of male parents not be able to growth in to the female parent style and only S_7 allele has the ability to enter the female parent style and so only genotypes with S genotype (S_xS_7) is expected. In addition, it indicated in table 4 and figures 1 to 6, only several percent of progenies are self-compatible and rest are self-incompatible, so this indicates that the S_1 allele under any circumstances does not exist in the female parents (native self-incompatible cultivars). Finally, results indicated that using PCR method in order to distinguish self-compatible genotypes and cultivar, also identification almond alleles, is a careful and scientific method.

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