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Identification of Self- incompatibility Alleles in Some Almond Genotypes by Degenerate S-RNase Primers

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Abstract

The almond, *Prunus dulcis* Miller which belongs to Rosaceae family, is one of the most important commercial and oldest cultivated tree nut crops. Almonds are classified as a 'nut' in which the edible seed is the commercial product. Therefore, pollination and fertilization are necessary in almond. The characteristic of cultivated almond to express gametophytic self- incompatibility discourages self-fertilization and favors cross pollination. Genetic control of pollen-pistil self-incompatibility is through a single gene (S) which exists in a series of alleles S_1 to S_x. Compatibility of pollen-pistil in almond is an important consideration in planning crosses in breeding program and in choosing pollinizers for orchard planting. Identification of self-(in) compatibility in almond carried out by molecular and controlled pollination methods. In this study, identification of S-alleles in 37 Iranian almond cultivars and genotypes was carried out by PCR method with using degenerate primers of EM-PC3consRDEMPC2cons FD, PaconsI-Fand EM-PC1consRD. In this way the size of S -alleles were estimated based on bands which amplified with second intron. The results confirmed self-incompatibility in cultivars and most genotypes. However, the S_f-like allele (in size) was observed in A_9 and A_{36} genotypes. If these results are confirmed by sequencing the S_f allele, it will be first time to identify self-compatible genotype in Iranian almond genotypes.

Keywords: Almond, Incompatibility, PCR, S-alleles.

Introduction

Almond is one of the most important nut trees in the *Prunus* genus. It belongs to the Rosaceae family. Most almond cultivars and genotypes are self-incompatible andsome are cross-incompatible (Socias I Company and Alonso, 2004). Pollination, fertilization and commercial production require compatible pollen (Socias I company, 1990). Selecting cross-compatible cultivars with high quality pollen is the most important practice in almond orchard establishment (Sharafi, 2014; Kester *et al*., 1994). This trait is controlled by a single locus with multiple alleles and is expressed within the styles of flowers as S-RNAs Glycoprotein (Barklay *et al*, 2006; Wiersma *et al*, 2001; Halasz *et al*., 2007). These glycoproteins are responsible for the inactivation of self-pollen tube growth in most species of *Prunus*, including almond (Socias I Company and Alonso, 2004; Alonso and Socias I Company, 2006), apricot (Hajilou *et al*., 2006), sweet cherry (Wunsch *et al*., 2004) and plum (Yamane *et al*., 1999 Sutherland *et al*., 2004; Tamura *et al*.,1999).

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In a gametophytic incompatibility system, two genotypes with similar S-alleles cannot fertilize each other, but the presence of different S-alleles in two genotypes will result in successful fertilization. Cross-incompatibility will take place when two similar S-alleles are presented in pollen and style (Yamane *et al*., 1999; Sonneveld *et al*., 2003; Alonso and Socias I Company, 2006). Therefore, identifying cross-compatible cultivars and genotypes with favorable traits will be very beneficial for growers and breeders. However, the identification of S-alleles is essential for almond breeding programs to maximize the efficiency of crosses (Alonso and Socias I Company, 2006). Recently, methods based on DNA techniques to identify S-alleles pattern have been incorporated into fruit breeding programs in order to accelerate and optimize the determination of the (in) compatibility situation and S-alleles of fruit trees cultivars and genotypes (Sanchez-Perez *et al*., 2004; Lopez *et al*., 2006). However, controlled field and laboratory pollination are needed to confirm the effects of pollens on fruit quality and for selecting suitable pollinizers of cross-compatible cultivars and genotypes identified by PCR based methods (Lopez *et al*., 2006). Identification of incompatibility alleles in almond was amplified using degenerate primers which were designed for different species of the *Prunus* genus. Different combinations of these primers for amplification of S-alleles in almonds (Sharafi *et al*, 2010 and 2012; Sutherland *et al*., 2004; Ortega *et al*., 2005, Mousavi *et al*., 2011), cherry (Sonneveld *et al*., 2001; Sutherland *et al*., 2004) and apricot (Halasz *et al*., 2005; Zhang *et al*., 2008) were introduced. Fallah *et al.* (2014), studied pollen-pistil compatibility relationships among cultivars Tuono, "Shokofeh", "Sahand" and five improved genotypes obtained from a breeding program based on their S-alleles profiles by PCR amplification using degenerate primers included Pa ConsI-F, EM-PC1consRD, EM-PC2consFD, and EM-PC3consRD. Theoretically, the use of the different degenerate andregular primers developed by Ma and Oliveira (2001a) should allow the amplification of the S_1 and S_3self-incompatibility alleles and the self-compatibilityallele S_f in the present progenies with just three primercombinations. Moreover, it should be mentioned that self-fertile cultivars are very important for establishment mono cultivar orchards to produce uniform nut production. The self (in) compatibility situation of Iranian almond cultivars and genotypes, especially those obtained from breeding programs, has been poorly studied. Therefore, the objective of this study was the assessment of self (in) compatibility alleles in 37 Iranian almond cultivars and genotypes obtained from different regions, based PCR amplification.

Materials and Methods

Plant material and Genomic DNA extraction

The plant materials included two middle bloom almond cultivars including $Shahroudi_{21}$ and $Shahroudi_{18}$ as control and 37 Iranian genotypes (obtained from hybridization of Touno ♂ cultivar by 230♀ genotype) planted in Shahed university collection. Genomic DNA was extracted using the procedure described by Doyle and Doyle (1987). The quantity of the DNA samples was determined using 2% agarose gel electrophoresis (Table 1).

Table 1. DNA extraction buffer information.

Autoclave	Chemical substance	Final concentration	Stock contraction	For 100cc
Yes	Trise-HCl pH 8.0	100mM	2M	5cc
Yes	EDTA	20mM	0.5M	4cc
Yes	NaCl	1.4M	5M	28cc
No	CTAB	---	---	1gr
No	PVP-40	---	---	2gr
No	B- mercaptoethanol	---	---	2cc

PCR Primers

A set of four specific degenerate primers were used to amplify S-alleles in the studied cultivars and genotypes. Degenerate primers Pa ConsI-F, EM-PC1consRD, EM-PC2consFD, and EM-PC3consRD were used (Table 2). For amplification, the second intron of the forward primers EM-PC2consFD and reverse primer EM-PC3consR(Sutherland *et al*., 2004) and amplification of the first intron of the forward primers PaConsI-F (Sonneveld *et al*., 2003) and reverse primer EM-PC1consRD (Ortega *et al*., 2005) were used.

Table 2. Primers information.

Primername	Forward (F)/ Reverse (R)	Intron	Sequence	Annealing temperature
PaConsI-F	F	First	5′(C/A)CTTGTTCTGT(C/G)TTT(T/C)GCTTTCTTC 3′	57°C
EM-PC1consRD	R	First	5′ GCCA(C/T)TGTTG(A/C)ACAAA(C/T)TGAA 3′	57 °C
EM-PC2consFD	F	Second	5′ TCAC(A/C)AT(C/T)CATGGCCTAT 3′	58 °C
EM-PC3consRD	R	Second	5′ A(A/T)(C/G)T(A/G)CC(A/G)TG(C/T)TTGTTCCATTC 3′	58 °C

S-alleles amplification

Amplification reactions were carried out in 20 µL volumes containing; 1x PCR buffer (100mMTrisHCl, pH 8, 500 mM KCl), 0.8 µL $MgCl_2$, 2 µL dNTPs, 1.2 µL of each primer (forward and reverse), 0.2 U Taq polymerase and 3 µL of genomic DNA. The PCR reaction program consisted of two minutes at 94°C for denaturation primary, in 34 cycles with 10 seconds, temperature 94°C,two minutes at 57°C and two minutes at 68°C, followed by a five minute extension at 72°C (Fallah *et al.,* 2014). After PCR, the products were stored at 4°C (refrigerator) until electrophoresis was performed (Fallah *et al.,* 2014).

Electrophoresis of PCR products

PCR products were separated in 2% agarose gel using 0.5 Tris-buffer-EDTA buffers and were stained with ethidium bromide. The gels were photographed using UV light with UVitec gel documentation. The molecular sizes of the PCR products were estimated based on 3 kbp DNA ladder plus (Fallah *et al.,* 2014).

Results

Degenerate primers used in this study were able to identify self-incompatibility alleles (S_1, S_2, S_3, S_5, S_7, S_9, S_{12}, S_{22}, S_{24}, and S_f) which reported in Fig. 1 and Fig 2. Fragments size was in the range of 330- 1300 bp in all studied genotypes. An unknown band was observed in some genotypes (Fig.1).

Fig 1. S allele pieces amplified with intron 2 amplification by EM-PC2consFD-EM-PC3consRD.

Table 3. Amplified S alleles in studied almond cultivars and genotypes

Number	Genotype	Product size (bp)	Object S allele
1	Shahroudi 18 (B1)	900 and?[4]	S_3, $S_?$
2	Shahroudi 21(B2)	1080 and 875	S_{13}, S_{24}
3	A1	400[1] and 570	S_{11}, S_6
4	A2	200-350[1,2] and 1300	S_A , S_{12}
5	A3	400[1] and 450	S_{11}, S_2
6	A4	330[1] and 800 [2,5]	S_5 , S_A
7	A5	400[1] and 620	S_{11}, S_4
8	A6	380 [2] and ?	S_A, $S_?$
9	A7	400[1] and 500	S_{11}, S_{21}
10	A9	1200 and 570	S_F, S_6
11	A10	750 and 1250[2]	S_A, S_1
12	A11	380[2] and?	S_A, $S_?$
13	A12	980[2] and 800	S_A, S_2
14	A13	500 and 570	$S_{21,}$ S_6
15	A14	400[1] and 600	S_{11}, S_{10}
16	A15	___	-----
17	A16	400[1] and 450	S_{11}, S_2
18	A17	620[1] and 550[2]	S_4, S_A
19	A18	? and 875	S?, S_{24}
20	A19	400[1] and 500	S_{11}, S_{21}
21	A20	? and 500	$S_?$, S_{21}
22	A21	260[1,2] and 500	S_A, S_{21}
23	A22	___	------
24	A23	330[1] and 530[2]	S_5, S_A
25	A24	450[1] and 700[5]	S_2, S_{11}
26	A25	450[1] and 530[2]	S_2, S_A
27	A26	450[1] and 600	S_2, S_{10}
28	A27	___	-----
29	A28	_	-----
30	A29	500[1] and 450	S_{21}, S_2
31	A30	1300 and 500	S_{12}, S_{21}
32	A31	450[1] and 500	S_2, S_{21}
33	A32	500[5,1] and 1050	S_{21}, S_{14}
34	A33	? and 500	$S_?$, S_{21}
35	A34	400[1] and 600	S_{11},S_{10}
36	A35	330[1] and 400	S_5, S_{11}
37	A36	1200 and 380	S_F, S_A

Alleles amplified with first (1) and second (2) intron primers, A; not amplified S alleles and,?: Shows unknown S- alleles.

Comparison of the allele's size with (Table 3) S-alleles in gene banks revealed that the most of the fragments were in the size range of S-alleles of the gene bank. The results showed that the second intron, which was amplified with primers (forward primer EM-pc2consFD and backward EM-pc3consR), identified cultivars and genotypes bands as being in the range 400bp to 1720 bp. Table 3 also shows that no bands were identified in four genotypes A_{15}, A_{22}, A_{27} and A_{28}, but at least one band identified in other 33 genotypes.

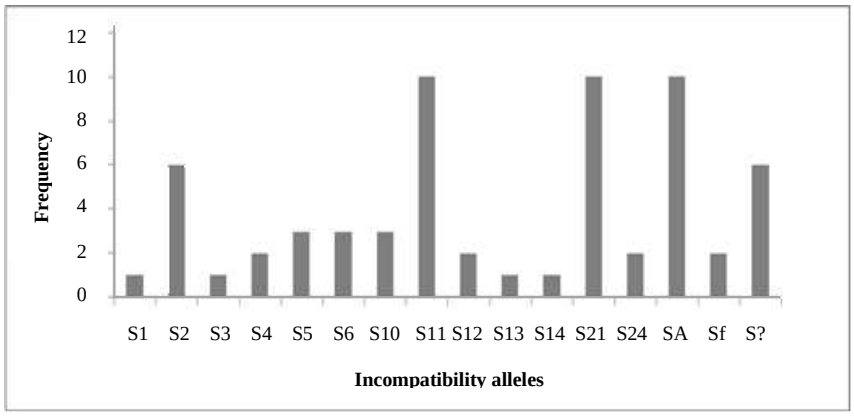

Fig 2. Frequency of S- allele's in studied almond cultivars and genotypes.

Alleles amplified with first (1) and second (2) intron primers, A; not amplified S alleles and, ?: Shows unknown S alleles.

Discussion

The fragments amplified by the four degenerate primers Pa ConsI-F, EM-PC1consRD, EM-PC2consFD, and EM-PC3consRD were in the size range of 330-1300 bp in all studied genotypes (Table 3). These four primer pairs could not amplify all S alleles in all studied genotypes. An unknown band was observed in some genotypes (Fig.1). However, in this research the primer for a second intron identified all genotypes and corresponded to research (Mousavi *et al*., 2011 and Mousavi *et al*., 2011).

Furthermore, according to these results Alonso *et al.*,(2006), studied S-genotype of some almond cultivars using four primer combinations of S3F/S3R2, S3F/ConR, ConF/S3R1 and ConF/S3R2 and amplified the S_3allele satisfactorily.

Fallah *et al*., (2014), studied pollen-pistil compatibility relationships among the cultivars Tuono, "Shokofeh", "Sahand" and five improved genotypes obtained from a breeding program based on their S-alleles profiles by PCR amplification using the same primers and reported similar results. Similar results have been reported by Mousavi *et al*., (2011) in other almond cultivars. Based on the results of amplification using degenerate primers alleles S in the first intron and second intron, all of bands obtained corresponded to the bands identified by similar research (Ortega *et al*., 2005; Mousavi *et al*., 2011). Mousavi *et al*., (2011) studied S alleles of 70 Iranian indigenous and foreign almonds using these primers and they could amplify 28 S alleles of studied cultivars.

Alonso *et al.*, (2006), studied S-genotype of some almond cultivars using four primer combinations of S3F/S3R2, S3F/ConR, ConF/S_3R1 and ConF/S3R2 and amplified the S_3allele satisfactorily. However, their study demonstrated that in S1Sf genotypes, the intensification of the S_1 piece usually covered S_f amplification. In addition, the amplification of the piece of the S_3 allele in the S_3S_f genotypes covered or silenced the intensification of the self-compatibility allele (S_f); two very close bands were sometimes seen since the molecular weights of the two fragments are very similar. Also these results show that the S_1S_f and S_1S_3 genotypes cannot be differentiated with the ConF/ConR primers. These two genotypes would not be expected to be found together in the present families, but in plants with those genotypes in which 'Tuono' is the mother plant, S_1S_f and S_fS_f could appear if accidental self-pollination occurred prior to 'Tuono' emasculation.

The specific primer mixture SfF/SfR, on the basis of the S_f intron sequences, suitably detected the self-compatibility allele (Sf) in many of the seedlings analyzed (Channuntapipat *et al*., 2003). A band corresponding to a 490-bp size piece was amplified in the self-compatible genotypes. Thus, Sf-allele specific recognition was attained using the SfF/SfR combina-

tion; the present work describes a proficient way to specifically recognize the S_3 allele.

Rahemi *et al* (2010) studied the S-alleles were in 96 wild almonds and related *Prunus* species from 10 taxonomic groups using six sets of primers including: three degenerate primer pairs (PaConsI-F(FAM)/EMPC1consRD, PaConsI-F(FAM)/EM-PC3consRD, EM-PC2consFD/EM-PC3consRD), one general primer pair AS1II/AmyC5R, one allele specific primer pair (CEBASf/AmyC5R), and one set of multiplex primers (AS1II/CEBASf/AmyC5R). Their results showed that the primers, including the allele specific (CEBASf/AmyC5R), did not amplify any self-compatibility allele (S_f) in the samples and that alleles S9, S2, S13, and S25 had the highest frequencies (12.26, 8.39, 7.74, and 7.74 percent respectively). This result was also reported by Sharafi *et al*. (2010) and Mousavi *et al*., (2014) It should be mentioned that primers and genotypes in this study was different in compared with same reported studies and differences in results is acceptable.

Conclusions

The results showed that 37 studied genotypes were clearly self-incompatible and only four genotypes were unrecognized. . These 4 primer pairs could not amplifiy all S alleles in all studied genotypes. According to many past studies it was demonstrated that the identification of S-alleles of new cultivars and genotypes, obtained from breeding programs, is very helpful for planning future breeding and orchard establishment programs, especially for speedy selecting of pollinizers. However our results, showed that as seen in many past studies the use of PCR for S-genotype detection in almond germplasm is slowly succeeding, but that more information on the behavior of the primers in different genotypes is required if efficiency is to increase. S-genotype determination by PCR in breeding progenies is most positive when the S-alleles of the parents are known and specific primers for them exist but presently this is not generally the case. However, a S_f-like allele (in size) was observed in A_9 and A_{36} genotypes. If these results are confirmed in the future studies by sequencing S_f allele, it will be the first identification of self-compatible genotype in Iranian almond genotypes.

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