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Investigation of root-knot nematode (*Meloidogyne* spp.) resistance in almond rootstocks with DNA markers

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Abstract: Seven almond varieties, Ferragnes, Nemaguard, AB3 (*Amygdalus orientalis* Mill.), GN22, GF677, Myrobalan (*Prunus cerasifera*), and *Pissardi nigra*, and F_1 crossbreed almonds, Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, Ferragnes × GN22, Nemaguard × GF677, Myrobalan × AB3 (*Prunus dulcis* Mill.), Myrobalan × Ferragnes, *Pissardi nigra* × AB3, and GF677 × Myrobalan, were used to determine resistance against root-knot nematode (RKN) (*Meloidogyne* spp.) using DNA markers. Parental lines and 316 F_1 plants were tested with SSR (plgms8, plgms9, and plgms19) and STS (STS-OPS14a, STS-834b) markers. It was found that plgms19 exhibited 76%, 77.01%, 73.13%, and 86.6% inheritance of resistance in the Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, Ferragnes × GN22, and Nemaguard × GF677 F_1 populations, respectively. No resistance was detected in the Nemaguard × GF677 population with the plgms8 marker. Since the plgms19 and STS-834b primers were found to be effective at high ratios in determining resistance to RKN in the Nemaguard × GF677 F_1 population, the use of these markers could be included in breeding studies. It was also detected that the STS-OPS14a primer is appropriate to use for determining the sensitivity to RKN in almond rootstocks.

Key words: Almond, breeding, DNA marker, nematode

1. Introduction

Almond (*Amygdalus communis* L. syn. *Prunus dulcis* (Miller) D.A.Webb) is placed within the subfamily Prunoideae of Rosaceae. It is consumed as fresh and dry nuts and is also used in the food industry (Kester et al., 1991). In Turkey, fresh and dry consumption of almond is very common and almond nuts are widely used in the food industry (Akalın, 1952; Bayrak and Yılmaz, 2009). Cultivation of almond in Turkey is increasing and almonds are grown in all the regions except for the Black Sea and high plateaus. A total of 69,838 t of fruit production was reported in 2011 and the yield has been stated to increase steadily since the 1990s (http://www.turkstat.gov.tr/). Almond was grown as a hedge plant before the 1990s and from that time orchards have been planted because of the government's increasing support for almond producers.

There are serious diseases and pests in almonds that cause considerable yield losses. Among them, root-knot nematodes (RKNs) *Meloidogyne arenaria*, *M. incognita*, and *M. javanica* have been reported from the Mediterranean countries that have large almond plantations (Sasser, 1977; Lamberti, 1979). *M. arenaria* was reported from the northern part of the Mediterranean countries while *M. incognita* and *M. javanica* are distributed throughout the southern Mediterranean (Trudgill and Blok, 2001). In Turkey, *M. incognita*, *M. javanica*, and *M. arenaria* were determined as the most widespread and economically important RKN species, while *M. hapla* and *M. chitwoodi* were reported as rare species (Söğüt and Elekçioğlu, 2000; Devran and Söğüt, 2009, 2011; Özarslandan and Elekçioğlu, 2010). Söğüt and Elekçioğlu (2010) used classical and DNA-based techniques to diagnose 79 RKN population collected from different regions and hosts. They identified *M. javanica*, *M. arenaria*, *M. incognita*, and *M. chitwoodi* at rates of 24.05%, 22.78%, 17.72%, and 10.13%, respectively. Devran and Söğüt (2009) collected 95 RKN samples from vegetable growing areas of the West Mediterranean region to determine *Meloidogyne* spp. with species-specific primers and the proportions were 64.2%, 28.4%, and 7.3% for *M. incognita*, *M. javanica*, and *M. arenaria*, respectively. *M. incognita* race 2 and *M. javanica* race 1 were determined to be widespread in protected vegetables grown in the West Mediterranean region (Devran and Söğüt, 2011).

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Meloidogyne spp. has been reported at a rate of 52% from the world's agricultural areas, including vegetables, fruit trees, and ornamental plants grown in Mediterranean climates (Trudgill and Blok, 2001). Besides the yield loss that *Meloidogyne* spp. cause in stone fruits orchards, their control is cumbersome since the banning of methyl bromide (MeBr) by the Kyoto protocol due to its high toxicity (Layne, 1987, Nyczepir, 1991). In Turkey MeBr usage was prohibited in 2007 except for critical use, and therefore other fumigant applications such as dazomet (98% a.i. Basamid) in combination with solarization have been used for controlling soil-borne fungal pathogens and nematodes (Yücel et al., 2007).

An effective and environmentally friendly approach in stone fruit trees for controlling nematodes is to develop resistant rootstocks (Boerma and Hussey, 1992; Vrain, 1999, Lopez-Perez et al., 2006). Resistance specificities to *Meloidogyne* spp. in plum, almond, and peach have been well documented (Esmenjaud et al., 1997), and the *Ma* gene from the Myrobalan plum (*Prunus cerasifera*) was cloned (Claverie et al., 2011). The *Ma* gene exhibits a complete-spectrum resistance to *M. arenaria*, *M. incognita*, and *M. javanica* with several R alleles having a dominant nature (Esmenjaud et al., 1996; Lecouls et al., 1997).

Simple sequence repeats (SSRs), namely plgms8, plgms9, and plgms19, were reported to exhibit tight linkage with the *Ma* gene; among them, plgms19 cosegregated with the *Ma* loci in Myrobalan plum (Claveire et al., 2004a, 2004b). It was found that plgms8 amplified a 316-bp band in Myrobalan and was mapped to the *Ma* loci (Claveire et al., 2004b). Sequence-tagged site (STS) markers were detected to be linked to *Mia*/*mia* and *Mja*/*mja* and among them STS-OPS14a and STS-834b exhibited close linkages (Yamamoto and Hayashi, 2002).

Marker-assisted selection has great potential in plant breeding studies and selection of agriculturally important characters with DNA markers has opened new possibilities (Collard et al., 2005). The DNA markers exhibiting tight linkage to desired genes have been used as molecular tools in many crop plants (Ribaut and Hoisington, 1998), called 'molecular breeding' in plant and animal breeding (Rafalski and Tingey, 1993). Exploitation of germplasm resources and characterization of genetic variation have been studied more effectively by use of molecular markers (Tanksley et al., 1989; Tanksley and McCouch, 1997; Gur and Zamir, 2004). Techniques in genomics also have useful applications in polymorphism detection and homozygosity testing besides protection of plant biodiversity (Heckenberger et al., 2006).

In Turkey, rootstock breeding against nematodes in almonds through controlled hybridization has not been conducted and such studies are very important since almond and peach plantation productions are increasing every year. DNA-based techniques may shorten the time necessary for RKN-resistant almond rootstock breeding. In this study 7 different parents and 316 F_1 plants were tested with 5 DNA-based markers to define the effectiveness of DNA markers in RKN resistance determination. Parental lines and F_1 individuals were screened with SSR (plgms8, plgms9, and plgms19) and STS (STS-OPS14a and STS-834b) markers to explore inheritance of resistance.

2. Materials and methods

2.1. Plant materials

Ferragnes, Nemaguard, AB3 (*Amygdalus orientalis* Mill.), GN22, GF677, Myrobalan plum (*Prunus cerasifera*), and *Pissardi nigra* were used as parental lines. *P. cerasifera*, *P. nigra*, Nemaguard, and GN22 lines used in this study were selected as resistant parental lines to RKN and Ferragnes, AB3, and GF677 as susceptible to RKN (Esmenjaud et al., 1993; Atlı, 2010).

The F_1 plants screened in this study were Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, Ferragnes × GN22, Nemaguard × GF677, Myrobalan × Ferragnes, *Pissardi nigra* × AB3, Myrobalan × AB3, and GF677 × Myrobalan. Leaves of 5-year-old parental lines and 2-year-old F_1 plants were collected from the Pistachio Research Station (Gaziantep, Turkey) experimental plots in April and May, frozen in liquid nitrogen, and kept at –80 °C until use.

2.2. Genomic DNA isolation

Young leaves were used for genomic DNA isolation and the modified CTAB protocol of Doyle and Doyle (1987) was used. The amount of DNA was measured by spectrophotometric absorbance at wavelengths of 260–280 nm and the concentration was adjusted to 40–60 ng/µL.

2.3. Markers and PCR analysis

Parents and F_1 plants were tested with SSR and STS markers that were reported to have tight linkages with *Ma* genes (Lecouls et al., 1997, 2004; Claverie et al., 2004a, 2004b). Sequences of SSR (plgms8, plgms9, plgms19) and STS (STS-OPS14a, STS-834b) markers, product sizes, and binding temperatures are given in Table 1. These markers were reported to be linked to RKN resistance by Lecouls et al. (2004), Claverie et al. (2004b), and Yamamoto and Hayashi (2002). Polymerase chain reaction (PCR) analyses of parents with SSR and STS markers were conducted on RKN-resistant and RKN-susceptible parental lines and band profiles were evaluated. According to band profiles of parental lines, F_1 individuals were tested with markers. PCR amplifications with STS and SSR markers were performed in a 20-µL final volume containing 40–60 ng of genomic DNA, 0.7 U Taq polymerase (Fermentas), 0.2 µM primer, 2 mM dNTPs (Fermentas), 1.5 mM $MgCl_2$, 1X reaction buffer, and ddH2O (Claveire et al., 2004a). PCR conditions consisted of denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 70 s, 56 °C for 15 s, and

Table 1. The sequence, product sizes and binding temperatures of DNA markers (Yomamoto and Hayashi, 2002; Claverie et al., 2004b; Lecouls et al., 2004).

Markers	Primer sequences (5′-3′)	Product size	Temperature
plgms8	F:AAACAGCCAGATCCGGAGTA	316	56 °C
	R:TATAAGTCCGCCATCGCTTG		
plgms9	F:GGTGGGAGAATTCGACTATCA	156	56 °C
	R:CAACCCAATACCACGTACCC		
plgms19	F:CGGGGTTCAAACTCAACAAG	327	56 °C
	R:AGACGTGCTGCTTTGTTCAC		
STS-OPS14a	F:TTACGTGAAACAGGGAAATGAA	402	55 °C
	R:GCCTTAGTACAGCTCCGCCC		
STS-834b	F:GCAGTCAAAAATTTCAAACC	227	55 °C
	R:TCCGATTCGAGCCCACTACA		

72 °C for 75 s, with final elongation at 72 °C for 4 min. PCR products were run on 2% agarose gel and visualized using a gel documentation system under UV light. Evaluations of paternal lines and F_1 individuals were conducted by the presence (+) or absence (–) of expected band profiles and coded as R for resistant or S for susceptible, respectively. Chi-square values were determined by using SPSS 16.

3. Results

Eight different crosses (Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, Ferragnes × GN22, Nemaguard × GF677, Myrobalan × Ferragnes, *Pissardi nigra* × AB3, Myrobalan × AB3, and GF677 × Myrobalan) were screened with DNA markers to define resistance to RKN. Genotypes and observed numbers are presented in Tables 2–6. Distribution of markers for resistance and susceptibility were calculated with chi-square and P values (Tables 2–5). Myrobalan × AB3, Myrobalan × Ferragnes, *Pissardi nigra* × *AB3*, and GF677 × Myrobalan hybrids were not included in chi-square analyses due to incomplete numbers of F_1 individuals (Table 6).

plgms19 segregated with resistance in the Ferragnes × *Pissardi nigra* F_1 populations. A total of 125 F_1 individuals were screened in this cross and the rate of resistance was 76% (Table 2). Similarly, segregation with resistance was higher with plgms19 in Ferragnes × Myrobalan, Ferragnes × GN22, and Nemaguard × GF677 F_1 individuals (Tables 3–5). The highest rate of resistance (86.6%) was obtained with Nemaguard × GF677 F_1 individuals (Table 5).

plgms8 and plgms9 segregated with susceptibility in the Ferragnes × *Pissardi nigra* and Ferragnes × Myrobalan F_1 populations (Tables 2 and 3). In Ferragnes × Myrobalan, plgms8 detected 87 (100%) F_1 individuals as susceptible and the rate of susceptibility was 99.2% in Ferragnes × *Pissardi nigra*. In Ferragnes × GN22, plgms8 exhibited 100% susceptibility, whereas plgms9 detected 68.6% resistance (Table 4).

P. cerasifera, *P. nigra*, and Nemaguard and GN22 have RKN resistance but AB3, GF677, and Ferragnes were reported to be susceptible to RKN (Esmenjaud et al., 1993; Atlı, 2010). PCR profiles of parental lines and hybrid individuals are presented in Figures 1 and 2. Hybrid individuals exhibited 327-bp and 156-bp fragments with the plgms19 and plgms9 primers, respectively.

Inheritance of resistance with STS-834b was 15.2%, 9.1%, and 93.3% in Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, and Nemaguard × GF677 F_1 individuals, respectively (Tables 2, 4, and 5). STS-834b detected 100% susceptibility in Ferragnes × GN22 F1 individuals (Table 4). STS-OPS14a found 11.5%, 16%, 31.4%, and 66.6% resistant F_1 individuals in the Ferragnes × Myrobalan, Ferragnes × *Pissardi nigra*, Ferragnes × GN22, and Nemaguard × GF677 populations, respectively (Tables 2–5).

4. Discussion

This study tested Ferragnes, Nemaguard, AB3 (*Amygdalus orientalis* Mill.), GN22, GF677, Myrobalan, and *Pissardi nigra* along with F_1 individuals with DNA markers to define the inheritance of resistance to RKNs. Three SSR (plgms8, plgms19, and plgms9) and two STS (STS-OPS14a and STS-834b) markers that were reported to have tight linkage to the RKN resistance specificities were used to screen 7 parental lines and 316 F_1 individuals (Yomamoto and Hayashi, 2002; Claverie et al., 2004a, 2004b).

Table 2. Distribution of RKN resistance and susceptibility in Ferragnes × *Pissardi nigra* F_1 individuals with chi-square and P values.

Markers	Genotype*	Number observed (%)	Percentage expected
	R	95 (76%)	50
plgms19	S	30 (24%)	
	Chi-square (P value)	33.800 (P < 0.0001)	
	R	1 (0.8%)	50
plgms8	S	124 (99.2%)	
	Chi-square (P value)	121.032 (P < 0.0001)	
	R	23 (18.4%)	50
plgms9	S	102 (81.6%)	
	Chi-square (P value)	49.928 (P < 0.0001)	
	R	20 (16%)	50
STS-OPS14a	S	105 (84%)	
	Chi-square (P value)	57.800 (P < 0.0001)	
	R	19 (15.2%)	50
STS-834b	S	106 (84.8%)	
	Chi-square (P value)	60.552 (P < 0.0001)	

*R: Resistant, S: Susceptible.

Table 3. Distribution of RKN resistance and susceptibility in Ferragnes × Myrobalan F_1 individuals with chi-square and P values.

Markers	Genotype*	Number observed (%)	Percentage expected
	R	67 (77.01%)	50
plgms19	S	20 (22.99%)	
	Chi-square (P value)	25.391 (P < 0.0001)	
	R	0 (0.0%)	50
plgms8	S	87 (100.0%)	
	Chi-square (P value)	87.000 (P < 0.0001)	
	R	16 (18.4%)	50
plgms9	S	71 (81.6%)	
	Chi-square (P value)	34.77 (P < 0.0001)	
	R	10 (11.5%)	50
STS-OPS14a	S	77 (88.5%)	
	Chi-square (P value)	51.59 (P < 0.0001)	
	R	8 (9.1%)	50
STS-834b	S	79 (90.9%)	
	Chi-square (P value)	57.94 (P < 0.0001)	

*R: Resistant, S: Susceptible.

Table 4. Distribution of RKN resistance and susceptibility in Ferragnes × GN22 F_1 individuals with chi-square and P values.

Markers	Genotype*	Number observed (%)	Percentage expected
	R	49 (73.13%)	50
plgms19	S	18 (26.87%)	
	Chi-square (P value)	14.343 (P < 0.0001)	
	R	0 (0.0%)	50
plgms8	S	67 (100.0%)	
	Chi-square (P value)	67 (P < 0.0001)	
	R	46 (68.6%)	50
plgms9	S	21 (31.4%)	
	Chi-square (P value)	9.328 (P = 0.002)	
	R	21 (31.4%)	50
STS-OPS14a	S	46 (68.6%)	
	Chi-square (P value)	9.328 (P = 0.002)	
	R	0 (0%)	50
STS-834b	S	67 (100.0%)	
	Chi-square (P value)	67 (P < 0.0001)	

*R: Resistant, S: Susceptible.

Table 5. Distribution of RKN resistance and susceptibility in Nemaguard × GF677 F_1 individuals with chi-square and P values.

Markers	Genotype*	Number observed (%)	Percentage expected
	R	13 (86.6%)	50
plgms19	S	2 (13.4%)	
	Chi-square (P value)	8.067 (P = 0.004)	
	R	0 (0.0%)	50
plgms8	S	15 (100.0%)	
	Chi-square (P value)	15 (P < 0.0001)	
	R	4 (26.6%)	50
plgms9	S	11 (73.4%)	
	Chi-square (P value)	3.267 (P = 0.071)	
	R	10 (66.6%)	50
STS-OPS14a	S	5 (33.4%)	
	Chi-square (P value)	1.667 (P = 0.197)	
	R	14 (93.3%)	50
STS-834b	S	1 (6.7%)	
	Chi-square (P value)	11.267 (P = 0.001)	

*R: Resistant, S: Susceptible.

Table 6. Distribution of RKN resistance in Myrobalan × AB3, Myrobalan × Ferragnes, *Pissardi nigra* × AB3, and GF677 × Myrobalan F_1 individuals with plgms19.

	Genotype*	Number observed**	Percentage expected
Myrobalan × AB3	R	2	50
	S	2	
Chi-square (P value)		n/a	
Myrobalan × Ferragnes	R	0	50
	S	4	
Chi-square (P value)		n/a	
Pissardi nigra × AB3	R	3	50
	S	4	
Chi-square (P value)		n/a	
GF677 × Myrobalan	R	6	50
	S	1	
Chi-square (P value)		n/a	

*R: Resistant, S: Susceptible. ** n/a: Not tested.

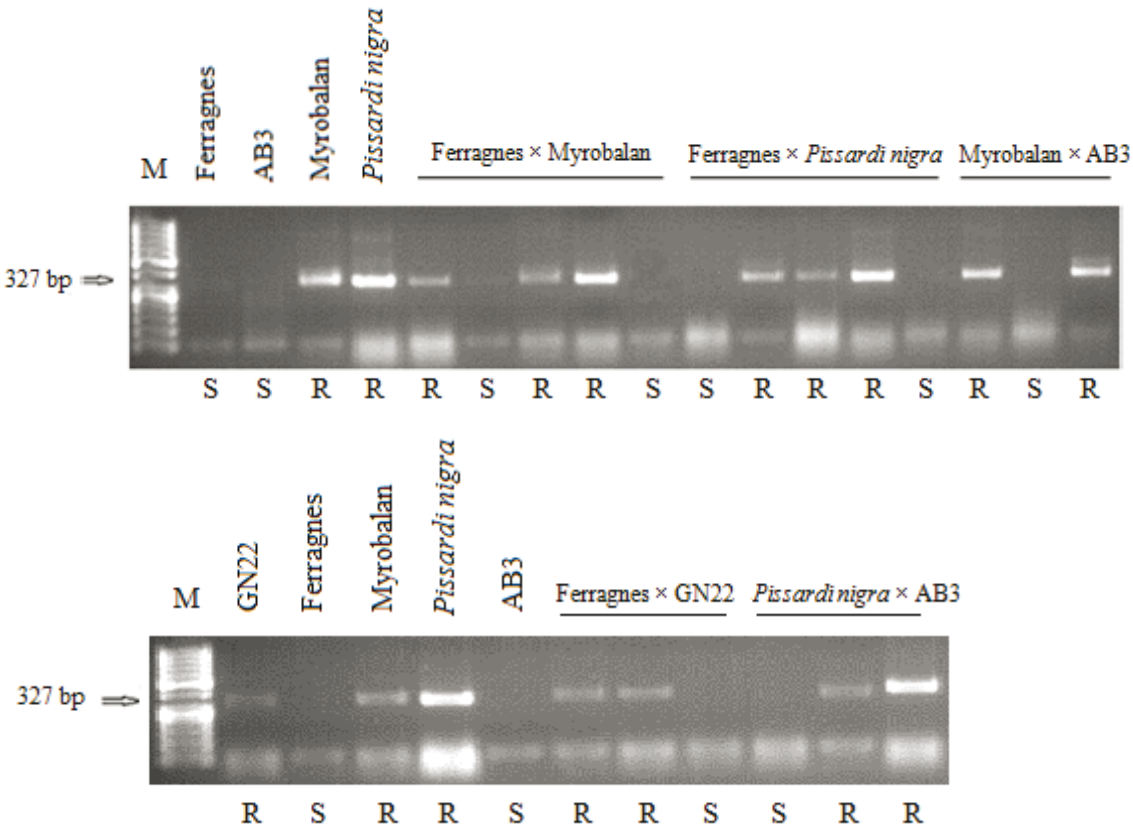

Figure 1. PCR profiles of parents and hybrid individuals with plgms19. M: 50-bp DNA molecular marker. R: Resistant, S: Susceptible.

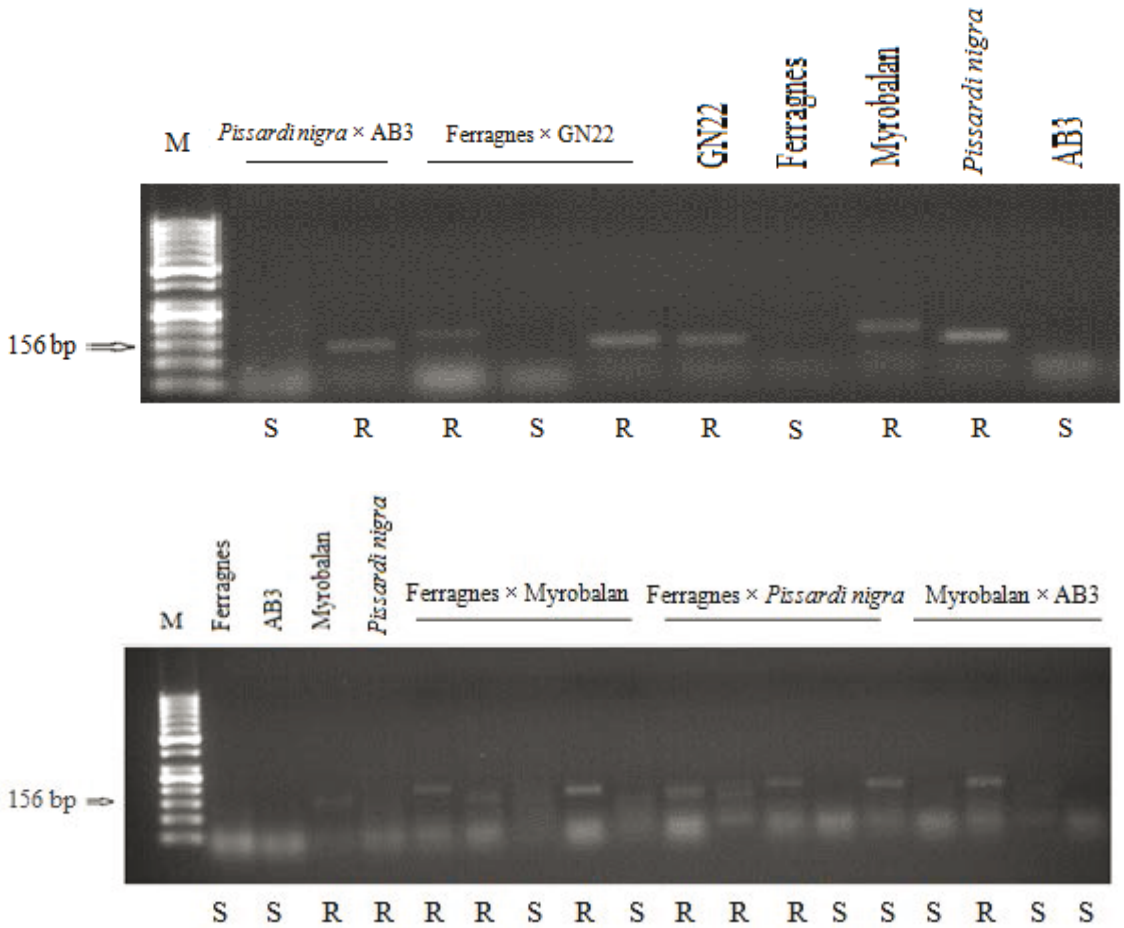

Figure 2. PCR profiles of parents and hybrid individuals with plgms9. M: 50-bp DNA molecular marker. R: Resistant, S: Susceptible.

The plgms19 marker that is linked to the *Ma* loci (Claverie et al., 2004b) resulted in statistically significant positive difference for RKN resistance in the Ferragnes × *Pissardi nigra* and Ferragnes × Myrobalan F_1 populations (Tables 2 and 3). The resistance rates were 73.13% and 86% in the Ferragnes × GN22 and Nemaguard × GF677 F_1 populations, respectively (Tables 4 and 5). Claverie et al. (2004b) used SSR markers linked to RKN resistance and determined the inheritance rate of markers with the *Ma* loci. The results obtained with the STS markers in our study exhibited rates of RKN resistance inheritance similar to that of SSR markers found by Claverie et al. (2004b).

plgms8 showed segregation with susceptible genotype in Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, and Nemaguard × GF677 F_1 populations (Tables 2, 3, and 5). Similarly, plgms9 segregation with susceptible genotype was observed in Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, and Ferragnes × GN22 F1 individuals (Tables 2–4). The plgms8 and plgms9 markers were mapped at both sides of the *Ma* region and away from the plgms19 marker defined with recombination (Claverie et al., 2004b). The segregation of plgms8 and plgms9 along with susceptibility in our F_1 population may be due to the distance of these markers from the *Ma* region compared to that of plgms19.

Yamamoto and Hayashi (2002) reported STS marker analyses to explore RKN resistance for marker-assisted analyses. Their study identified four STS markers that exhibited tight linkage in the Joseitou variety and STS-834b was reported to lie at loci that determined resistance to *M. javanica*. In our study, we have screened 125, 87, and 67 F_1 individuals with STS-834b in crosses of Ferragnes × *Pissardi nigra*, Ferragnes × Myrobalan, and Ferragnes × GN22, respectively (Tables 2–5). In all the crosses the marker segregated with RKN resistance as reported by Yamamoto and Hayashi (2002). STS-834b could be included in marker-assisted selection of almond rootstock breeding studies.

Chi-square and P value calculations were conducted for a total of 316 F_1 individuals derived from Ferragnes × Myrobalan, Ferragnes × *Pissardi nigra*, Ferragnes × GN22,

and Nemaguard × GF677 crosses (Tables 2–5). However, chi-square values were not calculated for Myrobalan × AB3, Myrobalan × Ferragnes, *Pissardi nigra* × AB3, or GF677 × Myrobalan F_1 individuals due to small numbers of F_1 individuals (Table 6). Hybridization studies should be conducted in future studies to define RKN resistance in these crosses.

We concluded that plgms19 could be used to determine RKN resistance in Ferragnes × *Pissardi nigra* and Nemaguard × GF677 hybrid individuals due to high rates of resistance genotypes obtained in this study. Further studies in F_2 and F_3 populations are necessary to determine Mendelian segregation of plgms19 in resistant individuals. In addition, in vitro RKN testing should also be conducted in these populations. Since plgms19 and STS-834b primers were found to be effective in determining resistance to RKN in the Nemaguard × GF677 F_1 population, usage of these markers could be beneficial in further studies. It was also detected that the STS-OPS14a primer is appropriate to use for determining sensitivity to RKNs in almond rootstock breeding.

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