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Reliability Assessment of Molecular Markers Linked to Resistance Genes against *Meloidogyne* spp. in Diverse Peppers Genotypes

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ABSTRACT

Root-knot nematodes (*Meloidogyne* spp.) resulted significant yield losses in peppers. It has been reported that at least 10 dominant genes (*N*, *Me1*, *Me2*, *Me3*, *Me4*, *Me5*, *Me6*, *Me7*, *Mech1* and *Mech2*) associated with resistance to *Meloidogyne* spp. are thought to act independently in gene-for-gene interactions in pepper. Several molecular markers were developed for these genes to be used in marker-assisted selection (MAS). The markers include *N*-SCAR, SCAR_B94, SCAR_CD, SCAR_PM6a, SCAR_PM6b, and SCAR_PM54. In this study, the pepper genotypes reported to carry different resistant genes against the nematodes were artificially inoculated with root-knot nematode (*Meloidogyne incognita* race 2) and compared via the linked molecular markers. Of all the markers tested, the SCAR_PM54 marker results were fully consistent with artificial nematode testing, correctly predicting resistant and susceptible genotypes. The results documented once again that the linked markers should be confirmed through classical testing before utilizing them in MAS.

Key words: Pepper**,** Root-knot nematodes, Resistance, Molecular marker.

INTRODUCTION

Root-knot nematodes (RKNs) (*Meloidogyne* spp.) are important pests significantly limiting world pepper production. Turkey ranks the third with 2 million tons/year pepper production in the world (31 million tons/year) (FAO, 2012). In field and other protected vegetable production areas, *M. arenaria* (Neal, 1889) Chitwood 1949, *M. javanica* (Treub, 1885) Chitwood 1949 and *M. incognita* (Kofoid & White, 1919) Chitwood 1949 were identified as the most common and economically important species (Sogut & Elekçioglu, 2000 and Ozarslandan & Elekçioglu, 2010). It is known that *M. incognita* is widespread over pepper cultivated areas. On the contrary, *M. javanica* was not able to propagate and damage peppers (Özarslandan & Elekçioğlu, 2003). Nematicides and fumigants are commonly used to fight against RKNs in vegetable cultivated lands. Wesemael *et al*. (2011) reported negative impacts of these chemicals on human health and environment. Researchers also indicated that crop rotation, non-host species and resistant cultivars could be effective in diminishing root-knot nematode infections, but host status should be known to get success in nematode control. Resistant pepper lines may restrict the reproduction of *M. incognita* and be effective in control of the nematode. Therefore, resistant lines and cultivars are used in integrated pest management programs together with solarization, crop rotation and other control procedures (Ozarslandan *et al*., 2015).

There are different non-allelic nematode resistance genes in pepper. The first gene is called *N*-gene. Pepper *N*-gene provided genetic resistance against *M. arenaria*, *M. incognita* and *M. Javanica* and showed that such a resistance mostly depends on nematode isolate and inoculum levels (Thies *et al*., 2008). N-gene was identified for the first time in Mississippi Nemaheart genotype and the resistance was transferred to sensitive Carolina Wonder and Charleston Bell pepper cultivars through backcross breeding. It was determined that such resistance was effective and was not broken up at even 32°C (Thies and Fery, 2002). The 10 dominant genes (*N*, *Me1*, *Me2*, *Me3*, *Me4*, *Me5*, *Me6*, *Me7*, *Mech1* and *Mech2*) that acting independently in gene-for-gene interactions were related to resistance against *Meloidogyne* spp. in pepper (Djian-Caporalino *et al*., 2001, 2007 and Wang & Bosland, 2006). The resistance against the nematode is generally characterized by a localized hypersensitive reaction, which prevents the development and reproduction of the nematode (Pegard *et al*., 2005 and Djian-Caporalino *et al*., 2007). Some highly species-specific *Me* genes for particular *Meloidogyne* species or populations exist but others provide resistance against a broad spectrum of RKNs (Djian-Caporalino *et al*., 1999). According to histological studies, these genes had different direct response patterns in root cells just based on pepper line and nematode species (Pegard *et al*., 2005). Such a response commonly varied based on plant genotype, resistance gene and RKN species (Williamson, 1999 and Pegard *et al*., 2005).

Various molecular markers and marker-assisted selection (MAS) procedures were developed to identify the genes for RKN resistance (Djian-Caporalino *et al*., 2001& 2007 and Farazi *et al*., 2012). *N*-gene reported in Mississippi Nemaheart (Hare, 1957), introduced and developed through two resistant inbred lines (Carolina Wonder and Charleston Bell) (Fery *et al*., 1998). Moreover, *N* and *Me3* genes are distinct R-genes using the allelism tests (Thies and Ariss, 2009). Wang *et al*. (2009), Farazi *et al*. (2012) and Gisbert *et al*. (2013) developed Sequence-Characterized Amplified Region (SCAR) markers for *N*-gene. The reliability of markers linked to pests and disease resistance traits should be tested at different genetic background before using them in MAS.

The objective of this study was to confirm the reliability of the molecular markers linked to resistance genes for RKN in pepper.

MATERIALS AND METHODS

Plant materials

They included 6 advanced lines developed in Alata Horticulture Research Institute, pepper breeding program, and Yolo Wonder B, California Wonder 300 (TMR), PM 687, PM 217, Criollo De morelos 331 and 2 Carolina Cayenne pepper genotypes were provided by world vegetable center (AVRDC, Taiwan) and reported to be resistant against *M. incognita*. Six known advanced resistant pepper lines that developed successive artificial nematode inoculations by Alata Horticulture Research Institute (Erdemli, Mersin, Turkey) pepper breeding program were tested with different markers developed for *Me* and *N* genes. Results were compared to identify useful markers for pepper breeding programs.

Nematode tests

Pepper seedlings were planted into pot soil mixture containing 80% sand, 5% silt and 15% clay, autoclaved at 121ºC for 1 hour. Testing was performed in growth chambers at 25±1ºC, 60±10% R.H., and 16/8 hours light/dark conditions. The root-knot nematode (*M. incognita* race 2) used in experiments was reproduced in a known nematode-susceptible pepper genotypes that belong to Alata Horticultural Research Institute. For root-knot nematode production, egg masses were removed from the roots of the plants under a stereo binocular and 2nd stage infective larvae were obtained using advanced Baermann-apparatus. Experiments were designed in randomized plots with 4 replications containing 5 plants when the pepper seedlings reached 2-leaf stage and around 15 cm height. About 1000 of the 2nd instar larvae were inoculated at 2 cm soil depth at four sides of pepper seedlings. After 60 days of root-knot nematode inoculation, plant roots of 14 pepper genotypes were assessed through "0-5 galls and egg masses rating scale" created by (Hartman & Sasser, 1985). According to assessments, the plants with a score of 0-2 were considered as resistant and with a score of 3-5 as sensitive. The scale was arranged as follows: 0 = no gall or egg mass development in roots; 1 = presence of 1-2 galls and egg mass developments in roots; 2 = presence of 3-10 galls and egg mass developments in roots; 3 = presence of 11-30 galls and egg mass developments in roots; 4 = presence of 31-100 galls and egg mass developments in roots and 5 = presence of more than 100 galls and egg mass developments in roots.

Molecular marker analyses

DNA isolations were performed from pepper leaves with *CTAB* method (Doyle and Doyle, 1987). Then, DNA amplification was carried out as described by (Wang *et al*. 2009) for the *N* gene and by (Djian-Caporalino *et al*. 2007) for *Me* genes, with the exception of the annealing temperature: 57°C for the *N*-SCAR marker, 54°C for SCAR B94 (*Me3-Me4*), 55°C for SCAR_CD (*Me1-Mech2*, *Me7-Mech1*), 49°C for SCAR_PM6a, 61°C for SCAR PM6b and 54°C for SCAR_PM54 (Farazi *et al*., 2012). Amplified products were separated on 1.5–3% agarose gels for *Me1*, *Me7* and *Me3* genes and 2% metaphor agarose gel for *N* gene in 1×TAE buffer stained with ethidium bromide. All PCR reactions were repeated twice.

RESULTS AND DISCUSSION

Breeding programs, especially breeding for disease resistance, benefited tremendously from the use of molecular markers that have reduced the laboratory cost and time requirements. However, before the initiation of breeding programs, molecular markers should be tested and the most reliable ones should be utilized in MAS. In the present study, 8 root-knot resistant pepper lines supplied by AVRDC and 6 advanced breeding lines were tested by 6 different molecular markers reported to have tight linkages to the genes and results of marker genotypes were compared against results of artificial nematode testing.

The pepper genotypes Yolo Wonder B, California Wonder 300 (VI014967) and California Wonder 300 (VI014977) showed galling score values around 5.0, indicating a high sensitivity (Table 1). The SCAR_CD, SCAR_N, SCAR_PM6b and SCAR_PM54 markers correctly determined susceptible genotypes except for Criollo De morelos 331. The SCAR_B94, SCAR_N, SCAR_PM6a and SCAR_PM6b markers, however, wrongly indicated

this genotype as resistant. Yolo Wonder was reported to carry *Me5* (*M. javanica* Abou Dhabi) gene and would be expected to be sensitive to other *Meloidogyne* spp. Therefore, it is concievable that the markers used, in this study, failed to detect *Me5* gene. In addition, the sensitivity of California Wonder genotypes was in agreement with the results of nematode testing and with the marker genotypes (Thies *et al*., 1997& 1998).

The galling index value was 0.00 for PM687 (VI011987) genotype. The PM687 genotype is known to contain *Me3* and *Me4* genes and is resistant to *M. incognita, M. javanica, M. arenaria* and *M. arenaria* Ain Taoujdate while sensitive against *M. chitwoodi* (Djian-Caporalino *et al*. 1999, 2001 and 2007). Although the marker genotypes obtained with SCAR_CD showed susceptibility, while SCAR_B94 marker as resistant. Because Djian-Caporalino *et al*. (2007) reported that SCAR_B94 marker linked to *Me3*/*Me4* genes that derived from PM687 genotype, and SCAR_CD marker is linked to *Me1* ve *Me-7* genes, so, SCAR_CD marker was not expected to detect the *Me3* and *Me-4* genes. Other markers predicted it correctly to be homozygous resistant (Table 1, Figs. 1 and 2). Current findings concur with the results of Djian-Caporalino *et al*. (2007) who tested 127 F3 families from the (PM687 X PM217) cross for resistance to *M. incognita* race 2. The researchers

Table (1): Resistance status of pepper genotypes with different markers and artificial tested nematode

No.	VI/Accession number	Genotype name	SCAR_CD	SCARB94	SACR_N	PM6a	PM6b	PM54	Galling score (0-5)
			Djian-Caporalino, *et al*. 2007		Wang *et al*., 2009		Farazi *et al*., 2012		
1	VI014374	Yolo Wonder B	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	4,2±0,56
2	VI014967	Calıfornia Wonder 300	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	5,0±0,00
3	VI014977	Calıfornia Wonder 300	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	5,0±0,00
4	VI011987	PM 687	Sensitive	Resistant	Resistant	Resistant	Resistant	Resistant	0,0±0,00
5	VI027997	Criollo De Morelos 331	Sensitive	Resistant	Resistant	Resistant	Resistant	Sensitive	5,0±0,00
6	VI014938	PM 217	Resistant	Sensitive	Sensitive	Sensitive	Heterozygote	Resistant	0,0±0,00
7	VI041276	Carolina Cayenne	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	0,0±0,00
8	VI044362	Carolina Cayenne	Sensitive	Sensitive	Sensitive	Resistant	Resistant	Resistant	0,0±0,00
9	PL1	Advanced lines	Resistant	Sensitive	Resistant	Heterozygote	Resistant	Resistant	0,0±0,00
10	PL2	Advanced lines	Resistant	Sensitive	Resistant	Heterozygote	Resistant	Resistant	0,0±0,00
11	PL3	Advanced lines	Resistant	Sensitive	Resistant	Heterozygote	Resistant	Resistant	0,0±0,00
12	PL4	Advanced lines	Resistant	Resistant	Resistant	Heterozygote	Resistant	Resistant	0,0±0,00
13	PL5	Advanced lines	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	0,0±0,00
14	PL6	Advanced lines	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	Sensitive	5,0±0,00

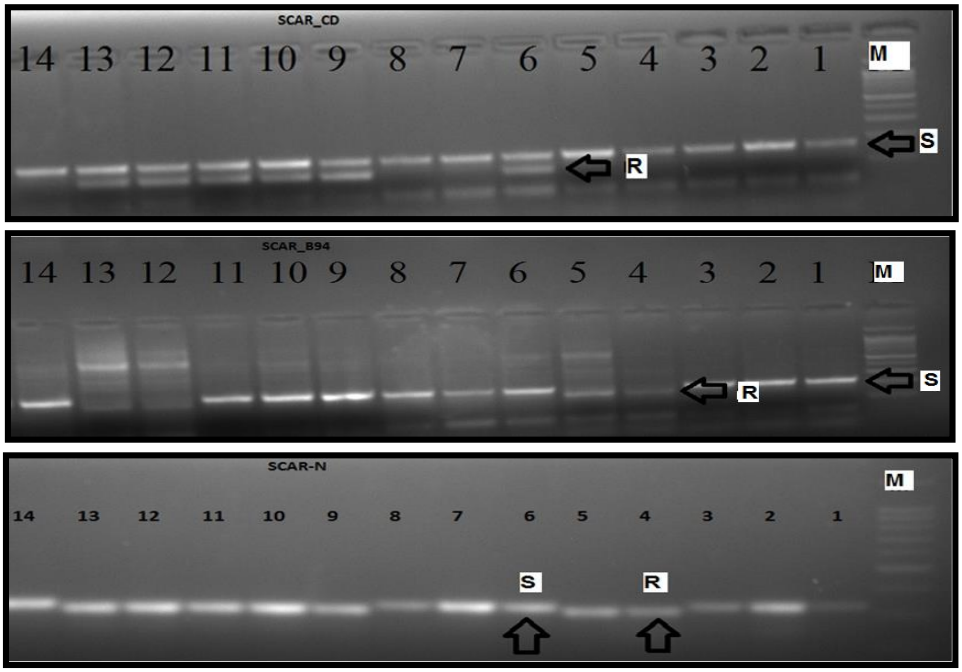

Fig. (1): SCAR_CD, SCAR_B94 and SCAR_N markers for pepper resistance against *Meloidogyne incognita* race 2. 1-14 are pepper genotypes: 1-14: 1. Yolo Wonder B, 2. California Wonder 300(VI014967), 3. California Wonder 300(VI014977), 4. PM 687, 5. Criollo De Morelos 331, 6. PM 217, 7. Carolina Cayenne(VI041276), 8. Carolina Cayenne (VI044362), 9. PL1, 10. PL2, 11. PL3, 12. PL4, 13. PL5, 14. PL6.

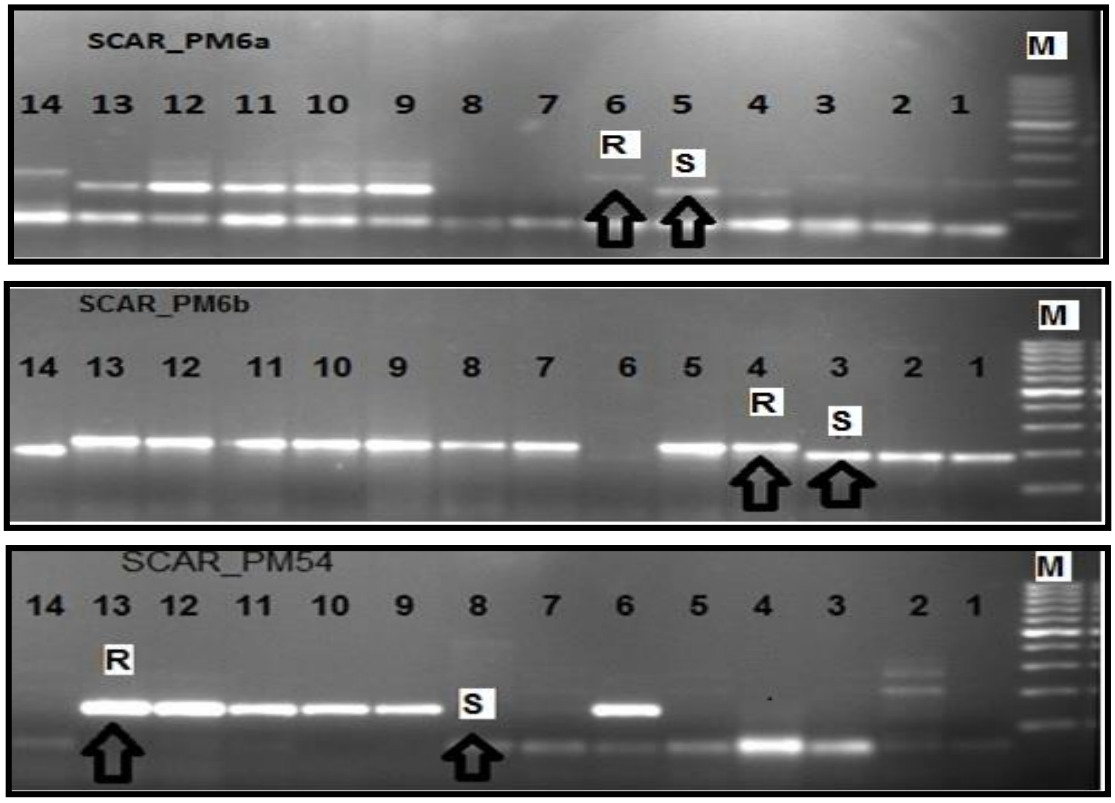

Fig. (2): SCAR_PM6a, SCAR_PM6b, SCAR_PM54 for pepper resistance against *Meloidogyne incognita* race 2. 1-14 are pepper genotypes: 1. Yolo Wonder B, 2. California Wonder 300 (VI014967), 3. California Wonder 300 (VI014977), 4. PM 687, 5. Criollo De Morelos 331 6. PM 217, 7. Carolina Cayenne (VI041276), 8. Carolina Cayenne (VI044362), 9. PL2, 10. PL4, 11. PL3, 12. PL5, 13. PL1, 14. PL6.

stated that PM687 (inbred line from PI 332719) had *Me3* and *Me-4* genes and showed that these two genes were not allelic, but linked with an estimated recombination rate of 9%.

The PM217 genotype carries *Me1/Me7* genes and was resistant to *M. incognita, M. javanica, M. arenaria* and *M. chitwoodi*, while it was sensitive against *M. arenaria* Ain Taoujdate (Djian-Caporalino *et al*., 1999, 2001& 2007). The SCAR_N marker predicted a susceptible genotype where SCAR_CD and PM54 markers indicated it as resistant and PM6b marker as heterozygote, while the remaining markers predicted it to be sensitive. SCAR_CD and PM54 markers linked to *Me1*/*Me-7* genes.

Carolina Cayenne (VI041276) and Carolina Cayenne (VI044362) pepper genotypes were identified as highly resistant in nematode testing, while SCAR_CD, and SCAR_N marker genotypes indicated homozygote sensitive genotypes. But PM6a, PM6b and PM54 markers showed them to be homozygote resistant (Table 1). Thies *et al*. (1997) stated that Carolina Cayenne genotypes carrying *N* gene. Fery and Dukes (1996) and de Souza-Sobrinho *et al*.(2002) exhibited exceptionally high resistance with minimal galling, minimal nematode reproduction and no yield reduction to *M. incognita*. Although SCAR_N markers linked to N genes, couldn't be identify resistance allele with this marker. Farazi *et al*. (2012) indicated that the rate of recombination between PM6a and PM6b was 2.5, 2.2%, respectively. Thus, PM6a and PM6b markers kept their linkage to the gene and to each other in these backgrounds.

Yolo Wonder B, two California Wonder 300 genotypes (VI014967 and VI014977) and Criollo De Morelos 331 (VI027997) and PL6, an advanced pepper line, were identified as sensitive in artificial nematode testing with galling index of 5.0 (Table 1). All the markers in this study correctly identified these genotypes to be homozygote sensitive while SCAR_N, SCAR_B94, PM6a and PM6b markers yielded a resistant genotype only in Criollo De Morelos 331 (VI027997). The marker genotypes correctly yielded sensitive alleles with SCAR_CD and SCAR_PM54 markers for Yolo Wonder B, California Wonder 300 (VI014967), California Wonder 300 (VI014977) and Criollo De Morelos 331 (VI027997) and for PL6 (Table 1).

In the present study, the 6 advanced pepper lines were tested with nematode and genotyped with the molecular markers. While the resistant lines PL1, PL2, PL3, and PL4 were identified as heterozygote resistant with SCAR_PM6a markers, they were identified as homozygote resistant with SCAR_B94, SCAR_N, SCAR_PM6b and SCAR_PM54 markers. The susceptible PL6 line was identified as sensitive with all the markers. The PL1, PL2 and PL3 advanced lines may have *Me1*/*Me7* and N genes, where SCAR_CD linked with Me1/Me7 genes and SCAR_N linked with *N* gene. Also the PL4, and PL5 lines possessed *N* and *Me3*/*Me4* genes, those linked with SCAR_N and SCAR_B94 markers but don't carry *Me1*/*Me7* resistance.

When marker genotypes were compared against artificial nematode testing results, SCAR_PM54 marker were in perfect agreement for both sensitive and resistance background in these pepper genotypes. The SCAR_PM6b marker yielded the resistant marker allele in susceptible Crillo De Morelos 331 genotype. The marker allele for susceptibility was obtained with SCAR_CD markers (linked to *Me1* and *Me7*) in resistant PM687 and two Carolina cayenne genotypes. The reason for discrepancy could be due to the fact that PM687 genotype carries *Me3* and *Me4* genes and Carolina genotypes the *N* gene (Fery and Dukes, 1996; de Souza-Sobrinho *et al*., 2002; and Djian-Caporalino *et al*., 1999, 2001 and 2007). In addition to that the rate of recombination of SCAR_PM6b was 0.9% (within 109 lines), 4.3% (within 92 lines) and 2.2% (within 132 lines) for *Me3*, *Me7*, and *N* genes, respectively (Farazi *et al*., 2012). Some of the differences could be attributed to recombination events between markers and the genes. Farazi *et al*. (2012) reported that there were recombination of SCAR_PM6a with 0.9% (within 110 lines), 5.6% (within 71 lines), and 2.5% (within 120 lines) for *Me3*, *Me7* and *N* genes, respectively. The nematode testing and marker genotypes of SCAR_N marker are in agreement in the pepper genotypes except for Crillo De Morelos 331 genotype.

Pepper breeding for resistance against plant parasitic nematodes is difficult. The combination of more than one *R* gene is needed for resistance against RKNs in pepper. Pyramiding of *Me* genes would prevent the resistance to be broken by root-knot nematodes (Djian-Caporalino *et al*., 2007). Hence, it is rather important to pyramid *Me* genes in breeding programs and to test these genotypes with artificial inoculation and with the most robust molecular markers. Pyramiding of *Me* genes is rather difficult in pepper breeding programs. That's why gene-specific markers tightly linked to *Me* genes are useful to screen a great number of recombinant genotypes.

In conclusion, 6 markers developed for *N* and *Me* genes were tested in 14 pepper genotypes. Results of marker genotypes data were compared against the results of artificial inoculation. Although different results were observed by the molecular markers, the most robust marker was SCAR_PM54 for pepper genotypes used. Molecular markers linked to *Me* and *N* genes should be first tested in breeding populations, confirmed by artificial nematode testing in a population specific manner and then be proceeded with the most reliable gene specific markers in a given breeding population. Yolo Wonder B, California Wonder 300 (TMR), PM 687, PM 217, Criollo De morelos 331 and Carolina Cayenne would be useful as control genotypes when employing molecular markers and/or nematode inoculations in breeding studies.

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