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The Screening of F_2 Plants for the Root-Knot Nematode Resistance Gene, Mi by PCR in Tomato

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Abstract: Root-knot nematodes are major pests of field and vegetable crops in Turkey and worldwide. They cause damage to many economically important horticultural crops like potato, cotton and tomato. Tomato is one of the crops in which genetic resistance has been especially effective against root-knot nematodes. In the 1940s the root-knot nematode resistance gene (Mi) was introgressed into the cultivated tomato from the wild species *Lycopersicon peruvianum*. Today, many commercial tomato varieties carry the Mi gene, which has been mapped. This gene confers resistance to *Meloidogyne incognita, M. javanica* and *M. arenaria*. The short arm of chromosome 6 and many markers linked to Mi have also been identified. The Mi gene has been isolated, cloned and sequenced. In this study, plants were infected with *M. incognita* race 2 and resistant and susceptible lines were determined. According to nematode resistance assays, the root-gall index was determined as > 2 and ≤ 2 for susceptible and resistant plants, and reproduction factors were 0 and > 1 for resistant and susceptible plants, respectively. In conjunction with traditional screening Mi gene specific primers were used to differentiate between resistant and susceptible plants with a 1.6 kb DNA band being detected in resistant plants but absent in susceptible plants. The data showed a clear correlation between traditional screening and the use of markers and support the possibilities of using marker assisted selection for *M. incognita* resistance breeding.

Key Words: Mi gene, root-knot nematodes, resistance

Domateste Kök-Ur Nematodlarına Dayanıklılık Geni (Mi) Taşıyan F_2 Bitkilerinin PCR ile Taranması

Özet: Kök-ur nematodları, Türkiye'de ve Dünya'da tarla ve sebze bitkilerinin en önemli zararlılarındandır. Domates, patates, pamuk ve gibi ekonomik öneme sahip bir çok bitkide zararlara neden olurlar. Domates, kök-ur nematodlarına karşı özellikle etkili genetik dayanıklılığı bilinen ürünlerden biridir. Kök-ur nematodlarına dayanıklılığı sağlayan Mi geni, 1940'lı yıllarda yabani domates olan *Lycopersicon peruvianum*'dan kültür domatesine aktarılmıştır. Bugün, ticari tomates çeşitlerinin çoğu bu geni taşımaktadır. Bu gen *M. incognita, M. javanica, M. arenaria* karşı dayanıklılık sağlamaktadır. Mi geni 6. kromozomun kısa kolu üzerine lokalize olmaktadır. Bu kromozom haritalanmış ve Mi genine bağlı bir çok marker tanımlanmıştır. Daha sonra, Mi geni, izole edilip klonlanarak dizilimi belirlenmiştir. Bu çalışmada, domates bitkisi *M. incognita* ile infekte edilip dayanıklı ve duyarlı hatları belirlenmiştir Nematoda dayanıklılık testleri sonucuna göre, Kök-ur indeksi değerinin duyarlı bitkilerde 2'den büyük, dayanıklı bitkilerde ise 2 ve 2'den küçük olduğu saptanmıştır. Üreme oranları ise dayanıklı bitkilerde 0, duyarlı bitkilerde ise 1'den büyük bulunmuştur. Ayrıca Mi genine özgü primerler kullanılarak dayanıklı ve duyarlı bitkiler PCR ile belirlenmiştir. 1.6 kb DNA bandı dayanıklı bitkilerde bulunurken duyarlı bitkilerde bulunmamıştır. Burada elde edilen veriler ışığında, geleneksel olarak kullanılan dayanıklılık testleri ile moleküler çalışmaların birbirlerini desteklediği ve bu primerlerin ıslah programında uygulanabileceği sonucuna varılmıştır.

Anahtar Sözcükler: Mi geni, kök-ur nematodları, dayanıklılık

Introduction

Root-knot nematodes, *Meloidogyne* spp., are obligate, sedentary endoparasites of many plant species. Their potential host range encompasses more than 3000 plant species (Abad et al., 2003). The most economically important species are *M. arenaria* Chitwood, *M. javanica* Chitwood and *M. incognita* Chitwood. They cause serious damage to tomato crops (Lamberti, 1979), especially in tropical, sub-tropical and warm climates. In Turkey, the Mediterranean region is the most important for protected

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and open field vegetables. *M. arenaria*, *M. javanica* and *M. incognita* are among the major economic problems facing crop production in this region (Elekçioğlu and Uygun, 1994; Elekçioğlu et al., 1994; Söğüt and Elekçioğlu, 2000).

Host-plant resistance to root-knot nematodes is a powerful tool for crop protection, and it is destined to play a more important role than ever before in managing nematode problems in sustainable agriculture. The most effective nematicides have been restricted in agriculture because of the high risk to human health and the environment (Veremis and Roberts, 1996).

Many commercial tomato varieties carry a single, dominant gene called Mi which confers resistance to 3 of the most damaging species of root-knot nematodes (*M. incognita*, *M. javanica* and *M. arenaria*). This resistance is associated with the localised death of host tissue near the invading nematode in the root tips. The Mi gene was discovered 50 years ago in an accession (P.I. 128657) of *L. peruvianum* (Mill.), a wild relative of the edible tomato (*L. esculentum* Mill.) that was grown in the western coastal region of South America (Cap et al., 1991). This resistance was transferred and expressed in F_1 plants derived from a cross between *L. peruvianum* P.I. 128657 and *L. esculentum* 'Michigan State Forcing' made by Smith (1944).

The Mi gene is located on the short arm of chromosome 6. This chromosome has been mapped in considerable detail, and multiple markers for other traits linked to Mi have been identified (Messequer et al., 1991; Williamson et al., 1994). Recently, the Mi gene was isolated by a positional cloning approach (Kaloshian et al., 1998; Milligan et al., 1998) and DNA sequence analysis was carried out to identify Mi candidates. Sequencing revealed 2 genes, Mi-1.1 and Mi 1.2, that were 95% identical to each other, and encoded proteins with a high similarity to previously cloned plant resistance genes. Complementation analysis showed that the introduction of Mi-1.2, but not of Mi 1.1, to susceptible tomato plants was sufficient to confer a nematode-resistant plant phenotype with the same spectrum resistance as that of Mi (Milligan et al., 1998).

The objective of this study was to screen F_2 plants' resistance to *M. incognita* race 2 derived from a cross of line A (resistant) with line B (susceptible) by PCR.

Materials and Methods

Plant Materials

F_2 plants were obtained from a cross of line A and line B. The tomato lines used in this study are listed in Table 1. Line A (resistant) is derived from a commercial F_1 hybrid (Astona ReNe, Nunhems Seed Grower) containing the Mi gene, and line B also comes from a commercial seed firm (Anamas Seed-Grower).

Nematode cultures

M. incognita race 2 was described based on perineal patterns and the host range by İ.H. Elekçioğlu (Çukurova University, Plant Protection Department, Adana). *M. incognita* race 2 culture was maintained on susceptible tomato (Simita F1, Nunhems, Seed Grower) in flowerpots containing a sterile, moist loamy soil (80% sand, 15% silt, and 5% clay) in a growth chamber for 6 weeks at 22-26 °C on a 16 h of light regime.

Screening Tests

The following methods were used for nematode inoculation: (1) two seeds were sown in a 15 cm diameter flowerpot containing a sterile, moist loamy soil (80% sand, 15% silt, and5% clay), (2) plants were thinned to one per pot at the second true leaf stage, (3)

Table1. Screening F_2 plants for nematode resistance.

Plant	Gall index (1-10 scale)	Response to root-knot nematode
Line A (parent)	1	R
Line B (parent)	5	S
Line AB_1	1	R
Line AB_2	1	R
Line AB_3	1	R
Line AB_4	1	R
Line AB_5	1	R
Line AB_6	2	R
Line AB_7	1	R
Line AB_8	1	R
Line AB_9	2	R
Line AB_{10}	1	R
Line AB_{11}	1	R
Line AB_{12}	6	S
Line AB_{13}	5	S

R: Resistant
S: Susceptible

M. incognita race 2 inoculum was produced in the growth chamber on susceptible tomato, (4) nematode eggs were extracted from roots, and (5) each plant was inoculated at the second-fourth true leaf stage with 1000 J_2 of the *M. incognita* race 2. Plants were harvested 8 weeks after the inoculation and evaluated according to the method of Barker et al. (1985). Plants were classified as resistant (≤ 2 root gall index) or susceptible (> 2 root gall index) based upon the distribution of F_2 plants infected with *M. incognita* race 2. In addition, reproduction factors were calculated as the final level of J_2 in soil / 1000 J_2 (the amount of J_2 in each flowerpot that was initially inoculated with *M. incognita* race 2).

DNA isolation

Total DNA was extracted by the method of Doyle and Doyle (1987) with minor modifications. Fresh leaf tissue was frozen in liquid nitrogen and ground using an Eppendorf tube and glass bar. The homogenate of the leaf tissue was placed in a tube together with 500 µl of DNA extraction buffer [2% (w/v) CTAB (hexadecyltrimethylammoniumbromide), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, and 100 mM Tris-HCl, pH 8.0], and incubated in a water bath at 60 °C for 30 min with occasional swirling. After incubation, the lysate was extracted once with chloroform:phenol (1:1, v/v). The aqueous phase was mixed with a two-thirds volume of cold isopropanol. The precipitated DNA was resuspended in TE (10 mM Tris pH 8.0, and 0.1 mM EDTA).

PCR analysis

PCR amplification was carried out in a 25 µl solution containing 10 ng of DNA, 2 mM $MgCl_2$, 200 µM dNTP (Bioline, UK), PCR Buffer (Bioline, UK), 0.4 mM each of the primers C1/2 (5'-cagtgaagtggaagtgatga-3') and C2S4 (5'-ctaagaggaatctcatcacagg-3') (Milligan et al., 1998), 1 unit of Taq DNA polymerase (Bioline, UK) and deionised water. PCR was conducted with a DNA Engine Dyad Peltier Thermal Cycler (MJ. Research, USA) using the following cycling profile: one cycle of 30 s at 94 °C, 30 s at 65 °C, and 60 s at 72 °C, followed by 11 cycles of 1 °C lower annealing temperature each and 24 cycles of 30 s at 94 °C, 30 s at 56 °C, 60 s at 72 °C. After completion of the PCR, 5 µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% sucrose) was added to each reaction tube. The samples were electrophoresed in 1.5% agarose gel in TAE buffer and stained with 0.5 µg/µl of ethidium bromide. The gel was illuminated by ultraviolet light, and photographed (Figure 1)

Results and Discussion

Root-knot nematodes of the genus *Meloidogyne* are economically important pathogens of a wide range of crops (Sasser and Carter, 1985). Infective second-stage juveniles of these obligate endoparasites penetrate the roots of the host and migrate intercellularly to the vascular cylinder (Williamson and Hussey, 1996). The primary symptom of root-knot nematode infection is the formation of typical root galls on the roots of susceptible

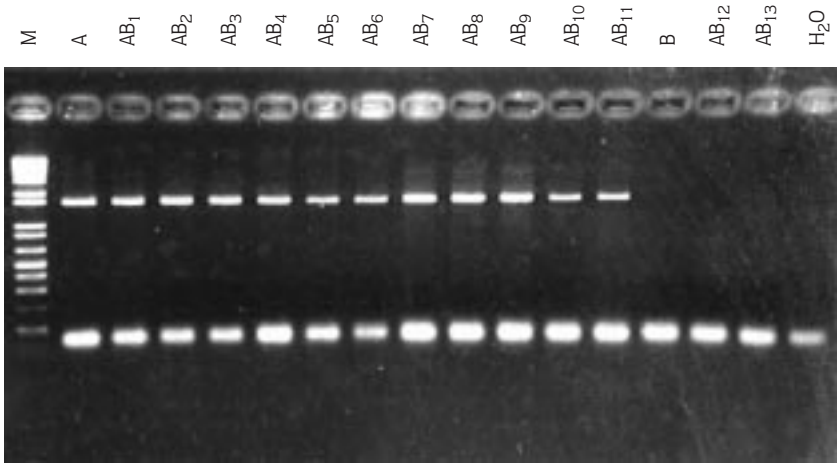

Figure 1. Mi gene amplification products obtained using C1/2 and C2S4 primers.

host plants. Their invasion of the root system of host plants results in a shallow, knotted root system and susceptibility to other pathogens. Nutrient and water uptake are substantially reduced because of the damaged root system, resulting in weak and poor yielding plants (Abad et al., 2003).

Recently, Turkish and foreign firms have developed fresh market tomato varieties with resistance to root-knot nematode, and these have become commercially available as an option for nematode management. In these resistant varieties, nematodes fail to develop and reproduce normally within the tomato root tissues, allowing plants to grow and produce fruit even though nematode infection of roots occurs.

In this study, the homozygous resistant line A carrying the Mi gene, the homozygous susceptible line B and 13 F_2 plants from a crossing of line A and line B were used to test resistance to *M. javanica* race 2. Fallowing bioassay, 11 resistant, and 2 susceptible strains were determined (Table 1). The resistant parent and F_2 plants had a mean gall index value of 1. The susceptible parent (line B) and F_2 plants had a gall index value that averaged 5 (Table 1). This is in agreement with similar studies under greenhouse conditions (Cap et al., 1993; Yaghoobi et al., 1995).

In molecular screening, A, B and 13 F_2 individuals of the AB family were examined with a C1/2 and C2S4 primer combination. The DNA banding patterns of PCR amplification products correlated well with the known resistant or susceptible phenotypes, which were previously evaluated in an inheritance study under greenhouse conditions. A 1.6 kb amplification which corresponded to a portion of the 3' region of the gene was detected in parent (parent) and F_2 plants containing the Mi-1.2 gene (Figure 1) by PCR. However, the 1.6 kb PCR product was absent in susceptible parents and F_2 plants. This is in agreement with the results obtained by Milligan et al. (1998) and Rossi et al. (1998).

REX-1 marker is very closely linked to the Mi gene. The parents and their F_2 progeny were examined with the PCR-based primers REX-F1 and REX-R2. One major DNA band (approximately 750 bp) was amplified for both resistant and susceptible plants. PCR products were digested by restriction enzyme TaqI. In TaqI digestion, heterozygous resistant plants displayed 3 bands, and homozygous resistant genotypes displayed 2 bands. However, susceptible genotypes were not digested by TaqI (Williamson et al., 1994). In this study, Mi gene specific primers (1/2 and C2S4 primers) were used and resistant and susceptible plants were distinguished from each other whereas resistant heterozygote and homozygote individuals were not distinguishable.

In breeding, the identification of the root-knot nematode resistance gene Mi in tomato plants depends on the traditional screening assay for resistance. If many recombinants are to be screened, this is time consuming, tedious and labour intensive. However, this situation can be overcome by using molecular markers in a marker assisted selection (MAS) programme. MAS dictates that the selection of one or more traits of interest be conducted indirectly by selecting for markers linked to the trait(s) (Melchinger, 1990). MAS is most efficient when selection for the marker is convenient and there is tight linkage between the marker and the trait of interest (Kelly, 1995). The major advantage of DNA molecular markers is that they are not influenced by environmental effects, have low negative selection pressure in populations and are developmentally stable. Nematode resistant genes, *Gro1* for *Globodera rostochiensis* (Woll.) in potato (Ballvora et al., 1995), *Cre3* and *Cre1* for *Heterodera avenae* in wheat (Eastwood et al., 1994; Ogbonnaya et al., 2001) and *Mij* for *M. javanica* and *M. incognita* in peach (Lu et al., 1999), are routinely used in breeding programmes to integrate these resistances. This study clearly demonstrates the tools of markers to incorporate key resistances of interest. Currently many tomato hybrids have the Mi gene incorporated using MAS belonging to foreign seed firms and there is limited application of MAS capacity in local seed firms. This paper clearly demonstrates the use of the Mi marker for incorporating Mi resistance that is reliable, timely and effective and supports the application hybrid of this tool for commercial tomato breeding companies.

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