Theor Appl Genet (2003) 106:478–484
DOI 10.1007/s00122-002-1106-y

J. S. S. Ammiraju · J. C. Veremis · X. Huang
P. A. Roberts · I. Kaloshian

The heat-stable root-knot nematode resistance gene *Mi-9* from Lycopersicon peruvianum is localized on the short arm of chromosome 6

Received: 27 December 2001 / Accepted: 16 May 2002 / Published online: 23 October 2002

Abstract The tomato gene *Mi-1* confers resistance to three species of root-knot nematodes, *Meloidogyne* spp. However, the resistance mediated by *Mi-1* is inactive at soil temperatures above 28 °C. Previously, we identified and mapped a novel heat-stable nematode resistance gene from the wild species *Lycopersicon peruvianum* accession LA2157 on to chromosome 6. Here we report further characterization of this heat-stable resistance against three *Mi-1*-avirulent biotypes of *Meloidogyne javanica*, *Meloidogyne arenaria* and *Meloidogyne incognita*. Screening segregating F_2 and F_3 progenies, derived from an intraspecific cross between susceptible LA392 and resistant LA2157, for nematode resistance at 25 °C and 32 °C, revealed a simple dominant monogenic inheritance with all the biotypes tested. We designate this gene as *Mi-9*. As a first step towards cloning of *Mi-9*, we constructed a linkage map around this gene. A total of 216 F_2 progeny from the cross between LA392 and LA2157 were screened with *M. javanica* at 32 °C and with CT119 and *Aps-1*, markers that flank the genetic interval that contains the *Mi-1* gene. DNA marker analysis indicated that these markers also flank *Mi-9*. Further mapping of recombinants with both RFLP and PCR-based markers localized *Mi-9* to the short arm of chromosome 6 and within the same genetic interval that spans the *Mi-1* region.

Keywords Heat-stable resistance · Mapping · *Meloidogyne* spp. · Tomato

Communicated by G. Wenzel

J.S.S. Ammiraju · J.C. Veremis · X. Huang · P.A. Roberts
I. Kaloshian (✉)
Department of Nematology, University of California,
Riverside, CA 92521, USA
e-mail: isgouhi.kaloshian@ucr.edu
Tel.: +909-787-3913, Fax: +909-787-3719

Introduction

Root-knot nematodes (*Meloidogyne* spp.) are important endoparasitic pests of numerous crop species including tomato (*Lycopersicon esculentum* Mill.). They have a worldwide distribution in warm temperate to tropical regions, and are also prevalent in greenhouse and other controlled environment production systems. The infective juveniles penetrate the root and initiate specialized feeding sites by modifying host cells in the vascular cylinder, from which they withdraw nutrients (Williamson and Hussey 1996). Nematode feeding results in damage to tomato plants, including poor fruit yield, stunted growth, wilting and susceptibility to other pathogens (Johnson 1998). Comparisons of tomato production between susceptible cultivars and resistant cultivars carrying gene *Mi-1* in the presence and absence of *M. incognita* revealed about 50% yield loss of susceptible cultivars due to nematode infection, while yield of resistant cultivars was unaffected (Roberts and May 1986).

Gene *Mi-1*, introgressed from *Lycopersicon peruvianum* L., is the only commercially available source of resistance to root-knot nematodes in tomato. This gene has been exploited extensively in the last two decades for modern tomato cultivar development. *Mi-1* confers resistance to three species of root-knot nematodes, *Meloidogyne arenaria*, *Meloidogyne incognita* and *Meloidogyne javanica* (Dropkin 1969a), as well as to the potato aphid (*Macrosiphum euphorbiae*) (Rossi et al. 1998). Genetic and physical mapping localized *Mi-1* in the introgressed region on the short arm of chromosome 6. *Mi-1* was cloned and shown to belong to the class of resistance genes that contains a leucine zipper, nucleotide binding site and leucine rich repeats (Milligan et al. 1998). Although *Mi-1* is a very effective source of root-knot nematode resistance in the field, *Mi-1*-mediated resistance is inactive above 28 °C soil temperature (Holtzmann 1965; Dropkin 1969b). Temperature sensitivity appears to be a characteristic of several *Meloidogyne* resistance genes as this trait is also described in other crop species such as alfalfa (Griffin 1969), sweet potato (Jatala and Russell

1972) and cotton (Carter 1982). Break-down of *Mi-1* mediated resistance due to high temperature has been reported in both greenhouse and field conditions, for example, in Cyprus and Florida (Philis and Vakis 1977; Tzortzakakis and Gowen 1996; Noling 2000). Therefore, the incorporation of heat-stable resistance to root-knot nematodes would be a valuable genetic improvement in tomato.

Several *L. peruvianum* accessions have been identified to possess heat-stable resistance (Ammati et al. 1986; Veremis and Roberts 1996b). *L. peruvianum* is cross-incompatible with cultivated tomato, *L. esculentum*, thus impeding efforts to introgress the resistance. However, in some instances these self-incompatible barriers could be overcome either by embryo rescue or bridge line methods (Ammati et al. 1986; Poysa 1990; Veremis and Roberts 1996c). In different accessions of *L. peruvianum*, several heat-stable resistance genes have been characterized and their allelic relationships have been determined (Veremis and Roberts 1996a, b, c). The accession LA2157, which belongs to the ancient Maranon race complex of *L. peruvianum* from Northern Peru, is highly resistant to *Mi-1*-aviruent root-knot nematodes at 25 °C and 32 °C, but does not confer resistance to *Mi-1*-virulent nematodes (Ammati et al. 1986; Veremis et al. 1999; Veremis and Roberts 2000). Genetic analysis using an interspecific population between LA2157 and cultivated tomato, generated by embryo rescue, revealed that the heat-stable resistance in LA2157 is governed by a single dominant gene. Using molecular markers this gene was mapped to chromosome 6 (Veremis et al. 1999). In this paper, we formally designate this gene *Mi-9*, due to its distinction from other named *Meloidogyne* resistance genes based on recombination and molecular data and phenotype specificity (see Veremis and Roberts 2000). The objective of this paper was to develop a linkage map around *Mi-9*, which would serve as a platform for the isolation of this gene and for understanding the molecular mechanism underlying the heat-stable resistance.

Materials and methods

Plant material

Plants used in this study were *L. peruvianum* LA2157 (Tunel Chotano), collected by the Lycopersicon expedition of 1980 in Tunel Chotano, Cajamarca, in northern Peru, field number SAL 5002, (Esquinas-Alcazar 1981) and LA392 (Llallan), both obtained from C.M. Rick, Tomato Genetics Resource Center, University of California, Davis. Pollen from the parents was collected and stored as described by McGuire (1952). One day before anthesis, when the petals changed color from green yellow to light yellow, flowers of *L. peruvianum* plants were emasculated and hand-pollinated. Crosses were made under greenhouse conditions between 11 a.m. and 1 p.m. Pollinated ovaries were enclosed with paper bags or gelatin capsules to prevent cross-pollination. Seeds were planted in seedling trays containing vermiculite at the top and steam-sterilized loamy sand at the bottom, and maintained in a mist chamber for at least 1 week. Clones were obtained agamically by making cuttings from young stems 3-nodes in length. Stem segments were dusted with rooting powder containing 2 g/l of 1-naphthaleneacetamide, 1 g/l of indole-3-butyric acid, and 4.40 g/l of Thiram. The dusted cuttings were planted in steam-sterilized loamy sand and maintained in a mist chamber for at least 1 week.

Nematode cultures

Cultures of *M. incognita* isolate Project 77, *M. arenaria* isolate W and *M. javanica* isolate VW4 (VW4 was obtained from V. M. Williamson, University of California, Davis) were grown on susceptible tomato cultivars Tropic and UC82B. The identity of the nematode isolates were confirmed as described previously (Cap and Roberts 1992). Eggs were extracted from infected roots by processing in 0.52% Na0Cl in a Waring blender, for 2 min at high speed (Hussey and Barker 1973). Eggs and root debris were collected on a 500-mesh sieve. Infective stage juveniles (J2s) were obtained by hatching the eggs in a modified Bearmann funnel, consisting of a wire mesh basket lined with a layer of paper towel, set in a glass Petri dish and filled with the egg mixture. The set-up was incubated at room temperature. Once the J2 hatches, it migrates through the paper towel and into the water in the Petri dish. Nematodes were collected every 48 h and used immediately or stored at room temperature for an additional 48 h with aeration.

Resistance screening

Heat-stable resistance screens were carried out in constant environment growth chambers where temperature was maintained at 32 °C for 5 days before and 30 days after inoculation, and then plants were moved to a greenhouse and were maintained at 25 ± 3 °C. The experiments with moderate temperatures were carried out in a glasshouse at 25 ± 3 °C. Six to eight week-old plants or well-rooted cuttings, growing in 10-cm pots filled with steam-sterilized loamy sand and fertilized with Osmocote, were inoculated with 3,000 J2 individuals per plant. Genotypes were tested in 2–4 replications per nematode isolate and were arranged in a completely randomized design. Screenings of F_3 families were made using from 5 to 20 individuals per F_3 family for families derived from susceptible F_2 plants, and from 20–40 individuals per F_3 family for families derived from resistant F_2 plants. Eight weeks after inoculation, nematode reproduction was evaluated by staining roots in 0.001% erioglaucine (Sigma). Plants were classified as resistant if the individual root system had less than 25 egg masses, or susceptible if the individual root system had 25 or more egg masses. Susceptible tomato cultivars UC82B *(mi-1/mi-1)* or Moneymaker (*mi-1/mi-1*) were included as controls for nematode infectivity, and *Mi-1*-containing cultivars VFNT (*Mi-1/Mi-1*) or Motelle (*Mi-1/Mi-1*) were included as controls for break-down of *Mi-1*-mediated resistance.

PCR-based markers

PCR was performed on DNA extracted from a single leaf according to Edwards et al. (1991) or from DNA preparation according to Williamson and Colwell (1991). The PCR reactions and thermal cycling conditions for the codominant CAPs marker *Aps-1* and the STS marker C8B were described previously (Kaloshian et al. 1998). The PCR reaction and thermal cycling conditions for STS markers REX-1 and CT119 were according to Williamson et al. (1994) and Dixon et al. (1995), respectively. Briefly, reactions were carried out in 25 µl volume in the presence of 0.5 µM of each of the primers, 0.2 mM dNTPs (Promega) in a buffer provided by the manufacturer, 1.5 mM $MgCl_2$, and 1.25–2.5 U of *Taq* polymerase (Promega). The *Aps-1* primer sequences were: APSF1 5′-GGAACGTGGGTAGCATATGA-3′ and APSR2 5′-GCCAATGCTCATCAATGTGA-3′. The C8B primer sequences were: C8BF 5′-TACCCACGCCCCATCAATG-3′ and C8BR 5′-TGCAAGAGGGTGAATATT-GAGTGC-3′. The REX-1 primer sequences were: REXF 5′-TCGGAGCCTTGGTCTGAATT-3′ and REXR 5′-GCC-

AGAGATGATTCGTGA-GA-3′. CT119 primer sequences were: CT119F 5′-TCAGGTATCGAACCAAAC-3′ and CT119R 5′-TA-AAAGGTTCATCCTAATA-3′.

When the PCR did not reveal polymorphisms, amplified reactions were restricted with six, 4-base-pair, cutting restriction enzymes, *Taq*I, *Hae*III, *BstN*I, *Mse*I, *Sau*3AI, *Hind*III (Promega). Ten microliters of PCR products with equal volume of 1 × restriction buffer (supplied by the enzyme manufacturer) were digested with 5 U of the respective enzyme. After incubation at appropriate temperature for 2 h, the reactions were resolved on 1.5–2% agarose gels and visualized on a UV box after staining with ethidium bromide.

RFLP analysis

DNA extractions were done according to Williamson and Colwell (1991). Five to ten Micrograms of total DNA were digested with six restriction enzymes (*Eco*R1, *Eco*RV, *Hind*III, *Dra*I, *Hae*III and *Taq*I), that previously were found to generate polymorphisms in *L. peruvianum* accessions (Kaloshian et al. 1998). Procedures for restriction digestions and DNA blotting were according to Kaloshian et al. (1998). Probes were prepared from inserts amplified from plasmid DNA using PCR and labeled with alpha ^{32}P dCTP by the random priming method (Feinberg and Vogelstein 1983) using a rediprime II kit (Amersham). Hybridization was carried out overnight in 50% formamide at 42 °C and final washes were done in 0.5 × SSC, 0.1% SDS at 55–60 °C.

Mapping

MapMaker version 3.0 (Lander et al. 1987) was used to analyze the segregation data and to establish a linkage map around *Mi-9* (LOD 3.0).

Results

Nematode resistance in *L. peruvianum* accessions LA2157, LA392 and F_1 plants derived from the cross LA392 × LA2157 were tested for resistance to *Mi-1*-avirulent biotypes of *M. incognita*, *M. arenaria* and *M. javanica* at 25 °C and 32 °C. All F_1 individuals were resistant to *Mi-1*-avirulent *Meloidogyne* spp. at 32 °C, confirming that LA2157 is homozygous for the heat-stable resistance. The *L. peruvianum* LA392 was homozygous susceptible, with all entries testing susceptible (Veremis and Roberts 2000). The inheritance of the heat-stable resistance from LA2157 was evaluated with F_2 and F_3 progenies. Screening the F_2 population for heat-stable resistance at 32 °C with *M. javanica* isolate VW4 resulted in 162 resistant and 54 susceptible individuals, indicating a 3:1 segregation ratio($x^2 = 0$), confirming the monogenic dominant inheritance of *Mi-9* (Veremis et al. 1999; Veremis and Roberts 2000). The number of egg masses per root system on resistant F_2 plants ranged between 0 and 15, while on susceptible F_2 plants the range was between 40 and 150 egg masses. Individual plants scored as resistant at 25 °C were also scored as resistant at 32 °C. Similarly, plants scored as susceptible at 25 °C were also scored as susceptible at 32 °C indicating either linkage of an *Mi-1* like gene (i.e., heat-unstable) to *Mi-9* or more likely that *Mi-9* confers resistance at both temperatures.

Subsets of this F_2 population were also screened with *Mi-1*-avirulent biotypes of *M. arenaria* and *M. incognita* at 32 °C. In these tests, a 3:1 segregation ratio for heat-stable resistance was also observed in the F_2 progeny to *M. arenaria* isolate W (59R:16S $x^2 = 1.17$) and *M. incognita* isolate Project 77 (123R:39S $x^2 = 0.06$). All F_2 individuals with heat-stable resistance to *M. javanica* were also scored as resistant to *M. arenaria* and *M. incognita* at high temperature, indicating that *Mi-9* confers heat-stable resistance to all three species.

A subset of 61 F_3 families derived from single F_2 plants were screened for heat-stable resistance at 32 °C with *M. javanica* isolate VW4. The F_3 families conformed closely to an expected 1:2:1 segregation ratio characteristic of a single dominant gene (16 all resistant: 30 segregating: 15 all susceptible). An example of a segregating F_3 family was F_3-99, that segregated 31R:11S. The high level of reproduction (more than 100 egg masses per root system) of *Mi-1*-avirulent biotypes on Motelle and VFNT (both carrying *Mi-1*) at 32 °C, compared to the resistance response at 25 °C (0–2 egg masses per root system), confirmed the high temperature inactivation of *Mi-1*-mediated resistance. High numbers (more than 100 per root system) of egg masses were observed on line Pixie and UC82B with all three species tested at both 25 °C and 32 °C, as expected in the absence of a resistance gene.

Polymorphisms generated by DNA markers

The marker CT119 produced a fragment of approximately 450 bp in both LA2157 and LA392. This marker revealed polymorphism between the two accessions after cleaving with restriction enzyme *Mse*I, distinguishing homozygous resistant (280 bp and 170 bp) and susceptible (250 bp and 170 bp), as well as heterozygous resistant individuals (280 bp, 250 bp, 170 bp), thus behaving as a codominant marker. Both REX-1 and *Aps-1* also behaved as codominant markers. PCR amplification of REX-1 resulted in a 750-bp fragment in both LA2157 and LA392. Upon restriction with the *Taq*I enzyme, the fragment in LA2157 was cleaved into two fragments of 570 bp and 162 bp in size, whereas the fragment in LA392 was not cleaved. *Aps-1* generated a 1.6-kb fragment in both LA2157 and LA392. Upon digestion with the *Taq*I enzyme, the fragment in LA2157 cleaved into two fragments of 1.0 kb and 0.6 kb in size, and the fragment in LA392 remained uncleaved. C8B produced a fragment of approximately 360 bp in LA392 and two fragments of 400 bp and 360 bp in LA2157, thus behaving as a dominant marker.

Most of the restriction enzymes used to prepare the DNA blots were able to generate polymorphisms between the parental accessions when tested with RFLP markers C264.2 and C32.1 (Table 1). The enzyme *Eco*RI was chosen for the DNA blot analysis of the progeny. Both C264.2 and C32.1 behaved as dominant markers in this *L. peruvianum* population. C264.2 produced 3.8-kb,

Table 1 RFLP, CAP, and STS markers used for fine scale mapping of *Mi-9*

Marker	Source	Restriction enzymes used to reveal polymorphism[a]
RFLP		
C32.1	Kaloshian et al.	All
C264.2	Kaloshian et al.	*Eco*RI, *Eco*RV, *Hind*III, *Dra*I
PCR-based markers		
CT119	Dixon et al.	*Mse*I (this study)
REX-1	Williamson et al.	*Taq*I
Aps-1	Kaloshian et al.	*Taq*I
C8B	Kaloshian et al.	

[a] *L. peruvianum* parental DNA was digested with the restriction enzymes *Eco*RI, *Dra*I, *Eco*RV, *Hind*III, *Taq*I and *Hae*III. Those enzymes that gave clearest polymorphism are listed. 'All' indicates that all enzymes tested gave clear polymorphism.

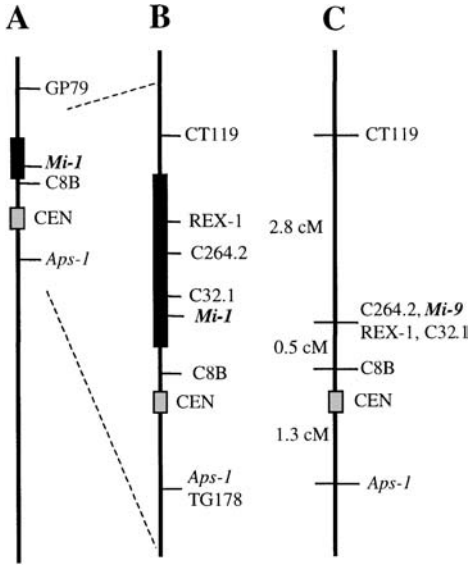

Fig. 1A–C Map of the *Mi-1* and *Mi-9* region of chromosome 6 of tomato. **A** Position of *Mi-1* and flanking markers on chromosome 6. The *thick bar* represents the introgressed region from *L. peruvianum* in the tomato line Motelle. **B** Expanded view of the introgressed *Mi-1* region with PCR-based (CT119, REX-1, C8B, *Aps-1*) or RFLP (C264.2, C32.1, TG178) markers (Dixon et al. 1995; Kaloshian et al. 1998). **C** Genetic map of *Mi-* 9 shown in genetic distances in cM derived from the cross between *L. peruvianum* LA392 and LA2157. *CEN* = centromere

2.5-kb and 1.25-kb fragments in LA2157, and 3.8-kb and 1.25-kb fragments in LA392, the 2.5-kb fragment cosegregating with resistance. The RFLP marker C32.1 produced 4.5-kb and 2.0-kb fragments in LA2157, and 4.5-kb, 3.0-kb and 2.0-kb fragments in LA392. The 3.0-kb fragment in LA392 cosegregated with homozygous susceptible and heterozygous individuals.

Linkage map surrounding *Mi-9*

In our previous study we mapped *Mi-9* to chromosome 6 using an interspecific F_2 population of 61 plants derived from a cross between LA2157 and Solentos using RFLP markers (Veremis et al. 1999). The closest linked markers were TG178 and TG118, located on the long arm of chromosome 6 (Messeguer et al. 1991) and over 20 cM from the gene. In order to identify markers closely linked to *Mi-9*, we initiated DNA marker analysis using existing markers located near TG178 and TG118 on chromosome 6 (Ho et al. 1992; Dixon et al. 1995). Initially we used *Aps-1* and CT119 markers located at the centromeric end of the long arm and the telomeric end of the short arm of chromosome 6, respectively (Fig. 1). It should be noted that *Aps-1* and CT119 flank the *Mi-1* gene in the *L. peruvianum* introgressed region in nematode-resistant *L. esculentum* genotypes (Fig. 1B) (Kaloshian et al. 1998). A total of 216 individuals were analyzed and 18 individuals were identified as carrying recombination events between CT119 and *Aps-1* (Table 2). Three recombinants (172, 228 and 259) were identified between *Aps-1* and the heat-stable resistance phenotype, indicating that *Mi-9* is above *Aps-1* (Table 2). Similarly, at least five plants (170, 210, 211, 212 and 213) were identified with recombination between CT119 and the heat-stable resistance phenotype. The identification of the recombination break points between the flanking markers and the phenotype indicated the presence of *Mi-9* in the genetic interval of CT119-*Aps-1* (Fig. 1C). All 18 recombinants were further analyzed with markers located between CT119 and *Aps-1* (Fig. 1B). Out of these, 11 recombinants were found between CT119 and REX-1 compared to seven recombinants between all other markers (Table 2). Both RFLP markers C264.2 and C32.1, as well as the STS marker C8B, behaved as dominant markers in this cross, making our analysis difficult to precisely identify the recombination break points. Based on F_3 progeny tests, there were two recombinants (108 and 228) between C32.1 and C8B. F_2 plant 228 was also a recombinant between C8B and heat-stable resistance, indicating that *Mi-9* is located above C8B (Fig. 1C). There were at least three recombinants (136, 172, 259) between C8B and *Aps-1* with the possibility for additional two recombinants (165 and 181) (Table 2). In F_2 plants 165 and 181, the recombination could be on either side of C8B and it was not possible to confirm genotypes in F_3 progeny because these plants died before setting fruit. All recombinants showed single cross-over events and we did not find any double recombinants. In this cross all markers segregated according to Mendelian expectations, contrary to our earlier study in which we found segregation distortion and skewing towards one of the parental alleles. In addition, the recombinant analysis indicated that the order of the markers in this region was consistent with previous reports (Dixon et al. 1995; Kaloshian et al. 1998) and suggested that *Mi-9* is in close physical linkage with gene*Mi-1*.

Table 2 Mapping of recombinants in the *Mi-9* region with markers flanking *Mi-1* gene

F2 plant #	Phenotype[a]	CT119[b]	REX -1	C264.2	C32.1	C8-B	*Aps-1*
8	R	1	3	n	n	1/3	3
108	R	1	1	1/3	1	3[c]	3
136	R	3	3	1/3	3[c]	3[c]	1
144	R	1	3	n	2/3	1/3	3
165[d]	R	3	3	1/3	2/3	1/3	1
168	R	1	3	1/3	2/3	1/3	3
170	R	2	1	1/3	1	1/3	1
172	S	2	2	2	2/3	2	3
173	R	3	1	1/3	1	1/3	1
181[d]	R	3	3	n	2/3	1/3	1
188	R	1	3	n	2/3	1/3	3
208	R	1	3	1/3	2/3	1/3	3
210	R	2	3	1/3	2/3	1/3	3
211	S	3	2	2	2/3	2	2
212	R	2	3	n	2/3	1/3	3
213	S	3	2	n	2/3	2	2
228	S	2	2	2	2[c]	3[c]	3
259	S	2	2	2	n	2	3
LA2157	R	1	1	1/3	1	1/3	1
LA392	S	2	2	2	2/3	2	2

[a] R, resistant; S, susceptible
[b] 1, homozygous resistant marker locus; 2, homozygous susceptible marker locus; 3, heterozygous marker locus; n, not determined
[c] Allele designation is based on F_3 progeny testing
[d] Plant died before setting fruit

Discussion

The fine-scale mapping of the gene in *L. peruvianum* LA2157 that confers heat-stable resistance to root-knot nematodes represents an important step toward developing markers useful in marker-assisted selection and breeding, and toward cloning the gene. Previously, using progeny from a *L. esculentum* × LA2157 interspecific cross, segregation for resistance in F_2 and F_5 families indicated that the resistance was inherited as a single dominant gene although a distorted F_3 segregation with fewer than expected heterozygous families was found (Veremis et al. 1999). Here, additional analysis of the phenotypic expression of this heat-stable resistance through screening F_2 and F_3 progenies from a *L. peruvianum* intraspecific cross at both moderate (25 °C) and high (32 °C) temperature, confirmed that this trait is inherited independently as a single dominant gene. There was no evidence of distorted segregation for resistance within this intraspecific cross. We designate this gene as *Mi-9* based on its distinction from other named *Meloidogyne* resistance genes according to recombination and molecular data, and phenotype specificity (see Veremis and Roberts 2000).

The resistance screens showed that *Mi-9* confers resistance to all three common warm-climate root-knot nematode species. Although the levels of reproduction of *M. arenaria*, *M. incognita*, and *M. javanica* differed slightly on resistant plants, the reactions were all classified as resistant. All the F_2 plants screened with the three nematode species showed exactly the same pattern of segregation with each species, in that all resistant plants were resistant to all species, and all susceptible plants were susceptible to all three species. Earlier work has also shown that *Mi-9* is not effective against *Meloidogyne hapla* and biotypes of *Meloidogyne* species that can parasitize tomato carrying the *Mi-1* gene (Veremis and Roberts 2000). Thus far, we can conclude that *Mi-9* has the same phenotypic expression as *Mi-1* in terms of *Meloidogyne* species and biotype specificity, and differs phenotypically from *Mi-1* only in the stability of the resistance at high temperature. There was no evidence of recombination between heat-stable and heat-sensitive resistance in F_2 and F_3 screens, suggesting that *Mi-9* confers resistance at both temperatures. However, the screening protocols do not allow us to detect the presence of an expressed *Mi-1*-like resistance gene also being present in LA2157 in the same region of chromosome 6 and in close linkage to *Mi-9*. Such a tightly linked gene could be present and expressed at moderate temperature, but because *Mi-9* is expressed at moderate and high temperature, it is difficult at this time to detect the phenotypic expression of a *Mi-1*-like gene. Cloning of *Mi-9* and its transfer into susceptible tomato will provide definite answers as to any pleiotropic role of this gene.

Our mapping analysis has revealed that *Mi-9* maps on the short arm of chromosome 6. The *L. peruvianum* intraspecific cross used here was useful in generating recombination in the region of *Mi-9*. A 4.16% recombination frequency was observed in the *Mi-9* region in this intraspecific population, with 18 of 216 individuals producing recombination between markers or between markers and the resistance phenotype. The recombination frequency in this region was comparable to that observed by Kaloshian et al. (1998) and significantly less than that observed by Ganal and Tanksley (1996) using different *L. peruvianum* intraspecific crosses. Also, in this cross, we did not observe suppression of recombination in a similar genomic location, the short arm of chromosome 6, as was reported in previous studies using *L. esculentum* crosses carrying introgressed genomic segments from *L. peruvianum* (Kaloshian et al. 1998; Ho et al. 1994; Ganal and Tanksley 1996). A strategy based on a *L. peruvianum* intraspecific cross was helpful in developing a high-resolution linkage map during the isolation of the *Mi-1* gene (Kaloshian et al. 1998).

Based on our recombination analysis the marker order in this region in LA2157 and LA392 was similar to what was reported in *L. esculentum*. The CT119-REX-1 interval was found to be highly recombinogenic in these accessions. It is possible that the distance between these markers is large in this particular cross or that a recombination hot spot is present at this genetic interval. Similarly, several recombinants were found between C8B and *Aps-1*. Differences in the genetic map distances between DNA markers in different *L. peruvianum* crosses were reported by Ganal and Tanksley (1996). No recombination was found between REX-1, C264.2, C32.1 and *Mi-9*

indicating that these markers are in close proximity to the gene (Fig. 1C). In the introgressed region of *Mi-1*, the distance between REX-1 and the gene was approximately 300 kb while the distance between C32.1 and the gene was 15 kb (Fig. 1B) (Kaloshian et al. 1998). Although we have not studied the physical distance in the intervals co-segregating with and adjacent to the *Mi-1*/*Mi-9* cluster in this mapping population, it appears that intervals closer to the *Mi-1*/*Mi-9* cluster (interval spanning REX-1–C32.1) undergo less recombination than adjacent intervals (between CT119 and REX-1 or C8B and *Aps-1*). Such regional differences in recombination frequencies were observed in *L. peruvianum* crosses where some regions showed significantly higher levels of recombination (Bonnema et al. 1997; Kaloshian et al. 1998).

Most disease resistance genes are members of multigene families and seem to be clustered in the plant genome. Duplication and subsequent divergence seem to be a common process in plant gene evolution. Such is the case of the *Mi-1* locus where two highly homologous genes were identified, *Mi-1.1* and *Mi-1.2*, but only *Mi-1.2* conferred resistance to root-knot nematodes (Milligan et al. 1998). On the other hand, two functional sequences encoding the same specificity were isolated from tomato; the *Cf2* locus contained two members of this gene family recognizing the same avirulence factor from the fungus *Cladosporium fulvum* (Dixon et al. 1996). Our work indicated that *Mi-9* mapped to a region in very close proximity to the *Mi-1* locus. We have identified several *Mi-1* homologues in this region (Kaloshian et al., unpublished), therefore it is possible that *Mi-9* is a member of the *Mi-1* family and that this member has evolved to confer heat-stable resistance. Future studies will shed light on the genetic organization of this complex disease resistance gene family.

There are many useful nematode resistance specificities available for tomato and most of these have been identified in the wild tomato *L. peruvianum* (Veremis and Roberts 1996a, b, 2000). A current challenge is to dissect these complex family members, at the molecular level, and access the useful variation either by conventional breeding approaches or transgenically. Markers like REX1 and C8B can be used to select the *Mi-9* trait and will be valuable for incorporating *Mi-9* into cultivated tomato using conventional breeding approaches, since phenotyping for nematode resistance is labor intensive and time consuming. It is now possible to pyramid *Mi-9* in a single genetic background along with other resistance specificities for durable resistance. Attempts have already been made to introgress *Mi-9* in to cultivated tomato (Veremis et al. 1999). Also, when we can understand the mechanisms generating new resistance specificities, we can attempt to emulate them *ex planta* to evolve genes with altered ligand binding characteristics and provide more effective resistance or new specificities. We have already identified four homologues of *Mi-1* that cosegregate with *Mi-9* and currently we are in the process of cloning these homologues.

Acknowledgements We are grateful for Dr. V. M. Williamson of University of California, Davis for providing the RFLP markers used in this study. We thank Dr. M. L. Roose, Department of Botany and Plant Sciences, University of California, Riverside, for helpful discussions. This work was supported in part by a United States Department of Agriculture postdoctoral fellowship to J.C.V and a USDA grant 98-35300-6350 to I.K. and P.R.

References

Ammati M, Thomason IJ, McKinney HE (1986) Retention of resistance to *Meloidogyne incognita* in *Lycopersicon* genotypes at high soil temperature. J Nematol 18:491–495

Bonnema G, Schipper D, van Heusden S, Zabel P, Lindhout P (1997) Tomato chromosome 1: high resolution genetic and physical mapping of the short arm in an interspecific *Lycopersicon esculentum* × *L. peruvianum* cross. Mol Gen Genet 253:455–462

Cap GB, Roberts PA (1992) A rapid and efficient method for the screening of acid phosphatase-1 in young tomato seedlings, and for the identification of root-knot nematode species using miniaturized polyacrylamide gel electrophoresis. Electrophoresis 13:295–299

Carter WW (1982) Influence of soil temperature on *Meloidogyne incognita* resistant and susceptible cotton. J Nematol 14:343–346

Dixon MS, Jones DA, Hatzixanthis K, Ganal MW, Tanksley SD, Jones JDG (1995) High-resolution mapping of the physical location of the tomato *Cf-2* gene. Mol Plant-Microbe Interact 8:200–206

Dixon MS, Jones DA, Keddie JS, Thomas CM, Harrisom K, Jones JDG (1996) The tomato *Cf-2* disease resistance locus comprises two functional genes encoding leucine-rich repeat proteins. Cell 84:451–459

Dropkin VH (1969a) Cellular responses of plants to nematode infections. Annu Rev Phytopathol 7:101–122

Dropkin VH (1969b) The necrotic reaction of tomatoes and other hosts resistant to *Meloidogyne*: reversal by temperature. Phytopathology 59:1632–1637

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19:1349

Esquinas-Alcazar JC (1981) Genetic resources of tomatoes and wild relatives. IBPGR Secretariat, Rome

Feinberg AP, Vogelstein B (1983) A technique for radiolabeling DNA restriction fragments to high specific activity. Anal Biochem 132:6–13

Ganal MW, Tanksley SD (1996) Recombination around the *Tm2a* and *Mi* resistance genes in different crosses of *Lycopersicon peruvianum*. Theor Appl Genet 92:101–108

Griffin GD (1969) Effects of temperature of *Meloidogyne hapla* in alfalfa. Phytopathology 59:599–609

Ho J-Y, Weide R, Ma HM, Wordragen MF, Lambert KN, Koornneef M, Zabel P, Williamson VM (1992) The root-knot nematode resistance gene (*Mi*) in tomato: Construction of a molecular linkage map and identification of dominant cDNA markers in resistant genotypes. Plant J 2:971–982

Holtzmann OV (1965) Effects of soil temperature on resistance of tomato to root-knot nematode (*Meloidogyne incognita)*. Phytpathology 55:990–992

Hussey R, Barker KR (1973) A comparison of methods of collecting inocula of *Meloidogyne* species including a new technique. Plant Dis Rep 57:1025–1028

Jatala P, Russell CC (1972) Nature of sweet potato resistance to *Meloidogyne incognita* and the effects of temperature on parasitism. J Nematol 4:1–7

Johnson AW (1998) Vegetable crops. In: Pederson GA, Windham GL, Barker KR (eds) Plant-nematode interactions. Agronomy Society of America, Madison Wisconsin, pp 595–635

Kaloshian I, Yaghoobi J, Liharska T, Hontelez J, Hanson D, Hogan P, Jesse T, Wijbrandi J, Simons G, Vos P, Zabel P, Williamson VM (1998) Genetic and physical localization of the root-knot nematode resistance locus *Mi* in tomato. Mol Gen Genet 257:376–385

Lander ES, Green P, Abrahamson J, Barlow A, Daly MJ, Lincoln SE, Newburg L (1987) MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174–181

McGuire DC (1952) Storage of tomato pollen. Proc Am Soc Hort Sci 60:419–424

Messeguer R, Ganal M, de Vicente MC, Young ND, Bolkan H, Tanksley SD (1991) High resolution RFLP map around the root-knot nematode resistance gene (*Mi*) in tomato. Theor Appl Genet 82:529–536

Milligan SB, Bodeau J, Yaghoobi J, Kaloshian I, Zabel P, Williamson VM (1998) The root-knot nematode resistance gene *Mi* from tomato is a member of leucine zipper nucleotide binding leucine-rich repeat family of plant genes. Plant Cell 10:1307–1319

Noling JW (2000) Effects of continuous culture of resistant tomato cultivars on *Meloidogyne incognita* soil population density and pathogenicity. J Nematol 32:452

Poysa V (1990) The development of bridge lines for interspecific gene transfer between *Lycopersicon-esculentum* and *L. peruvianum*. Theor Appl Genet 79:187–192

Philis J, Vakis N (1977) Resistance of tomato varieties to the root-knot nematodes *Meloidogyne javanica* in Cyprus. Nematol Medit 5:39–44

Roberts PA, May DM (1986) *Meloidogyne incognita* resistance characteristics in tomato genotypes developed for processing. J Nematol 18:353–359

Rossi M, Goggin FL, Milligan SB, Kaloshian I, Ullman DE, Williamson VM (1998) The nematode resistance gene *Mi* of tomato confers resistance against the potato aphid. Proc Natl Acad Sci USA 95:9750–9754

Tzortzakakis EG, Gowen SR (1996) Occurrence of resistance breaking pathotypes of *Meloidogyne javanica* on tomatoes in Crete, Greece. Fund Appl Nematol 19:283–288

Veremis JC, Roberts PA (1996a) Identification of resistance to *Meloidogyne javanica* in the *Lycopersicon peruvianum* complex. Theor Appl Genet 93:894–901

Veremis JC, Roberts PA (1996b) Relationships between *Meloidogyne incognita* resistance genes in *Lycopersicon peruvianum* differentiated by heat sensitivity and nematode virulence. Theor Appl Genet 93:950–959

Veremis JC, Roberts PA (1996c) Differentiation of *Meloidogyne incognita* and*M. arenaria* novel resistance phenotypes in*Lycopersicon peruvianum* and derived bridge lines. Theor Appl Genet 93:960–967

Veremis JC, Roberts PA (2000) Diversity of heat-stable genotype specific resistance to *Meloidogyne* in Maranon races of *Lycopersicon peruvianum* complex. Euphytica 111:9–16

Veremis JC, van Heusden AW, Roberts PA (1999) Mapping heat-stable resistance to *Meloidogyne* in *Lycopersicon peruvianum*. Theor Appl Genet 98:274–280

Williamson VM, Colwell G (1991) Acid phosphatase-1 from nematode resistant tomato – isolation and characterization of its gene. Plant Physiol 97:139–146

Williamson VM, Hussey RS (1996) Nematode pathogenesis and resistance in plants. Plant Cell 8:1735–1745

Williamson VM, Ho J-Y, Wu FF, Miller N, Kaloshian I (1994) A PCR-based markes tightly linzed to the nematode resistance gene, *Mi* in tomato. Theor Appl Genet 87:757–763