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Mapping a new nematode resistance locus in *Lycopersicon peruvianum*

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Abstract Accessions of the wild tomato species *L. peruvianum* were screened with a root-knot nematode population (557R) which infects tomato plants carrying the nematode resistance gene *Mi*. Several accessions were found to carry resistance to 557R. A *L. peruvianum* backcross population segregating for resistance to 557R was produced. The segregation ratio of resistant to susceptible plants suggested that a single, dominant gene was a major factor in the new resistance. This gene, which we have designated *Mi-3*, confers resistance against nematode strains that can infect plants carrying *Mi*. *Mi-3*, or a closely linked gene, also confers resistance to nematodes at 32 °C, a temperature at which *Mi* is not effective. Bulked-segregant analysis with resistant and susceptible DNA pools was employed to identify RAPD markers linked to this gene. Five-hundred-and-twenty oligonucleotide primers were screened and two markers linked to the new resistance gene were identified. One of the linked markers (NR14) was mapped to chromosome 12 of tomato in an *L. esculentum/L. pennellii* mapping population. Linkage of NR14 and *Mi-3* with RFLP markers known to map on the short arm of chromosome 12 was confirmed by Southern analysis in the population segregating for *Mi-3*. We have positioned *Mi-3* near RFLP marker TG180 which maps to the telomeric region of the short arm of chromosome 12 in tomato.

Key words *Mi-3* · Mapping · Root-knot nematodes *Meloidogyne* · Tomato

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Introduction

Root-knot nematodes (*Meloidogyne* spp.) cause severe damage to tomato and many other crops (Mai 1985; Eisenback and Triantaphyllou 1991). Some tomato varieties carry a dominant gene, *Mi*, that confers effective field resistance to these pests. *Mi* has been described as dominant, or semi-dominant, and confers resistance to three root-knot nematode species, *M. incognita*, *M. javanica* and *M. arenaria*, but not to a fourth, *M. hapla* (Roberts and Thomason 1986). This gene was introduced into cultivated tomato (*Lycopersicon esculentum*) from *Lycopersicon peruvianum* by embryo rescue of an interspecific cross (Smith 1944). A single F_1 plant is the sole source of nematode resistance in currently available fresh-market and processing tomato cultivars (Medina-Filho and Tanksley 1983). *Mi*-mediated resistance is characterized by localized necrosis of host cells near the infecting pathogen. A major effort is underway in several laboratories to isolate *Mi* by a positional cloning strategy (Messeguer et al. 1991; Ho et al. 1992).

There have been several reports of *M. incognita* populations that can infect tomato plants with *Mi* (Riggs and Winstead 1959; Triantaphyllou 1987; Roberts et al. 1990; Jarquin-Barberena et al. 1991). These populations include both field isolates and populations selected on *Mi*-containing plants in the greenhouse. Increased restrictions on the use of chemical controls have increased the reliance on *Mi* in recent years (Roberts and Thomason 1986). If *Mi* is cloned and incorporated into additional varieties, or perhaps even different plant species, the gene will be subject to additional pressure. The presence of "resistance-breaking" nematode populations makes obvious the need for incorporation of additional resistance genes. Incorporation of new resistance into domestic tomato using classical breeding or molecular techniques would relieve some of the selective pressure on *Mi*.

Screens of wild-species accessions for new sources of resistance have been carried out, and several resistant

accessions have been identified in the heterogeneous *L. peruvianum* complex (Ammati et al. 1986; Lobo et al. 1988). Resistance due to *Mi* is lost at soil temperatures above 28°C (Dropkin 1969). *L. peruvianum* accessions that are resistant at high soil temperature have been identified (Ammati et al. 1986; Roberts et al. 1990). Results of genetic segregation analysis indicate that the heat-stable resistance in accession PI270435 is due to a single, dominant locus, named *Mi-2*, that segregates independently of *Mi* (Cap et al. 1993). High-temperature resistance was also noted in accession PI126443, but whether resistance was due to the same gene in both accessions was not determined. Roberts et al. (1990) also observed that F_1 seedlings from a cross between the two *L. peruvianum* accessions PI270435 and PI126443 were resistant to some virulent selected isolates of *M. incognita* though the source of this resistance and its relationship to *Mi-2* was not determined.

In the present paper we describe the identification and mapping of a locus that confers resistance to an *Mi*-infecting nematode strain and present evidence for a correlation of this trait with high-temperature resistance to root-knot nematodes in accession PI126443. We also describe the development of a tightly linked DNA marker that can be used to aid in incorporation of this trait into cultivated tomato.

Materials and methods

Plant material

L. peruvianum accessions and cuttings were obtained from the sources listed in Table 1; PI128657 was the original source of *Mi* (Smith 1944). The pedigree of the segregating *L. peruvianum* family VWP2x4 is shown in Fig. 1. Seed of the susceptible control UC82B were obtained from Sunseeds, Holister, Calif., and VFNT cherry seeds were produced in our laboratory from a stock obtained from the C. M. Rick, Tomato Genetics Resource Center, Davis, Calif. Parental and 43 F_2 plants from a tomato mapping population, consisting of an interspecific cross between *L. esculentum* cv VF36-Tm2a and *L. pennellii* LA716 (Tanksley et al. 1992), were obtained from S. Tanksley, Cornell University, Ithaca, New York.

Nematode strains

M. javanica VW4 was used as the standard root-knot nematode strain in the resistance assays. This strain has been tested extensively and found to produce the expected response when applied to tomato with or without *Mi*. Isozyme (malate dehydrogenase and esterase) and perinneal patterns are characteristic for *M. javanica*. The strain has been maintained in hydroponic culture in our laboratory for several years (Lambert et al. 1992). The *M. incognita* strain, 557R, which is able to reproduce on plants with *Mi* was obtained from Dr. A. C. Triantaphyllou (North Carolina State University, Raleigh). Strain 557R has typical *M. incognita* isozyme patterns and chromosome number (A. C. Triantaphyllou, personal communication).

Nematode resistance assays

Second-stage juveniles (J2) that had hatched within a 24-h period were collected from a hydroponic culture system (Lambert et al. 1992) and used for the inoculation. Hydroponic solution was collected then poured though a 60-mesh screen to remove root material. Nematodes were collected on a 685-mesh screen.

Six-to-eight-week-old plants or well-rooted cuttings, grown in river sand in 11 Styrofoam cups with drainage holes and fertilized with Hoagland's nutrient solution, were infected with 3000 J2. Six to eight-weeks later, roots were removed from the sand, washed, then stained for 10 min in a solution of erioglaucine (0.05%; Aldrich Chemical Co., Milwaukee, Wis.), a dye which has been shown to specifically stain egg masses blue (Omwega et al. 1988). After staining, roots were rinsed and then examined under a magnifier and egg-mass number counted (up to 100 egg masses per root system). Control plants were UC82B, which is susceptible to nematode strains VW4 and 557R, and VFNT cherry, a culivar that carries *Mi* and is resistant to VW4, but not to 557R.

For the high-temperature assays, well-rooted cuttings were established in the greehouse. Five days before inoculation, plants were moved to a growth chamber (Conviron E15, Controlled Environments, Ltd., Winnipeg, Manitoba, Canada) maintained at 32 °C, 65% humidity on a 16-h light cycle. Plants were inoculated with 3000 J2 of *M. javanica* strain VW4 prepared from hydroponic culture as described above. VFNT cherry was included as a positive control. Six-to-eight weeks later plants were processed as above and the number of egg masses per root system determined.

DNA preparation

DNA extraction from leaves was done as previously described (Williamson and Colwell 1991); 10–15 g of fresh, young leaves were used for each extraction. For some PCR analyses, DNA was prepared as described by Edwards et al. (1991).

PCR procedures

PCR amplification to produce random amplified polymorphic DNA (RAPD) fragments from tomato was carried out as described by Williams et al. (1990.) Amplification, in a Perkin-Elmer Cetus DNA thermal cycler 480, was in a volume of 25-µl and was overlaid with mineral oil. The reaction consisted of 25–50 ng of template DNA, 0.2 mM primer, and 1.0 unit of *Taq* polymerase. A modified reaction mix consisting of the following was added: 10 mM Tris pH 8.3, 50 mM KCl, 2.0 mM $MgCl_2$, 0.01% gelatin, 0.1 mM each of dATP, dCTP, dGTP and dTTP. The decamer primers for RAPD markers were obtained from Operon Technologies (Alameda, Calif., Operon kits A through L and AA through AN). PCR conditions were: 94 °C, 1 min, followed by 40 cycles of 1 min at 94 °C, 1 min at 35 °C, and 2 min at 72 °C, then 5 min at 72 °C.

The PCR for the NR14 (or REX-1) marker was carried out as above except that the volume was 50 µl and 50 ng DNA together with 0.5 mM each of primers NR14A/R (5′-GTGGCAGGTATCTCATGGAA-3′) and NR14A/U (5′-GGGAACCTCCATATACAAG-3′) were used. The sequences of REX-1 primers were as previously reported (Williamson et al. 1994). In each reaction mix, 20 mM Tris pH 8.0, 25 mM KCl, 1.5 mM $MgCl_2$ and 0.05% Tween 20 was utilized. Samples were placed at 94 °C for 3 min, then amplification was carried out under the following conditions: 30 cycles of 1 min at 94 °C, 2 min at 60 °C, and 2 min at 72 °C. Following amplification, 8 µl of the reaction was transferred to a new tube and digested with five units of *Acc*I, or in the case of REX-1 with *Taq*I, (New England Biolabs, Beverly, Mass., USA) as recommended by the manufacturer in a total volume of 12 µl. The PCR of tomato RFLP markers was done as above except that the reaction mix was as for RAPD-PCR, and the annealing temperature was 50 °C. In all cases the amplification products were resolved on a 1.5–1.7% agarose gel.

Cloning of RAPD fragment NR14

The NR14 band was excised from the agarose gel and purified with a GeneClean kit (Bio101, Inc., La Jolla, Calif., USA) as recommended by the manufacturer. The purified product was cloned using a TA cloning kit (InVitrogen Corp., San Diego, Calif., USA). The ends of the insert were sequenced by the Nucleic Acid Facility, Iowa State University, Ames, Iowa.

Southern analysis

For RAPD reactions, 20 µl of each reaction was loaded onto a 2.0% agarose gel. After electrophoresis, the gel was stained and photographed, then transferred overnight with 10 × SSPE to Hybond N+ membranes (Amersham Corp., Arlington Heights, Ill. USA). The membrane was baked at 80 °C for 1 h. For genomic DNA, about 5–10 µg of each DNA sample was digested with the proper restriction enzyme and then loaded in the gel. Membranes were prepared as above.

PCR-amplified fragments were used as probes in hybridization. For the RAPD fragment NR14, the band was excised from the gel, purified using the GeneClean kit and then used in labeling. For the RFLP markers, the PCR amplification of each RFLP DNA was purified using the Magic-clean kit (Promega Crop., Wisc., USA) and used in labeling. The probe DNA was labeled with [^{32}P] dCTP by the random primer method (Feinberg and Vogelstein 1983) using a multipriming labeling kit from Amersham Co. Hybridization was done at 65 °C in an incubator/shaker. Blots were washed in low-stringency buffer (2 × SSC, 1% SDS) for 10–15 min at 65 °C, then in high-stringency buffer (0.2 × SSC, 1% SDS) for 10–15 min at 65 °C. Blots were exposed to film with intensifying screens at −70 to −80 °C. In cases where the same blots were used multiple times, they were stripped by boiling in high-stringency buffer for about 20 min and exposed to film overnight to ensure that counts had been removed.

Mapping

A collection of 1000 RFLP markers, mapped on the 12 tomato chromosomes, was supplied by S. Tanksley as plasmid clones. New markers were mapped using the tomato mapping population, MapMaker software for Macintosh (Lander et al. 1987) and a data file of the tomato map with more than 1000 mapped RFLP markers which was obtained through the Internet (see Tanksley et al. 1992).

Results

Nematode resistance in *L. peruvianum*

L. peruvianum accessions previously described as nematode resistant (Lobo et al. 1988), as well as additional accessions, were tested for resistance to *M. javanica* strain VW4 (Table 1). Lines that displayed resistance were screened with *M. incognita* strain 557R which infects plants with *Mi*. Also tested were plants from accession PI128657, the original source of *Mi*, and clones of plants reported to have high-temperature resistance to *M. incognita* (Ammati et al. 1986; Cap et al. 1993). In preliminary experiments we observed that gall size and the extent of galling was variable in *L. peruvianum* and difficult to score due to the diverse root morphology in this species. Since egg-mass number is a more direct measure of nematode reproduction, we selected this criterion to measure nematode resistance or susceptibility. Many lines tested showed resistance when infected with standard root-knot nematode strain VW4 (Table 1). VFNT cherry, a tomato line carrying the *Mi* gene, and UC82B, a susceptible processing tomato line, were used as controls in this experiment. VFNT cherry is resistant to typical root-knot nematode isolates (0–2 egg masses per root system after infection with VW4) but susceptible to 557R (> 100 egg masses per root system). UC82B was susceptible to both isolates. We chose the criterion of > 25 egg masses per root system as susceptible and ≤ 25 egg masses per root system as resistant. Based on this criterion we identified several accessions with potential resistance to 557R (Table 1).

Table 1 Screening *L. peruvianum* for nematode resistance

Plants	Source[b]	Response to root-knot nematodes[a]	
		Strain VW4	Strain 557R
Accessions			
LA98	1	S, S, S	S, S
LA103	1	R, S, S	S, S, S
LA110	1	R, R, R,	S, R, S
LA1537	1	—	S, S, S
LA2157	1	R, R, R	S, S, S
PI126448	2	R, R	R, S, R
PI128653	2	R, R	R, R
PI199380	2	R, R	S, R
Clones			
PI128657-G	2	R, R	S
PI128657-3R4	3	R, R, R	S, S, S
PI270435-3MH	3	R, R, R	R, R, R
PI270435-2R2	3	R, R, R	R, R, R
VWP2	4	R, R	R, R
VWP3	4	R, R	S, S
VWP4	4	R, R	S
VWP5	4	R, R	R, R, R

[a] For the accessions tested, each letter represents the score of a different plant from that accession; for the clones, each letter represents the score obtained from a vegetatively propagated cutting. "S" indicates > 25 egg masses per root system; "R" is ≤ 25 egg masses per root system

[b] Source 1 is C.M. RickTomato Genetics Resource Center, University of California, Davis, USA; 2 is USDA-ARS Germplasm Resources Unit, Geneva, New York; 3 is Dr. P. Roberts, University of California, Riverside; 4 is our own laboratory (see Fig. 1)

Segregation of novel resistance

Resistance in plant VWP2, which was the result of a cross of the 557R-susceptible plant (PI128657-G) with PI126443-1MH (Fig. 1), suggested that this trait was dominant and that it was inherited from PI126443-1MH. To investigate the inheritance, VWP2 was crossed to the susceptible plant VWP4 and the segregation of resistance in progeny plants (family VWP2x4) was scored (Fig. 1; Table 2). If the resistance was conferred

Fig. 1 Pedigree of segregating family VWP2x4. PI126443-1MH and PI126440-9MH were obtained as vegetative cuttings from P. Roberts, University of California, Riverside (Ammati et al. 1986)

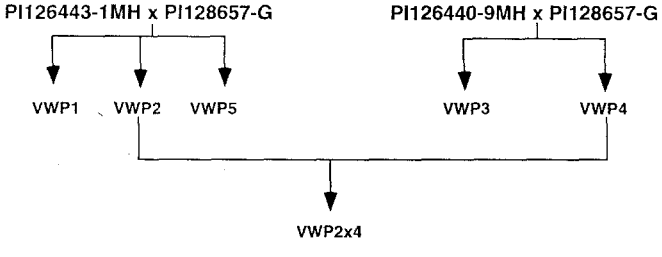


Table 2 Nematode resistance in segregating population VWP2x4

Plant	Response to strain 557		Response to strain VW4 at 32 °C		Linked markers			
	Egg masses[a]	R or S[b]	Egg masses[a]	R or S[b]	NR14	TG180	NR18	TG68
1	0, 0, 0	R	2, 2	R	+	+	+	+
2	0, 0, 0	R	6, 10	R	+	+	+	+
3	0, 0, 1	R	2, 25	R	+	+	+	+
4	0, 1, 8	R	2, 8	R	+	+	+	+
5	0, 5, 10	R	1, 11	R	+	+	+	+
6	1, 6, 17	R	2, 30	R	+	+	+	+
7	10, 14, 15	R	1, 1	R	+	+	+	+
8	0, 1, 41	R	0, 4	R	+	+	+	+
9	0, 18, 20	R	0, 4	R	+	+	+	+
10	0, 9, 29	R	15[c]	R	+	+	+	+
11	21, 23, 31	R	6, 8	R	+	+	+	+
12	0, 0, 0	R	6, 8	R	+	+	+	–
13	1, 1, 2	R	0, 3	R	+	+	+	–
14	0, 1, 9	R	3, 14	R	+	+	+	–
15	0, 0, 0	R	0, 0	R	+	+	–	–
16	0, 3, 3	R	0, 6	R	+	+	–	–
17	0, 2, 7	R	2, 8	R	+	+	–	–
18	18, 26, 38	S	44, 70	S	–	–	–	–
19	13, 23, 53	S	65, 100	S	–	–	–	–
20	10, 11, 70	S	92, 85	S	–	–	–	–
21	28, 31, 34	S	78, 90	S	–	–	–	–
22	0, 38, 73	S	100, 100	S	–	–	–	–
23	7, 35, 70	S	80, 84	S	–	–	–	–
24	13, 30, 76	S	85, 100	S	–	–	–	–
25	29, 39, 61	S	73, 79	S	–	–	–	–
26	16, 52, 60	S	54, 62	S	–	–	–	–
27	10, 37, 90	S	40, 60	S	–	–	–	–
28	34, 35, 70	S	73, 89	S	–	–	–	–
29	1, 42, 100	S	74, 100	S	–	–	–	–
30	27, 53, 83	S	85, 100	S	–	–	–	–
31	53, 62, 69	S	55, 80	S	–	–	–	–
32	31, 70, 80	S	82, 100	S	–	–	–	–
33	25, 59, 100	S	95, 95	S	–	–	–	–
34	31, 70, 85	S	100, 100	S	–	–	–	–
35	15, 80, 100	S	3, 100	S	–	–	–	–
36	27, 70, 100	S	58, 80	S	–	–	–	–
37	50, 56, 100	S	100, 100	S	–	–	–	–
38	70, 80, 100	S	75, 98	S	–	–	–	–
39	39, 100, 100	S	100, 100	S	–	–	–	–
40	70, 90, 100	S	100, 100	S	–	–	–	–
41	100, 100, 100	S	85, 100	S	–	–	–	–
42	50, 70, 90	S	50, 55	S	–	–	–	+
43	0, 1, 16	R	40, 75	S	+	+	+	–
44	0, 9, 14	R	16, 68	S	+	+	+	+
45	4, 9, 18	R	16, 36	S	+	+	+	+
46	0, 0, 0	R	42, 70	S	–	–	–	–
47	0, 3, 16	R	73, 89	S	–	–	–	–
48	7, 23, 39	R	70, 80	S	–	–	–	–

[a] Egg masses per root system (counted up to 100). Each number represents a replicate from a rooted cutting
[b] Susceptible (S), average number of egg masses per root system (n) > 25; Resistant (R), $n \leq 25$
[c] Replicate died

by a single, major locus derived from PI126443-1MH, it would be expected to yield a 1:1 segregation ratio of resistant to susceptible plants. Forty-eight progeny plants (VWP2x4-1 to VWP2x4-48) were grown in the greenhouse and each was propagated vegetatively as cuttings. Three cuttings of each of 48 F_2 were infected with *Mi*-infecting root-knot nematode variant strain 557R and, after 6–8 weeks, were scored for resistance (Table 2). The response was variable, but clear differences could be seen. Based on the criteria used above, 25 plants were scored as suceptible and 23 as resistant. This result corresponds to a 1:1 segregation ratio (chi-square not significant at the 0.05 level) which is consistent with a single dominant gene as the cause of this new resistance. We have designated this new gene *Mi-3*.

Two additional cuttings from each of the 48 progeny plants were infected with *M. javanica* strain VW4 at 32 °C and held at this temperature for 6 weeks. The number of egg masses per root system was determined (Table 2). At this temperature, VFNT cherry control plants were scored as suceptible. All individuals with high-temperature resistance had been scored as resistant to 557R, but, in a few cases, plants scored as resistant to 557R were found to be susceptible to VW4 at high

temperature (Table 2). The segregation ratio for high-temperature resistance was skewed (17 resistant to 31 susceptible plants). Overall, the data presented here indicates a strong correlation between *Mi-3* and the high-temperature resistance in this population.

Two cuttings from each of 20 progeny were inoculated with our standard root-knot nematode strain VW4 and scored for resistance after growth in the greenhouse at temperature of $< 28\,^{\circ}C$. Few egg masses were found (< 3 per root system compared to > 100 for susceptible control UC82B) in any of the 20 progeny, suggesting the presence of an additional resistance gene, possibly *Mi*, in all progeny.

Identification of DNA markers linked to new resistance

Bulked-segregant analysis (Michelmore et al. 1991) was employed to identify molecular markers associated with resistance to nematode strain 557R. From the first 20 progeny tested for 557R resistance, two pools of DNA were made, one with equal amounts of DNA from six plants scored as resistant and the other with DNA from six susceptible individuals. The two DNA pools were screened for RAPD polymorphisms using 520 decamer oligonucleotides as primers. On the average, eight bands (1–18) were detected per amplification. Potential polymorphisms were repeated to verify reproducibility. Overall about 4160 bands were compared and approximately 3% of the scorable bands were polymorphic between the two DNA bulks. Oligonucleotides found to generate reproducible polymorphisms between the bulks were used to amplify DNA from parental lines. Primers producing potentially linked bands were used to amplify DNA from each of the 48 VWP2x4 progeny. The two markers NR14 and NR18 correlated most strongly with resistance to 557R. NR14, a 750-bp band amplified in the resistant bulk and resistant parent VWP2 using primer OPF14, was present in 20 of 23 plants scored as resistant to nematode strain 557R and was not present in any of individuals scored as susceptible (Fig. 2; Table 2). NR14 was present in all 17 plants scored as resistant at high temperature and was present in 3 of 31 scored as S for the high-temperature resistance. The second marker, NR18, a band of 1.2 kb produced by primer OPE18 in the resistant bulk and resistant parent VWP2, correlated with marker NR14 in all but three cases (Fig. 2, Table 2).

The 750-bp PCR product NR14 was cloned and the DNA sequence of the ends of the insert was determined. A pair of 20-mer primers (NR14A/R and NR14A/U) was selected from this sequence and synthesized. These specific primers amplified a 620-bp PCR product from both parents. Screening for polymorphisms with restriction enzymes revealed that this region was conserved between the two parents, but a polymorphism was detected after cleavage with the restriction enzyme *Acc*I. *Acc*I digested the amplified product from the susceptible

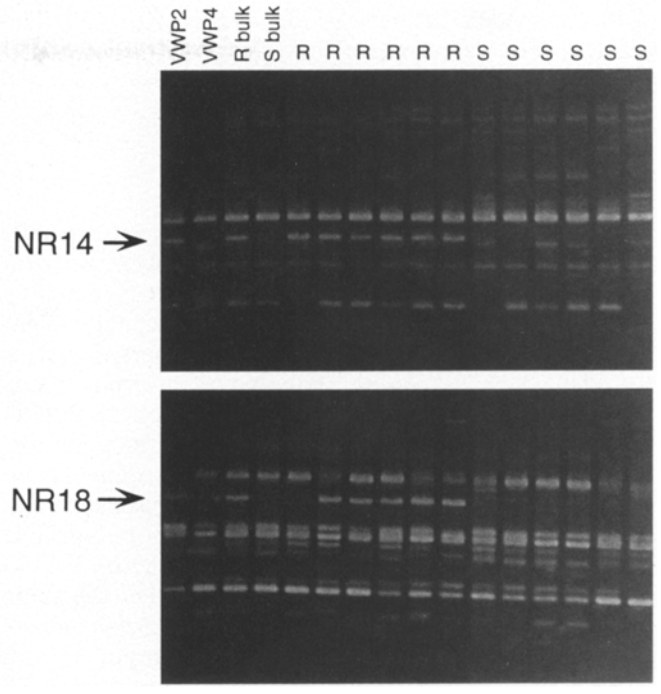
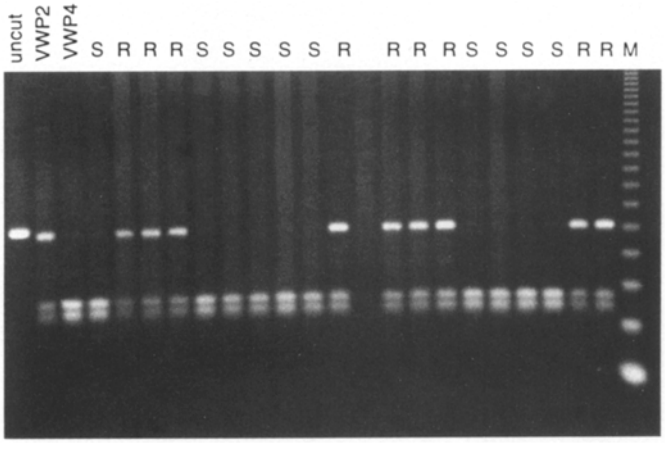


Fig. 2 RAPD markers linked to new nematode resistance. PCR amplification was carried out on DNA with primer OPF14 to produce RAPD marker NR14, and with primer OPE18 to produce RAPD marker NR18. *L. peruvianum* DNA was from VWP2, VWP4, resistant bulk (*R bulk*), susceptible bulk (*S bulk*), six resistant individual progeny (*R*), and six susceptible progeny (*S*)

plant into two fragments but did not digest the product linked to the resistant allele. Heterozygous resistant plants showed three bands; one allele cut into two fragments and the other was uncut (Fig. 3) yielding a co-dominant marker. The 48 progeny in the VWP2x4 population were scored after amplification of their DNA

Fig. 3 Segregation of co-dominant PCR marker NR14 in cross VWP2x4. DNA was amplified using primers NR14A/R and NR14A/U as described in Materials and methods. Amplified DNA in *lane 1* is undigested and DNA in the remaining lanes are digested with the restriction enzyme *Acc*I. Parental lines (*VWP2* and *VWP4*) and resistant (*R*) and susceptible (*S*) segregants are shown. *Lane M* contains molecular-weight markers



with the specific primers and cleavage with *Acc*I. The results were in complete agreement with the NR14 scoring presented in Table 2.

Mapping *Mi-3*

A mapping population developed from an interspecific cross of *L. esculentum* (cv VF36-Tm2a) and *L. pennellii* (LA716) (Tanksley et al. 1992) was used to genetically position *Mi-3*. DNA from the mapping-population parents was amplified with the RAPD primers that produced markers NR14 and NR18. No polymorphism was found corresponding to NR18. With the NR14 RAPD primer, a band of 750 bp, the same size as the polymorphism in the resistant *L. peruvianum*, was amplified from *L. esculentum* but not from LA716. The presence of this band segregated in the tomato mapping population. It was necessary to determine whether the 750-bp RAPD band amplified in *L. esculentum* represented the same locus as the band of the same size from *L. pervuianum* plant VWP2. For this purpose, the band amplified from VWP2 was excised from an agarose gel, purified, labeled, and probed against a Southern-blot of DNA from VWP2, VWP4, *L. esculentum*, *L. pennellii*, and 33 individual plants from the tomato mapping population that had been amplified with the OPF14 primer. In all cases where a 750-bp band was present, it hybridized to the probe, indicating that the *L. esculentum* band corresponded to locus NR14. With the NR14-specific primers a 620-bp band was amplified from both parents in the tomato mapping population. The restriction enzyme *Ava*I produced a polymorphism, cutting the *L. pennellii* product but not that of *L. esculentum*, and thus providing a co-dominant marker.

As a test of our material, we first mapped REX-1, a PCR-based marker known to be tightly linked to *Mi* on chromosome 6 (Williamson et al. 1994). REX-1 is a co-dominant marker and a polymorphism was detected between the tomato mapping-population parents by cleaving the amplified product with the restriction enzyme *Taq*I. The mapping-population progeny were scored for REX-1, the data were entered in the 1000 marker data file and the Map-maker program was used to position REX-1. As expected REX-1 mapped with a LOD score of > 5 to chromosome 6. We then scored the tomato mapping-population progeny with the NR14 co-dominant marker and mapped it accordingly. NR14 mapped near the telomeric region of the short arm of chromosome 12 with a LOD score of > 5. The nearest marker was RFLP marker TG180 which mapped 6 cM proximal to NR14. The segregation data for NR14, TG180, CT19, CD19, TG68, CT79 and CT211 (all markers from the region of interst in chromosome 12) were examined for individual plants in the tomato mapping-population file and all results were consistent with simple cross-over events.

Southern analysis was employed to prove the linkage of NR14 with markers on the short arm of chromosome 12 in the *L. peruvianum* VWP2x4 progeny. Southern blots were prepared of DNA from VWP2 and VWP4 digested with five enzymes (*Eco*RI, *Eco*RV, *Dra*I, *Hae*III, and *Bst*NI). Labeled DNA of the RFLP markers TG180, CT19, CD19, TG68, CT79, CT100, CT211, TG263 and TG360 were probed against the parental blots digested with the enzymes recommended for each RFLP marker. The polymorphic markers, TG68 and TG180, were probed against the blots with digested DNA from each of the 48 individuals in the VWP2x4 family (Fig. 4; Table 2). TG180 proved to be completely linked to NR14 (no recombination observed in 48 progeny) in the VWP2x4 family and 8 out of 48 individuals were recombinant between NR14 and TG68.

Discussion

We have mapped a gene that is a major factor in resistance to a strain of root-knot nematode capable of infecting tomato plants carrying the *Mi* gene. This gene, which we have designated *Mi-3*, is unlinked to *Mi* (*Mi-3* maps to chromosome 12 while *Mi* is on chromosome 6) and correlates with resistance at high temperatures where *Mi* is ineffective. We have identified a PCR-based marker, NR14, which is tightly linked to *Mi-3*. The closest RFLP marker to *Mi-3* and NR14 is TG180. In the *L. peruvianum* population segregating for *Mi-3*, TG180 was not separated from NR14 but, in the tomato

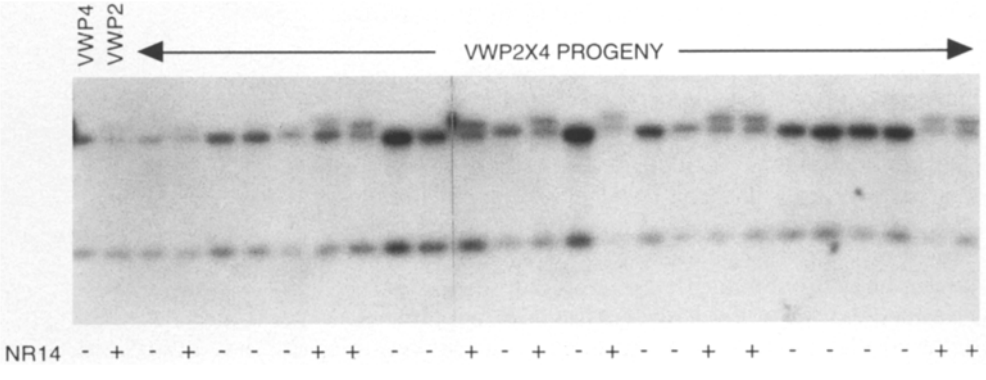


Fig. 4 Segregation of RFLP marker TG180 in cross VWP2x4. A Southern blot of DNA from VWP2, VWP4 and progeny VWP2x4 was digested with *Dra*I and probed with TG180. The NR14 phenotype is indicated below ("+" for presence of R allele and "−" for absence of this allele)

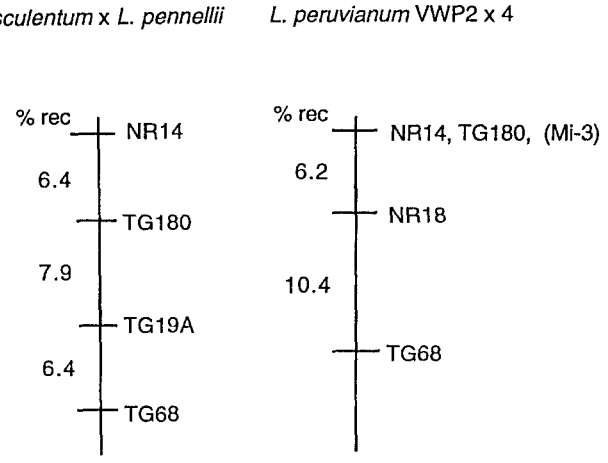


Fig. 5 Map position of *Mi-3*. Position of markers on the distal end of the short arm of chromosome 12. Map is shown for mapping population (*L. esculentum* x *L. pennellii*) and for the *L. peruvianum* cross VWP2x4 segregating for *Mi-3*

mapping population, NR14 maps 6.4 cM distal to TG180, which is the most distal molecular marker mapped on chromosome 12 (Fig. 5; Tanksley et al. 1992). In the *L. peruvianum* VWP2x4 population, the segregation ratio for markers below NR14 and TG180 was significantly skewed (see Table 2). The skewing increases as the centromere is approached, suggesting that there may be a factor on that part of chromosome 12 which is detrimental in the heterozygous form.

Scoring for resistance to 557R was not as straight forward as for *Mi*-mediated resistance to typical *Meloidogyne* strains. We found a broad range of egg-mass numbers per plant (see Table 2). A number of factors could explain this variablity. For example, root morphology and growth was highly variable among individuals, possibly affecting nematode infection. Cuttings were used in these studies, and root-regeneration rate varied considerably within this population. Thus the size of the root system at inoculation was likely to differ among individuals. Three individuals that were scored as resistant to 557R did not carry any of the four linked markers on chromosome 12. This could be due to recombination events between the markers and *Mi-3*, to the presence of other genetic factors for resistance in these accessions, or to mis-scoring of these individuals as resistant. In addition, all the plants in our segregating population are heterozygous for *Mi-3* and it is possible that resistance is incompletely dominant. We plan to generate homozygous resistant plants by sib mating and will test their resistance with 557R.

Our study of resistance derived from *L. peruvianum* accession PI126443 indicates a strong correlation between resistance to 557R and resistance at high temperature. Cap et al. (1993) showed that, in *L. peruvianum* accession PI270435, a gene, *Mi-2*, which is independent of *Mi*, is responsible for heat-stable resistance to typical strains of *M. incognita*. We find that PI270435 also carries resistance to the *Mi*-infecting nematode strain 557R (Table 1). We have designated the resistance from PI126443 as *Mi-3*. It is quite possible that *Mi-2* and *Mi-3* are the same gene. However, because of the large diversity within *L. peruvianum*, it is also possible that *Mi-2* and *Mi-3* represent different loci. PI126443 has been classified as *L. glandulosum* (Ammati et al. 1986) but is generally considered a "mountain race" of *L. peruvianum* or *L. peruvianum* var. *glandulosum* (Rick 1963). PI270435 does not fall into this grouping. In addition, it cannot be determined from our current data whether *Mi-3* and the high-temperrature resistance to VW4 derived from PI126443 are due to the same gene or to two different, but tightly linked, genes.

In the present study three plants that carry marker NR14 were scored as susceptible to strain VW4 at high temperature; all three had been scored as resistant to 557R. These three plants could be recombinants placing the heat-stable resistance gene, in the simplest case, distal to NR14 and *Mi-3* on chromosome 12. Alternatively, the susceptibility of these plants could indicate that *Mi-3* is necessary, but not sufficient, for high-temperature resistance. It is also worth pointing out that, for our high-temperature resistance screening, we used the nematode species *M. javanica* while previous studies had used *M. incognita* (Ammati et al. 1986; Cap et al. 1993). Thus the heat-stable resistance may protect against both nematode species or these resistances may be due to different genes. It will be interesting to check the segregation of high-temperature resistance to strain 557R. Obviously, further investigation is necessary to determine the relationship of these resistance factors, as well as their evolutionary and functional relationship with *Mi*. The genetic relationships among the nematode resistance factors in other *L. peruvianum* accessions (see Table 1) to the presently characterized genes is also not known.

Markers that we have identified and developed in this study, especially NR14, will be valuable for incorporation of *Mi-3* into cultivated tomato by traditional breeding, and also provide a tool for map-based cloning of this gene. *Mi*, which has already been incorporated into *L. esculentum* lines, behaved as a single locus during the introgression process. Whether *Mi-3* will behave in a similar fashion in a *L. esculentum* background or will require additional factors from the original source remains to be seen. Incorporation of *Mi-3* (and *Mi-2* or other genes) into cultivated tomato would broaden the basis of root-knot nematode resistance. Map-based cloning of *Mi* from *L. esculentum* lines has proved challenging due to the suppression of recombination in the region of the introgressed gene. Repression of recombination may be less problematic for *Mi-3* as this gene appears to be located in the sub-telomeric region of chromosome 12, a region that is not dense in markers, suggesting that recombination is not suppressed (Tanksley et al. 1992). This study provides another example of the rich resource of resistance genes present in the *L. peruvianum* complex (Rick and Yoder 1988).

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