A Molecular Marker Correlated with Selected Virulence Against the Tomato Resistance Gene *Mi* in *Meloidogyne incognita*, *M. javanica*, and *M. arenaria*

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ABSTRACT

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Root-knot nematodes of the genus *Meloidogyne* are economically important pathogens of a wide range of crops. The tomato resistance gene *Mi* typically confers resistance to the three major species, *M. incognita*, *M. javanica*, and *M. arenaria*. However, virulent populations completely overcoming the *Mi* resistance still occur. In an attempt to develop molecular markers for virulence against *Mi* and gain insights into the genetic relationships among virulent populations of different species and origins, random amplified polymorphic DNA (RAPD) analyses of laboratory-selected virulent, field virulent, and avirulent populations of *M.*

incognita, *M. javanica*, and *M. arenaria* were carried out. A RAPD marker, specific for selected virulent populations, was identified, and subsequently, converted to a sequence characterized amplified region (SCAR). Sequence characterization of the SCAR locus showed that alleles from laboratory- and field-selected virulent populations were highly similar to each other and clearly different from alleles from natural virulent and avirulent populations. This result suggests that the genetic mechanism for virulence against *Mi* may be similar among selected virulent populations of the three *Meloidogyne* spp., but different between selected and natural virulent populations. Based on the nucleotide polymorphisms at the SCAR locus, codominant and dominant polymerase chain reaction-based markers were developed enabling rapid diagnosis of selected virulent genotypes in *M. incognita*, *M. javanica*, and *M. arenaria*.

Root-knot nematodes of the genus *Meloidogyne* are economically important pathogens of a wide range of crops (16). Infective second-stage juveniles of these obligate endoparasites penetrate the roots of the host and migrate intercellularly to the vascular cylinder, where they settle down and transform several host cells into specialized feeding cells (giant cells) (20). The continuous feeding by the nematodes adversely affects the normal physiological processes of the host root system, which is phenotypically manifested by the formation of the characteristic disease symptom of root galls. To date, approximately 70 species of *Meloidogyne* have been described, of which the three mitotic parthenogenetic species, *M. incognita*, *M. javanica*, and *M. arenaria*, are distributed worldwide and account for most of the crop losses due to root-knot nematodes.

Use of crop resistance constitutes an important component in many control programs for root-knot nematodes. In tomato, the dominant resistance gene *Mi* from the wild species *Lycopersicon peruvianum* has been incorporated into many cultivated tomatoes to combat root-knot nematode infestations (14). *Mi* typically confers high resistance to *M. incognita*, *M. javanica*, and *M. arenaria*. The resistance is phenotypically characterized by a hypersensitive response at sites where nematodes attempt to feed. However, use of this resistance could be hampered by the occurrence of resis-

tance-breaking virulent populations. In fact, field populations that completely overcome the *Mi* resistance have been reported for these three *Meloidogyne* spp. from different regions of the world (2,7,11,13,19).

Mi-virulent populations can be differentiated into different types, based on their origin. These include those detected from fields where resistant tomatoes with *Mi* had been grown for extended time or selected under laboratory-controlled conditions (i.e., selected virulent populations) (7,10,11,13,19), and those detected from places where resistant tomatoes with *Mi* had not been exposed in recent times (i.e., natural virulent populations) (4,10,11,17). The genetic relationships among virulent populations of different *Meloidogyne* spp. and different types of virulence are currently poorly characterized. It is still not clear whether *M. incognita*, *M. javanica*, and *M. arenaria* employ a similar mechanism to overcome the *Mi* resistance. Several studies have compared the reproduction characteristics of laboratory selected and natural virulent populations of *M. incognita*. They showed differences in virulence specificity, virulence stability, and reproduction rate between the two types of virulent populations, suggesting that the genetic determinants for virulence against *Mi* may be different between them (3,4,15).

Molecular characterization of various *Mi*-virulent and avirulent populations should increase our understanding of the genetic relationships among virulent populations of different origins and provide the basis for the development of rapid procedures for the accurate diagnosis of virulent genotypes. For these reasons, several attempts have recently been made to investigate the genotypic relationships among virulent and avirulent populations of these

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three species (1,17,18). Those studies, using the techniques of random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphism (AFLP), demonstrated general DNA polymorphisms among different populations tested, but they fell short of revealing markers correlated with the virulence character. The current study constitutes a continued effort toward the molecular characterization of *Mi*-virulent and avirulent populations. We employed RAPD to analyze the genotypic differences among laboratory-selected virulent, field virulent, and avirulent populations of *M. incognita*, *M. javanica*, and *M. arenaria* from different geographical locations. Our objectives were to identify RAPD markers that correlate with virulence, exploit the RAPD markers to develop sequence characterized amplified region (SCAR) for the rapid identification of virulent genotypes, and gain insights into the genetic relationships among virulent populations of different species and origins.

MATERIALS AND METHODS

Nematode populations. Nematode populations used in this study are listed in Table 1. All the populations were derived from single egg-masses from field populations. The species identity of the populations was determined by means of morphological observation of female perineal patterns and isozyme analyses (8). Virulence of the populations toward *Mi* was determined according to their relative reproduction rates on resistant tomato cv. Momotaro with *Mi*, and susceptible tomato cv. Kyoryokubeiju without *Mi*, following the procedure of Narabu and Momota (13). Populations producing a comparable number of egg-masses on the resistant and susceptible cultivars were typed as virulent (VI); populations producing no or very few egg-masses (less than 1% of the initial inoculum) on the resistant cultivar were typed as avirulent (AV). Of the populations, *M. javanica* MJON-VI and MJON-

AV constitute near isogenic virulent and avirulent lines, respectively. MJON-VI was derived from MJON-AV by continuous selection for 16 generations on cv. Momotaro at 25°C in a greenhouse.

DNA extraction. Clean egg-masses were hand picked from infected tomato roots, and juveniles were hatched in water. Total genomic DNA was extracted from juveniles according to the miniprep method of Cenise (5), except that an incubation of the nematode solution for 1 h at 65°C was added following the grinding of nematodes. The quality and quantity of extracted DNA samples were checked by subjecting them to electrophoresis and ethidium bromide staining together with uncut λ DNA standards.

RAPD analysis. Altogether, 202 10-base and 12-base primers of random sequences (OP-26, OPF, and OPG; Operon Technologies, Alameda, CA: UBC Set 2; Biotechnology Laboratory, University of British Columbia, Canada: and NG-A1, NG-B1, and NG-C1; Wako Chemicals, Osaka, Japan) were used in the study. RAPD analysis was initially performed on near isogenic *Mi*-virulent and avirulent lines MJON-VI and MJON-AV of *M. javanica*. Primers displaying polymorphisms between the two lines were further tested on other virulent and avirulent populations of *M. incognita*, *M. javanica*, and *M. arenaria* to verify the correlation of the polymorphic bands to virulent or avirulent populations. RAPD-polymerase chain reaction (PCR) was conducted in 25- μ l reaction volume containing 10 ng of template DNA, 0.8 μ M primer, 0.2 mM dNTPs, 1 $\times$ PCR buffer (10 mM Tris, pH 8.3, 50 mM KCl, and 2 mM MgCl₂), and 1 unit of Takara *Taq* DNA polymerase (Takara Biomedicals, Otsu, Japan). For 10-mer primers, reaction mixtures were subjected to a preheating at 94°C for 4 min, followed by 45 cycles of 30 s at 94°C, 1 min at 36°C, and 2 min at 72°C, and a final incubation at 72°C for 5 min using a thermal cycler (9700; Perkin-Elmer Applied Biosystems, Foster City, CA). For 12-mer primers, similar cycling conditions were used, except that the annealing temperature was adjusted to 40°C.

TABLE 1. Populations of *Meloidogyne* spp. used in this study

Species	Population	Code	Origin	<i>Mi</i> virulence ^a	Codominant marker		Dominant marker
					429/444 bp	873 bp	1,032 bp
<i>M. incognita</i>	CHB9601	MIAS-VI	Chiba, Japan	VI*	+	–	+
	CHB9505	MICK-VI	Chiba, Japan	VI*	+	–	+
	FKS9601	MIFS-VI	Fukushima, Japan	VI*	+	–	+
	KAN9501	MIKN-VI	Kanagawa, Japan	VI*	+	–	+
	NGN91	MINN-VI	Nagano, Japan	VI*	+	–	+
	YMN9001-29	MIYN-VI	Yamanashi, Japan	VI*	+	–	+
	INGD1-1	MIGD-VI	Guangdong, China	VI**	–	+	–
	CHB89-27	MICB-AV	Chiba, Japan	AV	–	+	–
	IBA9401	MIIK-AV	Ibaraki, Japan	AV	–	+	–
	MYZ9626	MIKS-AV	Miyazaki, Japan	AV	–	+	–
	IRI9416	MION-AV	Okinawa, Japan	AV	–	+	–
	SIZ9001	MISO-AV	Shizuoka, Japan	AV	–	+	–
	INHB1-2	MIHB-AV	Hubei, China	AV	–	+	–
	INH1-1	MIHN-AV	Henan, China	AV	–	+	–
	INJS1	MJS-AV	Jiangsu, China	AV	–	+	–
	INSH1	MISH-AV	Shanghai, China	AV	–	+	–
<i>M. javanica</i>	ISI9206-25	MJON-VI	Okinawa, Japan	VI***	+	–	+
	UFL9702	MJFL-VI	Florida, U.S.	VI*	+	–	+
	ISI9206-28	MJON-AV	Okinawa, Japan	AV	–	+	–
	CHB9501	MJCB-AV	Chiba, Japan	AV	–	+	–
	KGO9635	MJKS-AV	Kagoshima, Japan	AV	–	+	–
	JAFJ-1	MJFJ-AV	Fujian, China	AV	–	+	–
	JAGD-1	MJGD-AV	Guangdong, China	AV	–	+	–
<i>M. arenaria</i>	FKS9301-24	MAFS-VI	Fukushima, Japan	VI*	+	–	+
	HOK9701	MAHK-VI	Hokkaido, Japan	VI*	+	–	+
	FKS9701	MAOG-VI	Fukushima, Japan	VI*	+	–	+
	FKS9611	MAYG-VI	Fukushima, Japan	VI*	+	–	+
	MYG9404	MAMG-AV	Miyagi, Japan	AV	–	+	–
	MYK9212-7	MAMG-AV	Okinawa, Japan	AV	–	+	–
	ARYN2-4	MAYN-AV	Yunnan, China	AV	–	+	–

^a VI = virulent; *, **, and *** indicate populations detected from fields where resistant tomatoes had been grown (field-selected virulent populations), population detected from a field where resistant tomatoes had not been grown (natural virulent population), and population selected from an avirulent population in the laboratory (laboratory-selected virulent population), respectively. AV = avirulent populations.

Cloning and sequencing of RAPD fragments. Candidate RAPD marker bands were excised from low-melting-temperature agarose gels, reamplified, and purified with a purification kit (UltraClean 15; MO BIO Laboratories, Solana Beach, CA). The gel-purified fragments were then cloned into the pGEM-T Easy vector (Promega, Madison, WI). The authenticity of the cloned fragments was checked by hybridizing inserts to gel blots of the original RAPD products. Sequencing of clones was done on an ABI Prism 310 genetic analyzer using a cycle sequencing reaction kit (Rhodamine Terminator FS; Perkin-Elmer Applied Biosystems).

SCAR analysis. Initial SCAR analysis was based on the sequence of the RAPD marker generated with primer UBC-193, which is specific for selected virulent populations. To facilitate amplification and comparison of allelic sequences of the marker locus from both virulent and avirulent populations, an additional sequence bordering one terminus of the original RAPD marker was determined by thermal asymmetrical interlaced (TAIL)-PCR (12). The extended sequence formed the basis for subsequent SCAR primer design and analysis.

All SCAR primers were designed with the aid of the software Amplify (version 1.2, B. Engels, University of Wisconsin). The SCAR allelic fragments amplified with primer set MVC-F2/R1 from representative virulent and avirulent populations were gel-purified, cloned, and sequenced as described previously. The sequences were deposited in the DDBJ/EMBL/GenBank databases (Accession Nos. AB047761 to AB047767). Sequence alignment, pairwise similarity calculation, and phylogenetic tree construction of the SCAR alleles were performed with CLUSTAL W. The nucleotide polymorphisms revealed among the SCAR alleles were exploited to design primer sets MVC-F3/R2 and MVC-VF/R1 to display codominant and dominant markers, respectively, for selected virulent populations. The consistency of the markers was examined by amplification tests of DNA from all the populations listed in Table 1. SCAR amplification reactions in 25- μ l volume included 10 ng of template DNA, 0.2 μ M each primer, 0.2 mM dNTPs, 1 $\times$ PCR buffer, and 1 unit of Takara *Taq* DNA polymerase. Specific amplification conditions were: 4 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at the annealing temperature, and 1 min at 72°C. The annealing temperatures for amplifications with primer sets MVC-F2/R1, MVC-VF/R1, and MVC-F3/R2 were 55, 62, and 60°C, respectively.

Hybridization. Agarose gel-separated DNA products were transferred to Biodyne Plus membranes (Pall Gelman Laboratory, Port Washington, NY) with a VacuGene XL blotting unit

(Pharmacia Biotech, Uppsala, Sweden). Hybridization was performed with an enhanced chemiluminescence direct nucleic acid labeling and detection system (Amersham, Little Chalfont, England) according to the manufacturer's protocols.

RESULTS

RAPD analysis. RAPD analysis was initially carried out on the near isogenic virulent and avirulent lines MJON-VI and MJON-AV of *M. javanica*. Altogether, 202 10-mer and 12-mer primers were screened for their ability to detect polymorphisms between the two lines. Seven primers (OP26-13, OP26-23, UBC-123, UBC-150, UBC-172, UBC-182, and UBC-193) amplified eight distinct and reproducible polymorphic fragments, of which seven were specific for the virulent line and one was specific for the avirulent line (Fig. 1). To further confirm the association of these polymorphic fragments with the virulence and avirulence phenotypes, primers that generated these polymorphic fragments were tested on additional virulent and avirulent populations of *M. javanica* and also on *M. incognita* and *M. arenaria*. This confirmation step revealed that, whereas most of the primers failed to produce the relevant polymorphisms in *M. incognita* and *M. arenaria* (data not shown), primer UBC-193 consistently amplified a fragment of $\approx$ 1.03 kb, specifically from the selected virulent populations of all three species but not from the natural virulent population MIGD-VI. Figure 2 shows an example of the RAPD patterns generated with UBC-193 in *M. incognita*. This specific fragment, which we designate MVC-1.03 kb (*Mi*-virulence correlated), was therefore chosen for further development as a marker for selected virulence against *Mi* in *M. incognita*, *M. javanica*, and *M. arenaria*.

Fig. 1. Random amplified polymorphic DNA patterns of the near isogenic *Mi*-virulent and avirulent lines MJON-VI (V) and MJON-AV (A) of *Meloidogyne javanica* amplified with primers OP26-13, OP26-23, UBC-123, UBC-150, UBC-172, UBC-182, and UBC-193. The arrow indicates the $\approx$ 1.03-kb fragment specific for the virulent line amplified with UBC-193. M: 100-bp DNA ladder (New England Biolabs, Beverly, MA).

Fig. 2. Random amplified polymorphic DNA patterns of 13 populations of *Meloidogyne incognita* amplified with UBC-193. MIYN-VI, MIAS-VI, MICK-VI, MIKN-VI, MIFS-VI, and MINN-VI are selected *Mi*-virulent populations; MIGD-VI is a natural *Mi*-virulent population; and MICB-AV, MIK-AV, MISO-AV, MIJS-AV, MISH-AV, and MIKS-AV are *Mi*-avirulent populations (Table 1). The arrow indicates the $\approx$ 1.03-kb fragment specific for selected virulent populations. M: 100-bp DNA ladder (Gibco BRL, Gaithersburg, MD).

TABLE 2. Random amplified polymorphic DNA and sequence-characterized amplified region primers used in this study

Primer	Sequence (5'→3')
UBC-193	TGCTGGCTTT
MVC-F1	TGCTGGCTTCCACACAGGT
MVC-R1	TGCTGGCTTTCGGAGAACAC
MVC-F2	ACCTTCATCGCACCGCTGTTC
MVC-F3	AAGGGCCTGTTCGTGATGGACGG
MVC-R2	CATGCACGTCGTGCGTGTTC
MVC-VF	TGCTGGGCTGGCTTTCCACA

Conversion of the RAPD marker to a SCAR. To convert the RAPD marker into a more reliable SCAR, the MVC-1.03-kb fragment from population MINN-VI of *M. incognita* was cloned and sequenced. The resulting 1,027-bp sequence was used to design the primer set MVC-F1 and MVC-R1, which complement the terminal sequences of the fragment (Table 2; Fig. 3). As expected, MVC-F1/R1 amplified the original RAPD fragment from all the selected virulent populations. However, this primer set also amplified a weak band of comparable size from most of the avirulent populations, indicating that the original RAPD polymorphism was due to one or a few base mismatches at one or both primer binding sites. To facilitate amplification and comparison of allelic sequences of the MVC locus from both virulent and avirulent populations, we used TAIL-PCR to determine a 900-bp sequence bordering the MVC-F1 binding terminus of the RAPD fragment for population MINN-VI (Fig. 3). Based on the extended sequence of 1,927 bp, several forward and reverse primers were designed and tested for their ability to amplify allelic fragments from various populations. Finally, the primer set MVC-F2 and MVC-R1 (Table 2; Fig. 3) was able to amplify a fragment of ≈ 1.35 kb from both virulent and avirulent populations. This fragment, MVC-1.35 kb, covers the original RAPD marker fragment and ≈ 330 -bp border sequence (Fig. 3).

Sequence characterization of MVC-1.35-kb alleles. The MVC-1.35-kb fragments amplified from three selected virulent populations (MINN-VI, MJON-VI, and MAYG-VI of *M. incognita*, *M. javanica*, and *M. arenaria*, respectively), one natural virulent population (MIGD-VI of *M. incognita*), and three avirulent populations (MIIK-AV, MJON-AV, and MAMG-AV of *M. incognita*, *M. javanica*, and *M. arenaria*, respectively) were cloned, and three clones were sequenced for each population. No intrapopulation sequence variations were found. The seven allelic sequences (Accession Nos. AB047761 to AB047767) were actually 1,357 to 1,371 bp in size, with G+C contents of 61 to 65%. Pairwise sequence similarities among the alleles are given in Table 3. The relationships among the alleles can be represented as a neighbor-joining tree (Fig. 4). The three alleles from the selected virulent populations of the three species formed a tight group, showing only 1 to 2% sequence divergence from each other, but 9 to 14% sequence divergence from the alleles from the natural

Fig. 3. Schematic representation of the MVC locus. Numbers indicate the relative positions of the sequence characterized amplified region primers used in the study on the $\approx 1,357$ -bp sequence.

TABLE 3. Percentage of nucleotide sequence similarities among seven alleles of the MVC locus from virulent (VI) and avirulent (AV) populations of *Meloidogyne incognita*, *M. javanica*, and *M. arenaria*

Population	VI				AV		
	MINN	MJON	MAYG	MIGD	MIK	MJON	MAMG
MINN-VI	...	98	99	91	90	86	91
MJON-VI	...	...	98	91	90	86	91
MAYG-VI	...	...	...	91	91	86	91
MIGD-VI	...	...	...	...	99	88	96
MIK-AV	...	...	...	...	...	88	96
MJON-AV	...	...	...	...	...	...	87
MAMG-AV	...	...	...	...	...	...	...

virulent and avirulent populations. In comparison, the three alleles from the avirulent populations were more divergent from each other (4 to 13% sequence divergence). Overall, the alleles from the selected virulent populations were clearly distinguishable from the rest of the alleles by nucleotide differences spread across the whole length of the sequences. These differences involved nucleotide substitutions and small deletions (data not shown). Using BLAST searches of public databases, no significant matches were found for the MVC-1.35-kb sequences at the nucleic acid level. However, at the deduced amino acid level, part of the MVC-1.35-kb sequences showed significant homology to the C-terminal domains of a group of zinc-metallo proteinases with the characteristic zinc-binding motif HEXXH. The closest matches were the yeast gene product ST24 (Score = 151, E value = $2e - 35$ for the MVC-1.35-kb sequence from MINN-VI) and the hypothetical gene product C04F12.10 from *Caenorhabditis elegans* (Score = 124, E value = $3e - 27$ for the MVC-1.35-kb sequence from MINN-VI). Because a complete open reading frame was not contained in the sequences, we were unable to determine whether the sequences really represent part of a functional zinc-metallo proteinase.

Development of codominant and dominant markers for selected virulent populations. Alignment of the seven MVC-1.35-kb allelic sequences revealed several polymorphisms that were exploited to develop PCR-based markers for selected virulent populations. We designed two procedures that would display codominant and dominant markers, respectively. The codominant marker was based on PCR-restriction fragment length polymorphism involving the restriction enzyme *Nde*I. Using this approach, a fragment of ≈ 873 bp was amplified from various populations with primers MVC-F3 and MVC-R2, which anneal to conserved regions among the seven alleles (Table 2; Fig. 3), and was digested with *Nde*I. Fragments from selected virulent populations were cut into two fragments of 429 and 444 bp, whereas fragments from natural virulent and avirulent populations were not restricted (Fig. 5). The dominant marker was based on specific amplification of part of the MVC-1.35-kb fragment. The forward primer MVC-VF was designed to anneal specifically to a sequence of the three alleles from the selected virulent populations (Table 2; Fig. 3). Using the combination of MVC-VF and MVC-R1, a fragment of 1,032 bp was amplified from selected virulent populations, whereas no product was amplified from either natural virulent or avirulent populations (Fig. 6). The consistency of the markers was evaluated by applying the PCR assays to all the populations (Table 1). Consistent with the original RAPD data, all selected virulent populations showed the expected markers and were unambiguously differentiated from the rest of the popula-

Fig. 4. Neighbor-joining tree showing the relationships among seven alleles of the MVC locus from *Mi*-virulent and avirulent populations of *Meloidogyne incognita*, *M. javanica*, and *M. arenaria*. The tree is based on an alignment of the $\approx 1,357$ -bp sequences from the seven alleles. MINN-VI, MJON-VI, and MAYG-VI are selected virulent populations of *M. incognita*, *M. javanica*, and *M. arenaria*, respectively; MIGD-VI is a natural virulent population of *M. incognita*; and MIIK-AV, MJON-AV, and MAMG-AV are avirulent populations of *M. incognita*, *M. javanica*, and *M. arenaria*, respectively.

tions by the marker phenotypes described previously (Table 1). The sensitivity for detection of the markers from selected virulent populations was examined by amplification tests using sequentially diluted miniprep DNA and crude DNA extracts from individual juveniles. Both the codominant and dominant markers were readily amplified from ≈ 0.1 ng of miniprep DNA, but not from a single juvenile (data not shown).

DISCUSSION

To identify molecular markers that correlate with virulence against *Mi*, we carried out two rounds of RAPD analyses of virulent and avirulent populations from *M. incognita*, *M. javanica*, and *M. arenaria* with 202 random primers. This revealed a RAPD marker specific for selected virulent populations. Conversion of the dominant RAPD marker into a SCAR enabled us to characterize the nucleotide differences at the marker locus MVC between selected virulent and avirulent populations. As indicated by the sequence difference between the near isogenic virulent and avirulent lines MJON-VI and MJON-AV, respectively, this locus experienced extensive nucleotide mutations (substitutions and small deletions) during the process of virulence selection. The high homology among the sequences from the selected virulent populations of the three *Meloidogyne* spp. (Table 2) suggests that the MVC locus was influenced by positive selection (for virulence against *Mi*). It can be argued that if the mutations resulted from neutral evolution, the level of polymorphisms among the virulent populations would be near that between the virulent and avirulent populations. In this connection, it is likely that the MVC locus is close to a genetic determinant for virulence against *Mi*. Positive selection of such a closely linked virulence locus could have caused the low level of nucleotide polymorphisms at the MVC locus among the selected virulent populations through the effect of genetic hitchhiking (9). The sequences of the MVC locus characterized for the current study were short (≈ 1.35 kb), and as such, we were not able to determine the identity of the sequences through BLAST searches of databases. It would be of interest to characterize the genomic regions adjacent to the MVC locus to see whether open reading frames exist that show coding or regulatory sequence differences between selected virulent and avirulent populations.

Virulent populations that break the *Mi* resistance have been reported for all three *Meloidogyne* spp. (2,7,11,13,19). Of the reported virulent populations, the majority were detected from fields where resistant tomato cultivars with *Mi* had been extensively grown for successive seasons, and hence, they have been selected

from avirulent populations. This seems logical because several studies have demonstrated that under laboratory-controlled conditions virulent lineages can be rapidly selected from some avirulent populations by repeated propagation on resistant tomatoes (10). However, the genetic relationships among selected virulent populations of different *Meloidogyne* spp. and different geographical origins have not been tested. In this study, we analyzed laboratory- and field-selected virulent populations of *M. incognita*, *M. javanica*, and *M. arenaria* from different geographical locations. Sequence characterization and amplification tests with the codominant and dominant markers indicated that the genomes of all the selected virulent populations experienced similar mutational events at the MVC locus under the selection pressure of *Mi* (Tables 1 and 2). Supposing that the MVC locus is closely linked to a virulence determinant, the results suggest that avirulent populations under selection develop virulence against *Mi* through a similar genetic mechanism in *M. incognita*, *M. javanica*, and *M. arenaria*.

Sequence characterization and amplification tests with the markers also revealed that at the MVC locus the natural virulent population was significantly different from the selected virulent populations (Tables 1 and 2), indicating that the genetic events leading to the acquisition of virulence against *Mi* may be different between the two types of virulent populations. This result can be explained by assuming that the genetic determinants for virulence against *Mi* are different between natural and selected virulent populations. Although there is a need to confirm the result by analyzing more natural virulent populations from the three *Meloidogyne* spp., such a hypothesis is in agreement with the results of several biological studies (3,4,15) that showed differences in virulence specificity, virulence stability in the absence of the selection pressure, and reproduction rate between laboratory-selected and natural virulent populations in *M. incognita*. The three *Meloidogyne* spp. studied here reproduce by obligate mitotic parthenogenesis, and as such, the genetic basis in different types of virulent populations can not be analyzed by traditional genetic approaches. However, in the case of the amphimictic soybean cyst nematode (*Heterodera glycines*), controlled crossing experiments revealed independent nematode genes for parasitism on the same resistant host (6).

Based on the nucleotide polymorphisms at the MVC locus, we have developed PCR-based codominant and dominant markers that distinguish selected *Mi*-virulent populations from avirulent populations in *M. incognita*, *M. javanica*, and *M. arenaria*. Consistent with the RAPD analysis result, the markers showed complete correlation with the trait of selected virulence when they

Fig. 5. Codominant sequence characterized amplified region marker for selected *Mi*-virulent populations of *Meloidogyne incognita*, *M. javanica*, and *M. arenaria*. Fragments of ≈ 873 bp were amplified from various populations by primers MVC-F3 and MVC-R2 and cut with the restriction enzyme *Nde*I. MIYN-VI, MICK-VI, MINN-VI, MJON-VI, MJFL-VI, MAYG-VI, and MAHK-VI are selected virulent populations of *M. incognita*, *M. javanica*, and *M. arenaria*; MIGD-VI is a natural virulent population of *M. incognita*; and MICB-AV, MIIK-AV, MISH-AV, MJON-AV, MJFJ-AV, MAMG-AV, and MAYN-AV are avirulent populations of *M. incognita*, *M. javanica*, and *M. arenaria* (Table 1). M: 100-bp DNA ladder (New England Biolabs, Beverly, MA).

Fig. 6. Dominant sequence-characterized amplified region marker for selected *Mi*-virulent populations of *Meloidogyne incognita*, *M. javanica*, and *M. arenaria* amplified with primers MVC-VF and MVC-R1. MIYN-VI, MICK-VI, MINN-VI, MJON-VI, MJFL-VI, MAYG-VI, and MAHK-VI are selected virulent populations of *M. incognita*, *M. javanica*, and *M. arenaria*; MIGD-VI is a natural virulent population of *M. incognita*; and MICB-AV, MIIK-AV, MISH-AV, MJON-AV, MJFJ-AV, MAMG-AV, and MAYN-AV are avirulent populations of *M. incognita*, *M. javanica*, and *M. arenaria* (Table 1). M: 100-bp DNA ladder (Gibco BRL, Gaithersburg, MD).

were tested on a number of populations of different origins (Table 1). Furthermore, the markers could be easily amplified from ≈ 0.1 ng of miniprep DNA, although not from a single juvenile. Thus, amplification of the markers described here should have the potential to be optimized as a rapid procedure for the detection and monitoring of selected virulent genotypes in field populations of *M. incognita*, *M. javanica*, and *M. arenaria*, which will undoubtedly contribute to the efficient and durable management of the *Meloidogyne* spp. using the *Mi* resistance. It must be noted that almost all the populations analyzed in this study were of East Asian origin. In future studies, it is necessary to examine more populations from other parts of the world to confirm the general applicability of the markers. It should also be noted that the markers developed here do not distinguish between natural virulent and avirulent populations. Therefore, efforts should also be made to identify molecular markers that correlate with natural virulence and avirulence in the three *Meloidogyne* spp. Combining dominant markers for the three types of populations in a multiplex PCR assay would enable the rapid diagnosis of both selected and natural virulent genotypes in field populations.

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