A Set of Genes Differentially Expressed Between Avirulent and Virulent *Meloidogyne incognita* Near-Isogenic Lines Encode Secreted Proteins

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A cDNA-amplification fragment length polymorphism (AFLP)-based strategy has been used to identify genes differentially expressed between two pairs of near-isogenic lines (NIL) of the root-knot nematode *Meloidogyne incognita* either avirulent or virulent against the tomato *Mi* resistance gene. Gene expression profiles from infective second-stage juveniles (J2) were compared, and 22 of the 24,025 transcript-derived fragments (TDF) generated proved to be differential, i.e., present in both avirulent NIL and absent in both virulent NIL. Fourteen of the TDF sequences did not show any significant similarity to known proteins, while eight matched reported sequences from nematodes and other invertebrates. The differential expression of nine genes was confirmed by reverse transcription-polymerase chain reaction (RT-PCR) experiments. In situ hybridization conducted with five of the sequences showed that two were specifically expressed in the intestinal cells (HM10 and PM1), one in the subventral esophageal glands (HM1), and two in the dorsal esophageal gland of J2 (HM7 and HM12). Analysis of full-length cDNA sequences revealed the presence of a signal peptide for HM1, HM10, and HM12, indicating that the encoded proteins are putatively secreted. Since secreted products in general and esophageal gland secretions in particular are thought to be among the main *M. incognita* pathogenicity factors, this result suggests a possible dual role for some of the genes encoding such secretions, i.e., they could be involved in both pathogenicity and virulence or avirulence of these biotrophic parasites.

Additional keywords: mitotic parthenogenesis, plant-parasitic nematode, selection.

Due to the current restrictions in the use of chemical nematocides, host resistance is being considered as the most efficient method to control plant-parasitic nematodes. Plants are defined as resistant when they block the nematode life cycle, thus reducing or suppressing its reproduction, and resistance has been used successfully in a number of diverse crops (Trudgill 1991; Williamson and Hussey 1996). Resistance genes against root-knot nematodes from the genus *Meloidogyne*, which constitute a leading cause of crop losses due to plant-parasitic nematodes,

have been identified in many plant species (Roberts 1995). In particular, the tomato *Mi* gene, which confers effective resistance to the three major root-knot nematode species *M. arenaria*, *M. incognita*, and *M. javanica*, is, so far, one of the best characterized nematode resistance genes (Williamson 1998). Resistance was identified in the wild tomato species *Lycopersicon peruvianum*, and a single, interspecific hybrid plant was produced with the cultivated tomato *L. esculentum* using embryo rescue (Smith 1944). Up to now, all the available tomato cultivars with resistance to *Meloidogyne* spp. originated from this hybrid plant. The expression of *Mi*-mediated resistance is characterized by a localized hypersensitive reaction that consists of plant cell necrosis at the nematode infection site (Williamson and Hussey 1996). The *Mi* gene was isolated by positional cloning and shown to belong to the leucine zipper, nucleotide binding, leucine-rich repeat family of plant resistance genes (Milligan et al. 1998).

Although satisfactory from a conceptual point of view, plant resistance may lose its practical utility when resistant cultivars are challenged with virulent nematode populations. For example, natural *Meloidogyne* biotypes virulent against the tomato *Mi* gene have been described from a number of recent reports (Eddoudi et al. 1997; Ornat et al. 2001). So far, the molecular mechanisms involved in virulence or avirulence to plant resistance genes are largely unknown in nematodes. In a previous study, in order to identify markers correlated with virulence or avirulence, we assembled a collection of natural root-knot nematode isolates either avirulent or virulent against the *Mi* gene and assessed their molecular diversity by amplification fragment length polymorphism (AFLP) analysis. However, no correlation was found between virulence phenotypes and DNA markers, which suggested that genetic polymorphism was independent of virulence (Semblat et al. 2000). This result also indicated that another strategy needed to be developed to isolate genes involved in virulence or avirulence in root-knot nematodes. Although reproduction in the major species occurs by mitotic parthenogenesis (Triantaphyllou 1985), the selection of *Mi*-virulent lines from progenies of single avirulent nematodes was demonstrated for *M. incognita* under laboratory conditions (Bost and Triantaphyllou 1982; Jarquin-Barberena et al. 1991). In such conditions, the avirulent and virulent lines should theoretically be nearly isogenic, just differing in their ability to overcome the resistance gene. Therefore, it can be hypothesized that any molecular difference between them is related to the character of interest, and thus, such near-isogenic lines (NIL) constitute an ideal starting point for the identification of virulence or avirulence genes by means of differential analysis. Recently, a cDNA encoding an amphid-secreted putative avirulence protein from *M. incognita* was cloned through

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Nucleotide sequence data reported are available in the EMBL/GenBank/DBJ databases under the accession numbers listed in Table 1.

the genomic fingerprinting of three pairs of such NIL (Semblat et al. 2001). The similarity of the fingerprints validated the near-isogenicity of these genotypes and their utility for the development of a differential cloning strategy.

The goal of the present study was the identification of genes that are differentially regulated in avirulent and virulent nematodes, in order to understand the molecular basis of *M. incognita* virulence or avirulence in response to the selective pressure of the tomato *Mi* resistance gene. For that purpose, we have used a previously established model system involving selection of pairs of NIL originating from isolates from very different geographic origins (Jarquin-Barberena et al. 1991; Semblat et al. 2001), with the objective to find genes whose regulation would be the same in both avirulent lines and different from both virulent lines. Since cDNA-AFLP (Bachem et al. 1996) has often been described as an extremely efficient method for isolation of genes differentially expressed in plant-microbe interactions (Durrant et al. 2000; Qin et al. 2000; Simoes-Araujo et al. 2002), it was used to compare such avirulent and virulent NIL at the transcriptome level. In the work reported here, several transcript-derived fragments (TDF) differential between avirulent and virulent *M. incognita* NIL of were isolated, and some clones of interest were investigated in more detail, in order to validate the cloning strategy described and to gain insight into their putative biological function.

RESULTS

Isolation of differentially expressed genes.

To analyze differential gene expression associated with nematode virulence or avirulence, cDNA-AFLP was carried out on infective second-stage juveniles (J2) from the two pairs of *M. incognita* avirulent and virulent NIL from Kursk and Morelos. The number and length, varying from about 100 to 800 bp, of the observed TDF were dependent on both the restriction enzymes and primer combination used (Fig. 1). In order to validate the initial steps of the experimental procedure, we looked for a TDF corresponding to the *map-1* gene, previously shown to be differentially expressed between the same NIL (Semblat et al. 2001). The primer combination H+G and M+TG was used, which

should amplify a 820-bp fragment in the avirulent line from Morelos and should produce no amplification in the corresponding virulent NIL. In agreement with our previous results, the resulting cDNA-AFLP showed an amplification product at the expected size in the avirulent line (data not shown), indicating that the procedure worked correctly. Approximately 8,325, 7,700, and 8,000 bands per line were generated, using *HindIII/MseI*, *PstI/MseI*, and *EcoRI/TaqI* digestions, respectively. A very low proportion of these TDF proved to be differential, i.e., present in both avirulent NIL and absent in both virulent NIL (13, 4, and 5, respectively). Some examples of such TDF are presented in Figure 1. No differential TDF was found to be present in the virulent NIL and absent in the avirulent NIL. The 22 differential TDF were cloned and sequenced, and the hypothetical proteins deduced from the six-frame translated fragments were analyzed by BLASTX. The sequence similarities found are shown in Table 1. Fourteen of the TDF sequences did not show any significant similarity with known proteins, while eight matched reported sequences from nematodes (*Caenorhabditis elegans* and the plant-parasitic species *M. incognita* and *Heterodera glycines*) and other invertebrates (the segmented tube worm *Chaetopterus variopedatus* and the insect *Anopheles gambiae*). For example, homologies were found with two proteases from *C. elegans* and *H. glycines*, cuticular precollagen from *M. incognita*, innexin (a gap-junction channel protein involved in intercellular communication) from *C. variopedatus*, or the *unc-52* gene from *C. elegans*. Half of the 22 differential TDF showed homology to *M. arenaria* or *M. incognita* expressed sequence tags (EST), and five of the 14 sequences with no BLASTX homology did match to *Meloidogyne* EST, although no function has been described for them. Finally, nine TDF did not show significant matches to any known gene or EST sequence from databases. They may represent yet uncharacterized genes, but this result has to be considered carefully, since the DNA fragment lengths are rather short, which may limit the homology search analysis.

Reverse transcription-polymerase chain reaction (RT-PCR) expression analysis.

Transcription of the genes corresponding to the 22 differential TDF was analyzed by RT-PCR on mRNA from infective J2

Table 1. Summary of the 22 transcript-derived fragments (TDF)^a

TDF name	Accession no.	TDF/full-length cDNA size (bp)	Highest homology	BLASTX E value	EST homology ^b	BLASTN E value	RT-PCR ^c
HM1	AJ544623	149/525	No homology	...	<i>M. incognita</i> rd23f01.y1	e-138	+
HM2	AJ544624	97	Ribosomal protein <i>Caenorhabditis elegans</i>	3 e-05	<i>M. incognita</i> rb06c09.y1	2 e-35	–
HM3	AJ544625	559	Cuticle precollagen <i>M. incognita</i>	1 e-08	<i>M. arenaria</i> rm39d10.y1	0.0	–
HM4	AJ544626	169	No homology	...	<i>M. incognita</i> rd15a10.y1	3 e-51	+
HM5	AJ544627	80	No homology	...	...	...	–
HM6	AJ544628	274	UNC-52 protein <i>C. elegans</i>	5 e-20	<i>M. incognita</i> rd08A06.y1	1 e-122	–
HM7	AJ544629	205	No homology	...	...	...	+
HM8	AJ544630	221	Innexin <i>Chaetopterus variopedatus</i>	5 e-05	...	...	–
HM9	AJ544631	650	Aspartic protease <i>C. elegans</i>	2 e-27	...	...	–
HM10	AJ557572	394/1152	Cathepsin L-like <i>Heterodera glycines</i>	1e-140	<i>M. incognita</i> ra23h02.y1	0.0	–
HM11	AJ544633	300	No homology	...	...	...	+
HM12	AJ544634	205/927	SacI homology domain <i>C. elegans</i>	5 e-67	...	...	+
HM13	AJ544635	204	No homology	...	<i>M. arenaria</i> rm16h10.y1	1 e-54	–
PM1	AJ544636	413/672	Hypothetical protein <i>C. elegans</i>	2 e-42	<i>M. incognita</i> rd04g08.y1	0.0	+
PM2	AJ544637	183	No homology	...	...	...	+
PM3	AJ544638	201	No homology	...	...	...	–
PM4	AJ544639	169	No homology	...	...	...	+
ET1	AJ544640	321	No homology	...	<i>M. incognita</i> ra03d09.y2	1 e-126	–
ET2	AJ544641	319	Hypothetical protein <i>Anopheles gambiae</i>	9 e-21	<i>M. arenaria</i> rm39c10.y1	5 e-76	–
ET3	AJ544642	167	No homology	...	...	...	+
ET4	AJ544643	223	No homology	...	<i>M. incognita</i> MD0049	8 e-99	–
ET5	AJ544644	167	No homology	...	...	...	–

^a Differential between *Meloidogyne incognita* avirulent and virulent near-isogenic lines (NIL).

^b TDF/full-length cDNA sequences were compared with available *Meloidogyne* spp. expressed sequence tags using BLASTN.

^c + or – indicates a differential or nondifferential amplification pattern between the avirulent and virulent NIL.

from the two pairs of *M. incognita* NIL. In these experiments, repeated at least twice for each TDF, nine genes showed the same pattern of amplification as that obtained in cDNA-AFLP experiments, i.e., presence of a band of the expected size in the two avirulent lines and absence in the two virulent lines (Table 1). An example of differential expression is shown in Figure 2. Conversely, 13 TDF presented comparable expression levels in both avirulent and virulent NIL (Table 1). Among the nine differentially expressed clones, three matched to *M. incognita* EST (HM1, HM4, and PM1), and one (PM1) to a *C. elegans* hypothetical protein. The six other TDF had no homology at all in the databases.

In situ hybridization of selected TDF

In order to determine which nematode organs express the differential TDF, some of them were selected for further in situ hybridization experiments. Sense and antisense cDNA probes were made by asymmetric PCR and were used against *M. incognita* J2. Four TDF were selected, based on RT-PCR confirmation of their differential expression between avirulent and virulent NILS: HM7 and HM12 had no homology in databases, HM1 showed a significant match to a *M. incognita* EST sequence, and PM1 showed homology to a hypothetical protein from *C. elegans* and a *M. incognita* EST

(Table 1). HM10 was homologous to a *H. glycines* cathepsin L-like protease (Table 1), a gene shown to be specifically transcribed in nematode intestinal cells (Gao et al. 2001), and was used as internal control. The in situ hybridization patterns are shown in Figure 3. Labeling was observed in the intestinal cells with the antisense probe of PM1. As expected, the same localization of expression was detected for HM10, used as control. Antisense probes of HM1 and HM7 specifically labeled the subventral esophageal glands and the dorsal esophageal gland of J2, respectively. Labeling of the dorsal gland ampulla was observed with the antisense probe of HM12 after 16 h of staining. When staining was extended to 72 h, hybridization was detected in both the basal part and the ampulla of the dorsal gland, with a signal intensity higher in the ampulla. No labeling was detected for the control sense probes of all TDF (data not shown). The same results were obtained using independent batches of nematodes and newly synthesized hybridization probes.

Full-length cDNA cloning and predicted subcellular localization of mRNAs possibly encoding nematode-secreted proteins.

Since the presence of an N-terminal signal peptide may indicate that a protein is secreted and in order to get more sequence information, the sequence of the full-length cDNA was determined for the TDF previously localized in the intestinal (HM10 and PM1) or esophageal gland cells (HM1, HM7, and HM12). Only full-length sequences of HM1, HM10, HM12, and PM1 could be obtained. BLASTX sequence analysis confirmed that HM10 and PM1 encoded proteins with homology to a cathepsin L-like protease from *H. glycines* and a hypothetical protein from *C. elegans*, respectively. It also revealed that HM12 encoded a protein with homology to a SacI domain containing protein from *C. elegans*, the biological function of which may include the coordination of the activities of the secretory pathway. The computer algorithm SignalP (Nielsen et al. 1997) predicted the presence of a signal peptide for secretion for the proteins encoded by HM1 (in the subventral glands), HM10 (in the intestine), and HM12 (in the dorsal gland). Subcellular localization of the proteins encoded by HM1, HM10, HM12, and PM1 was evaluated using the PSORT II program (Nakai and Horton 1999). Proteins encoded by HM10 and HM12 were predicted to be extracellular, whereas the protein encoded by PM1 was predicted to be nuclear. For HM1, the prediction was less conclusive, the protein being possibly either cytoplasmic or within the vesicles of the secretory system.

Fig. 1. Typical cDNA-amplification fragment length polymorphism patterns from *Meloidogyne incognita* near-isogenic lines (NIL) generated with H+I/M+2 primer combinations. **A**, Conserved patterns generated with different primer combinations. **B** through **E**, Selected parts of gels showing differential transcript-derived fragments: **B**, HM1, obtained with H+A/M+AC; **C**, HM4, obtained with H+T/M+GT; **D**, HM7, obtained with H+G/M+CC; and **E**, HM12, obtained with H+T/M+TG. 1, 2 and 3, 4 = cDNA from avirulent and virulent NIL from Kursk and Morelos, respectively.

Fig. 2. Reverse transcription-polymerase chain reaction expression analysis of selected transcript-derived fragments. Equivalent amounts of cDNA from *Meloidogyne incognita* near-isogenic lines (NIL) were used (a1, v1 and a2, v2 = avirulent and virulent NIL from Kursk and Morelos, respectively). M = size molecular marker.

Fig. 3. In situ hybridizations with selected transcript-derived fragment antisense probes from *Meloidogyne incognita*. Labeling was done in avirulent infective J2s. Labeling patterns with **A**, HM10, **B**, PM1, **C**, HM7, **D**, HM1, **E**, HM12 (after one day of staining), and **F**, HM12 (after 3 days of staining) antisense probes. DG = dorsal gland; DGA = dorsal gland ampula; H = head; I = intestine; M = metacarpus; and SvG = subventral gland.

DISCUSSION

In this paper, a comparative strategy based on cDNA-AFLP analysis of NIL is described, which resulted in the identification of 22 genes differentially expressed between *M. incognita* lines avirulent or virulent against the tomato *Mi* resistance gene. Such differences between avirulent and virulent nematodes may indicate that some transcripts are missing in the virulent NIL but may also result from qualitative differences in mRNA sequence (mutations, deletions) between avirulent and virulent NIL. Clearly, further studies are needed to clarify that point. Based on sequence homology with known proteins, on localization of the transcripts in infective J2, or both, a putative function could be assigned to the products of some of these genes. Some other of these candidates were considered as pioneer sequences, but in both cases, the direct implication of these 22 sequences in nematode virulence or avirulence remains to be established. Since the NIL used in this study had been selected for 40 generations, a possible explanation would be that some of these differential bands are due to secondary effects or independent mutations not related to virulence or avirulence. An alternative explanation for the detection of genes polymorphic between avirulent and virulent lines and not related to the mechanism of virulence or avirulence is that such genes are located close to the avirulence genes on the chromosome and are lost together with the avirulence gene in case of a large deletion. However, two arguments suggest that they are not due to strong bias in the selection procedure. First, the fact that the same differences were found in two independent pairs of NILS tends to indicate that they are, indeed, correlated with the biological character of interest. Second, RT-PCR analysis performed on RNA isolated from NIL of the same origin but selected for only 10 generations confirmed the differential patterns for a set of selected TDF (HM1, HM12, PM1, and PM2) (data not shown).

In our study, mRNA expression profiles from two pairs of *M. incognita* NIL from very different geographic origins were generated, using three restriction enzyme combinations, in order to maximize the coverage of the transcriptome of the nematode. Three six-cutter (*EcoRI*, *HindIII*, and *PstI*) and two four-cutter enzymes (*MseI* and *TaqI*) were used, and *MseI* was associated with the six-cutter enzyme in two of the three combinations, as it cuts very frequently in most eukaryotic genomes (Vos et al. 1995). In each case, all the possible (+1/+2) primer combinations ($4 \times 4^2 = 64$) were used and generated equivalent numbers of TDF, for a total of about 24,025. Sequencing of the 97-Mb genome of the model nematode *C. elegans* predicted the occurrence of 19,099 protein-coding genes (The *C. elegans* Sequencing Consortium 1998), and recent data brought this number to 20,448 predicted proteins (McCarter et al. 2002). Considering the size of the *M. incognita* genome (51 Mb) (Pableo and Triantaphyllou 1988), its expressed gene content, in the absence of other evidence, is expected to be in the same range as that of *C. elegans* (i.e., from 10,000 to 20,000 genes). In the closely related cyst nematode *Globodera rostochiensis*, the number of expressed genes in juveniles was estimated to be about 12,000 (Qin et al. 2000). Therefore, and taking into account the possible limitations inherent in the cDNA-AFLP methodology (redundancy of some TDF, under-representation of transcripts lacking a recognition site for the restriction enzymes used), it seems, nevertheless, reasonable to consider that the 24,025 TDF analyzed in this study represent a large fraction of the *M. incognita* transcriptome. Overall, a very low proportion of these TDF were shown to be differential (about 0.09%), i.e., present in the two avirulent lines and

absent in the two virulent lines. We never observed the opposite situation, i.e., a TDF present in the two virulent lines and absent in the two avirulent lines. Occasionally, other kinds of polymorphisms were observed (TDF present in the two lines from the same geographic origin and absent in the two others, TDF differential between the avirulent and virulent lines of a single origin only), but they were not taken into account in this study. If we consider that virulence or avirulence is due to several genes, then differences observed within a single pair of NIL may be of interest. Moreover, since our experiments were exclusively conducted on preparasitic J2, the possibility that the transcripts of some virulence or avirulence genes expressed later during parasitism have been missed must not be excluded.

In order to further characterize the differential TDF, we focused on some of those that proved to be differential in RT-PCR experiments, although the lack of a differential RT-PCR amplification does not mean that the corresponding TDF are false differential clones (e.g., nucleotide polymorphism at the restriction enzyme recognition site). The PM1 clone, homologous to a *C. elegans* hypothetical protein, was shown to be specifically expressed in intestinal cells of *M. incognita* J2. The same expression pattern was detected for another TDF, HM10, homologous to a cathepsin L-like protease from *H. glycines* (Gao et al. 2001), which was not differential in RT-PCR but was used as a standard control in situ hybridizations. Although the function of the genes corresponding to these two TDF remains to be determined, the fact that two independent putative intestinal proteins are found differential between avirulent and virulent NIL in cDNA-AFLP analysis suggests a possible link between food digestion by the nematode and its ability to develop or not on resistant plants. Interestingly, it has been shown in the parasitic nematode *Haemonchus contortus* and trematode *Fasciola hepatica* that gut-secreted cysteine proteases are not restricted to a digestive function in the intestine but are actively secreted from intestinal cells into the worm's external environment, where they interact with the extracellular matrix components of the host (Carnevale et al. 2001; Rhoads and Fetterer 1995). Further characterization of the full-length cDNA clones for HM10 and PM1 revealed the presence of a secretion signal peptide for HM10 only, which confirms that the encoded protein is putatively secreted. However, the presence or absence of a signal peptide is not a definitive argument, since some proteins lacking such a signal peptide have been shown to be secreted by potato cyst nematodes, *Globodera* spp. (Fioretti et al. 2001; Robertson et al. 2000).

Stylet secretions synthesized in the esophageal gland cells of root-knot nematodes are considered the main determinants of the pathogenicity of these organisms (Davis et al. 2000). Changes in esophageal gland activity during the parasitic cycle have been documented, i.e., active subventral glands in infective J2 and predominant activity of the dorsal gland in the later parasitic stages (Hussey and Mims 1990). Interestingly, the transcripts corresponding to three TDF were specifically localized to the subventral and the dorsal glands of J2. The in situ localization of HM1 mRNA in the subventral gland cells of *M. incognita* may indicate a role for the putative encoded protein in the early stages of interaction between the nematode and its host plant. However, its function remains unknown, since no homology was found in databases for this TDF. Localization of the transcripts corresponding to two other TDF, HM7 and HM12, was exclusively observed in the dorsal gland of the infective J2. This result suggests that the putative encoded proteins could be involved in later events of the parasitic cycle. In the case of HM12, mRNA concentrated into the region of the ampulla of

the dorsal gland. In *M. incognita*, secretory proteins are produced in the nuclear region of the gland cell and stored into spherical granules that are transported to the cell ampulla before their excretion into the esophagus lumen by exocytosis. In parasitic J2, the dorsal gland ampulla is enlarged and contains many secretory granules (Hussey and Mims 1990). Our observation that HM12 transcripts are preferentially located in the region of the dorsal gland ampulla of infective pre-parasitic J2 may indicate a possible role of the gene in both early and late parasitic interactions with the plant. No homology in databases was found for HM7 and HM12, and thus, their precise implication in nematode parasitism has not been resolved. Characterization of the full-length cDNA clones for HM1 and HM12 revealed the presence of an N-terminal signal peptide, which confirms the putative secretion of these proteins from the subventral and dorsal glands, respectively. However, functional analyses will have to be performed to demonstrate the implication of these genes in *M. incognita* virulence or avirulence against the *Mi* gene. In that respect, the RNAi procedure, shown to be efficient for cyst nematodes (Urwin et al. 2002), would be of interest in our model system. Indeed, changes in the (resistant) plant-nematode interaction phenotype after inactivation of the candidate genes identified here would be strong arguments in favor of their role in *M. incognita* virulence or avirulence.

The strategy developed here to identify genes differentially expressed between avirulent and virulent root-knot nematodes did not require any a priori knowledge about the functions to be involved. However, it resulted in the characterization of a set of genes putatively coding for esophageal gland secretions, which are thought to be among the main *M. incognita* pathogenicity factors (Davis et al. 2000; Huang et al. 2003; Jaubert et al. 2002). This observation could suggest a possible dual role for such secretions and is in line with current accumulating evidence that most avirulence genes found in biotrophic pathogens are or once were pathogenicity genes that encode protein signals that trigger transduction pathways into plant cells (Gabriel 1999; White et al. 2000).

MATERIALS AND METHODS

Nematodes.

Two *M. incognita* isolates from very different geographic origins (Kursk, Russia and Morelos, Mexico, respectively), both avirulent against the tomato *Mi* resistance gene, were used. They were multiplied on the susceptible tomato cv. Saint Pierre. From each avirulent isolate, a NIL was selected for virulence on the *Mi*-resistant tomato cv. Piersol, according to the procedure originally described by Jarquin-Barberena and associates (1991). In this study, we used avirulent and virulent NIL that were selected on Saint Pierre and Piersol, respectively, for 40 successive generations.

Eggs were collected after treatment of the egg masses in 0.5% NaOCl and were rinsed with distilled water. J2 were then hatched in vitro on 10- μ m sieves in 0.7% streptomycin sulphate and were directly used for in situ hybridization experiments or were concentrated by centrifugation and were stored at -80°C until RNA extraction.

RNA isolation.

For each nematode line, total RNA was isolated from 200 μ l of J2 with TRIzol Reagent (Invitrogen, Cergy-Pontoise, France), following the manufacturer's instructions, after a preliminary step of grinding in liquid nitrogen. Subsequently, mRNA was purified from total RNA with the Micro-Poly(A)Purist kit (Ambion, Huntingdon, U.K.), according to the manufacturer's recommendations.

cDNA-AFLP procedure.

The two pairs of avirulent and virulent *M. incognita* NIL were compared with cDNA-AFLP derived markers. cDNA-AFLP was performed as described by Bachem and associates (1996) with minor modifications. First-strand cDNA was synthesized from 1 μ g of mRNA with a T₁₆VN oligonucleotide primer (V = A, C, or G; N = A, T, C, or G). Double-stranded cDNA synthesis was performed with the Superscript double-stranded cDNA synthesis kit (Invitrogen, Cergy-Pontoise, France). Each cDNA sample was digested by two restriction endonucleases, and three pairs of restriction endonucleases were successively tested: *HindIII/MseI*, *PstI/MseI*, and *EcoRI/TaqI*. After digestion, the restriction fragments were ligated with their corresponding adapters (Semblat et al. 2000; Thomas et al. 1995; Vos et al. 1995), and preamplification was carried out for 25 cycles (94°C, 30 s; 57°C, 45 s; 72°C, 60 s), using primers without selective nucleotide (E+0: 5'-GTAGAC TCGGTACCAATTC-3', H+0: 5'-GACTGCGTACCAGCTT-3', P+0: 5'-GACTGCGTACCAGTGCAG-3', M+0: 5'-GATGA GTCCTGAGTAA-3', and T+0: 5'-GATGAGTCCTGAGCGA-3'). The PCR products were diluted (10 $\times$) in Tris-EDTA (TE) buffer, and 2 μ l were used for selective amplification, with one selective base extension at the 3' end of the primers H+0, E+0, and P+0 and two selective base extensions at the 3' end of the primers M+0 and T+0, using a standard AFLP touchdown-selective amplification program. All the possible primer combinations ($4 \times 4^2 = 64$) for each pair of restriction endonucleases were tested in these experiments. The PCR products were electrophoresed in 4.5% denaturing polyacrylamide gels. The gels were dried on 3MM paper (Whatman, Maidstone, U.K.) under vacuum and were exposed to Kodak Biomax film (Amersham, Buckinghamshire, England) at room temperature for 24 h.

TDF analysis and sequencing.

Because more than one DNA fragment can be present in a single band, purification of the differential TDF was performed as follows. Bands of interest (i.e., those that were present in the two avirulent and absent in the two virulent NIL) were cut from the dried polyacrylamide gel and were soaked in 25 μ l of TE buffer. The same procedure was applied to the corresponding regions of the same size in the virulent NIL lanes in which no band was present. DNA was recovered after three cycles of "freeze and thaw" in liquid nitrogen. Diffusate (2 μ l) from avirulent and virulent NIL were reamplified, using new selective primer combinations with one additional nucleotide in the primers corresponding to the six-cutter enzyme adaptor (+2/+2 instead of +1/+2 in the initial cDNA-AFLP step), and only TDF displaying the same differential expression pattern as that exhibited in the cDNA-AFLP gel were analyzed further. They were cloned into pGEM-T easy vector (Promega, Charbonnières, France) and sequenced by Genome Express (Grenoble, France). Database searches were performed using the BLAST Network Service (National Center for Biotechnology Information). Each TDF sequence was compared against all sequences in the nonredundant databases, using the BLASTX algorithm (Altschul et al. 1997). In addition, TDF sequences were also compared against the EST databases using the BLASTN algorithm.

In order to get full-length sequence information, 5' and 3' ends of some TDF of interest were isolated by RNA ligase-mediated rapid amplification of cDNA ends, using the GeneRacer Kit (Invitrogen) according to the manufacturer's instructions. Bioinformatic analysis of the obtained sequences was performed as described previously. Moreover, the amino acid sequence of each predicted protein was analyzed for the presence

of an N-terminal secretion signal peptide, using the program SignalP (Nielsen et al. 1997), and its putative subcellular localization was predicted using PSORT analysis (Nakai and Horton 1999).

RT-PCR amplification.

RT-PCR experiments were performed using oligonucleotide primers that were designed from each TDF sequence of interest. For each nematode avirulent and virulent NIL, first-strand cDNA was synthesized from 200 ng of mRNA, using a T₁₆VN oligonucleotide primer (V = A, C, or G; N = A, T, C, or G) and the Superscript II reverse transcriptase (Invitrogen) in a reaction volume of 20 µl. PCR reactions were performed using 2 µl of the first-strand reaction volume as template, 1 U of Taq polymerase (Appligene Oncor, Illkirch, France), and 0.25 mM dNTPs, each, and primers (0.3 µM each) for 3 min at 94°C, 30 cycles at 94°C for 45 s, 57°C for 45 s, 72°C for 60 s, and a final elongation of 5 min at 72°C.

In situ hybridization.

For in situ hybridization, specific primers designed from the cloned TDF of interest were used to amplify the cDNAs in the plasmids. These PCR products were then used to synthesize digoxigenin-labeled sense and antisense cDNA probes by asymmetric PCR. In situ hybridizations were performed on J2 from avirulent NIL essentially as described previously (De Boer et al. 1998). Briefly, freshly hatched J2 were fixed in 2% paraformaldehyde for 16 h at 4°C and 4 h at room temperature. Nematodes were permeabilized with proteinase K, acetone, and methanol. They were then hybridized at 45°C with the sense and antisense single-strand cDNA probes for 16 h. Bound cDNA probes were detected by alkaline phosphatase immunostaining at 4°C for 16 h. The stained J2 were then examined with differential interference microscopy.

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