A Profile of Putative Parasitism Genes Expressed in the Esophageal Gland Cells of the Root-knot Nematode *Meloidogyne incognita*

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Identifying parasitism genes encoding proteins secreted from a nematode's esophageal gland cells and injected through its stylet into plant tissue is the key to understanding the molecular basis of nematode parasitism of plants. *Meloidogyne incognita* parasitism genes were cloned by microaspirating the cytoplasm from the esophageal gland cells of different parasitic stages to provide mRNA to create a gland cell-specific cDNA library by long-distance reverse-transcriptase polymerase chain reaction. Of 2,452 cDNA clones sequenced, deduced protein sequences of 185 cDNAs had a signal peptide for secretion and, thus, could have a role in root-knot nematode parasitism of plants. High-throughput in situ hybridization with cDNA clones encoding signal peptides resulted in probes of 37 unique clones specifically hybridizing to transcripts accumulating within the subventral (13 clones) or dorsal (24 clones) esophageal gland cells of *M. incognita*. In BLASTP analyses, 73% of the predicted proteins were novel proteins. Those with similarities to known proteins included a pectate lyase, acid phosphatase, and hypothetical proteins from other organisms. Our cell-specific analysis of genes encoding secretory proteins provided, for the first time, a profile of putative parasitism genes expressed in the *M. incognita* esophageal gland cells throughout the parasitic cycle.

Additional keywords: gland-cell cDNA library, plant-parasitic nematode, stylet secretions.

Root-knot nematodes, *Meloidogyne* spp., synthesize secretory proteins in their dorsal and subventral esophageal gland cells and release these proteins through a hollow, protrusible stylet (feeding structure) during migration within plant roots and subsequent modification of root cells into the elaborate permanent feeding cells, called giant-cells (Hussey and Grundler 1998). In preparasitic second-stage juveniles, the most active esophageal glands are the two subventral gland cells, while the single dorsal gland cell becomes the predominate source of secretions released through the stylet in subsequent parasitic stages, indicating that gland function and their

secreted proteins change during the parasitic cycle (Davis et al. 2000, Hussey 1989). With very few exceptions, the identities of these secretory proteins are completely unknown and the mechanism of *Meloidogyne* parasitism of plants remains obscure. The cloning and identification of parasitism genes encoding esophageal gland proteins secreted from the nematode stylet into host tissue is now increasing our understanding of the molecular mechanisms of nematode parasitism of plants.

The first nematode parasitism genes expressed in the esophageal gland cells to be cloned encoded β -1,4-endoglucanases (cellulases) and were identified in cyst nematodes, *Heterodera* and *Globodera* spp. (Smant et al. 1998; Yan et al. 1998). Soon thereafter, cellulase parasitism genes also were identified in root-knot nematodes (Rosso et al. 1999). Cellulases are developmentally expressed in the subventral gland cells of cyst and root-knot nematodes and are secreted within host root tissue by infective second-stage juveniles during the migration phase of the infection process, which confirmed the in planta secretion of a parasitism gene product (Wang et al. 1999).

Differential gene expression, cDNA library screening, and direct analysis of secreted proteins have been used to clone parasitism genes expressed in *Meloidogyne* spp. esophageal gland cells, but with only limited success (Ding et al. 1998; Doyle and Lambert 2002; Jaubert et al. 2002; Lambert et al. 1999; Rosso et al. 1999). Whole nematode expressed sequence tag (EST) analysis also has been used to identify gland expressed genes. However, this approach has limited potential because it predominately identifies only genes whose translation products are obviously related to parasitism, like cell-wall digesting enzymes (Dautova et al. 2001). Recently a novel method for directly cloning expressed nematode parasitism genes was developed by microaspirating the cytoplasm from the esophageal gland cells of parasitic nematode stages to provide mRNA to construct gland cell-specific cDNA libraries by reverse-transcriptase polymerase chain reaction (RT-PCR) (Gao et al. 2001; Wang et al. 2001). Suppression subtractive hybridization (Gao et al. 2001) and signal peptide selection (Wang et al. 2001) of gland-cell cDNA libraries from *Heterodera glycines* were conducted to enrich for expressed parasitism gene products and resulted in the cloning of several putative parasitism genes expressed in the subventral and dorsal esophageal gland cells. Coupling large-scale EST analysis of a gland-cell cDNA library, identification of cDNAs encoding an N-terminal signal peptide sequence for secretion, and high-throughput in situ mRNA hybridization in nematode developmental stages has provided here a suite of 37 candidate parasit-

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Nucleotide and/or amino acid sequence data have been submitted to the DDBJ/EMBL/GenBank databases.

ism genes expressed in the esophageal gland cells of *Meloidogyne incognita* during the parasitic cycle.

RESULTS

Analysis of the *M. incognita* gland-cell library.

A gland-cell cDNA library was constructed by long-distance (LD)-PCR (SMART System) using mRNA from the cytoplasm microaspirated directly from the esophageal gland cells of 43 different *M. incognita* specimens representing all parasitic life stages. Gel analysis of 20 clones randomly selected from the gland-cell LD-PCR cDNA library showed that insert sizes ranged from 350 bp to 3 kb. A cDNA library prepared similarly from intestinal cells of *M. incognita* hybridized to a macroarray of 6,144 clones of the *M. incognita* gland-cell cDNA library identified common housekeeping and structural genes and enabled the efficient isolation of candidate parasitism genes among the nonhybridized gland-cell cDNA clones. High-quality sequences from the nonhybridized gland-cell cDNA clones were obtained for 2,452 sequences which produced 307 contigs representing 1,239 sequences. SignalP analyses predicted protein sequences of 185

cDNAs from 2,452 cDNA sequences to contain N-terminal signal peptides for secretion. The presence of a signal peptide prioritized these esophageal gland cell proteins as candidates for being secreted by *M. incognita* and potentially having a biological function in the nematode–host interaction. High-throughput in situ mRNA hybridizations were conducted on the 185 clones to confirm expression within the esophageal gland cells of *M. incognita* specimens. Probes of 37 distinct cDNA clones specifically hybridized to transcripts accumulating within the subventral (13 clones) or dorsal (24 clones) esophageal gland cells of *M. incognita* (Table 1; Fig. 1). By fulfilling the two criteria of gland expression and encoding proteins with signal peptides, these clones can be regarded as candidate parasitism genes. Probes of a few clones hybridized to mRNA in tissues adjacent to the esophageal gland cells (e.g. nerve ring, metacarpus, and esophago-intestinal cells) while the remainder of the probes did not hybridize within specimens of *M. incognita*. Full-length cDNAs sequences with predicted open reading frames for these candidate parasitism genes ranged in size from 362 bp to 2.2 kb (Table 1). The predicted open reading frames were determined by the presence of i) translation initiation

Table 1. Summary of 37 distinct putative parasitism genes encoding proteins preceded by a signal peptide for secretion and expressed exclusively within the esophageal gland cells of *Meloidogyne incognita*

Clone	Accession no. ^b	FL/ORF (bp) ^c	Highest homology	Blastp score/E value	Gland expression ^a		
					Pre-J2	Par-J2	J3-A
2E07	AF531160	694/522	Pioneer ^d		...	DG	DG
2G02	AF531161	776/630	Pioneer		...	SvG	...
2G10	AY135363	999/384	Pioneer		...	DG	DG
4D01	AF531162	785/522	Hypothetical protein— <i>Caenorhabditis elegans</i>	51/7e ⁻⁰⁶	...	SvG	...
4D03	AY135364	864/573	Hypothetical protein— <i>C. elegans</i>	132/2e ⁻³⁰	...	DG	DG
5G05	AY135362	977/813	Hypothetical protein— <i>C. elegans</i>	165/5e ⁻⁴⁰	...	SvG	...
6F06	AF531163	1339/990	Hypothetical protein— <i>Methanosarcina mazei</i>	54/2e ⁻⁰⁶	...	DG	DG
6G07	AF531164	762/291	Pioneer		...	DG	DG
7A01	AF531165	778/540	Pioneer		...	DG	DG
7E12	AF531166	757/528	Pioneer		...	DG	DG
7H08	AF531168	1091/900	Pioneer		...	DG	DG
8D05	AF531169	1282/990	Pioneer		SvG	SvG	SvG
8H11	AF531170	1228/954	Hypothetical protein— <i>C. elegans</i>	62/1e ⁻⁰⁸	SvG	SvG	SvG
9H10	AF531167	737/534	Pioneer		...	DG	DG
10A08	AY142117	656/498	Sodium/calcium/potassium exchanger— <i>Tursiops truncatus</i> (99% identity to 5' end of 8H11)	58/4e ⁻⁰⁸	SvG	SvG	SvG
10G02	AY135365	1499/1242	Hypothetical protein— <i>Leishmania major</i>	54/5e ⁻⁰⁶	...	DG	DG
11A01	AY134431	1266/447	Pioneer (89% identity to 2E07) ^e		...	DG	DG
12H03	AY134432	723/552	Pioneer (97% identity to 7A01)		...	DG	DG
13A12	AY134433	824/522	Pioneer (90% identity to 2E07)		...	DG	DG
14E06	AY134434	696/525	Pioneer (98% identity to 7E12)		...	DG	DG
16D10	AY134435	364/129	Pioneer		...	SvG	SvG
16E05	AY134436	1687/1311	Pioneer		...	DG	...
17H02	AY134437	783/516	Pioneer		...	DG	DG
19F07	AY142116	798/645	Pioneer		...	DG	DG
21E02	AY134438	704/528	Pioneer (95% identity to 7E12)		...	DG	DG
25B10	AY142118	414*/333	Pioneer		...	DG	...
28B04	AY142119	748/519	Pioneer (95% identity to 7E12)		...	DG	DG
30G11	AY134440	1511/1374	Acid phosphatase— <i>C. elegans</i>	214/2e ⁻⁵⁴	SvG	SvG	...
30H07	AY134439	994/783	Pioneer		SvG	SvG	...
31H06	AY134441	362/150	Pioneer		SvG	SvG	...
34C04	AF527788	1124/813	Pectate lyase— <i>M. javanica</i>	477/1e ⁻¹³⁴	SvG	SvG	...
34D01	AY134442	782/540	Pioneer (89% identity to 7A01)		...	DG	DG
34F06	AY134443	801/357	Pioneer		...	DG	DG
35A02	AY134444	2210/1848	Pioneer		SvG	SvG	SvG
35E04	AY142121	836/441	Pioneer (99% identity to 5' end of 19F07)		...	DG	DG
35F03	AY142120	398/141	Pioneer		...	DG	...
42G06	AF049139	860/609	Cellulose binding protein— <i>M. incognita</i>	352/1e ⁻⁹⁶	SvG	...	...

^a In situ hybridization of cDNA probes to mRNA specifically within the *M. incognita* dorsal esophageal gland cell (DG) or subventral esophageal gland cells (SvG) in preparasitic second-stage juveniles (Pre-J2), parasitic J2 (Par-J2), or later stages (J3-A). ... = Not detected.

^b New sequences submitted to GenBank with the exception of AF049139, which was already in the database.

^c Size of the full-length clone with predicted open reading frame (ORF) size; * indicates not full length.

^d Novel transcript with no homology to any genes in the public databases.

^e Percent identity in the amino acid residues of predicted protein.

and termination signals, ii) a polyadenylation signal sequence, and iii) a putative signal peptide at the N-terminal end of the predicted protein. Most interesting, PSI-BLASTP analyses revealed 73% of the putative parasitism genes to encode novel protein sequences specific to *M. incognita* (E value < 0.005).

The predicted proteins of several putative parasitism gene contig members differed slightly in their amino acid identity and therefore, represented gene families (Table 1). For example, predicted proteins of clones 2E07, 11A01, and 13A12 shared 89 to 98% sequence identity. Interestingly, predicted proteins of the dorsal gland-expressed clones 19F07 (228 amino acids) and 35E04 (147 amino acids) had identical sequences for the first 146 amino acids from the 5' end. Similarly, sequences of proteins encoded by the subventral gland-expressed clones 8H11 (318 amino acids) and 10A08 (166 amino acids) were 99% identical for the first 165 amino acids from the 5' end.

Only four candidate *M. incognita* parasitism genes had homologues with known functions in other organisms. The predicted amino acid sequence of the full-length cDNA of 34C04 had significant similarity to pectate lyases found in plant-parasitic nematodes and fungi. Clone 30G11 encoded an acid phosphatase with homologues in the free-living nematode, *Caenorhabditis elegans*, and other organisms. The predicted protein of clone 10A08 was similar to sodium/calcium/potassium exchanger from other organisms. Clone 42G06 encoded a cellulose-binding protein with a novel N-terminal domain, which was previously cloned from *M. incognita* (Ding et al. 1998).

Four other predicted proteins encoded by the putative parasitism genes were similar to hypothetical proteins of *C. elegans*. Although 64% of all 2,452 gland cDNA predicted protein sequences had homologues (E value < 0.05) in *C. elegans*, only 19% of the products of the putative parasitism genes were homologous with *C. elegans* proteins. Only one of the *M. incognita* putative parasitism gene cDNAs (clone 34C04) was trans-spliced with the *Meloidogyne* 22 nucleotide leader sequence (SL1M) or derivatives thereof (Blumenthal and Steward 1997; Ray et al. 1994).

One of the predicted proteins (7H08) of the putative parasitism genes contained a canonical nuclear localization signal (SV40-like seven-residue pattern class) and was predicted by PSORT II analyses to be targeted to the nucleus. mRNA of this novel predicted nuclear protein was detected exclusively within the dorsal gland cell (Table 1). Domain analysis using the SMART database revealed the predicted proteins of six cDNA clones (5G05, 6F06, 8D05, 8H11, 10A08, and 30H07) contained coiled-coil domains and three clones (7H08, 8H11, and 35A02) had internal repeats.

Developmental expression.

Variations in developmental regulation as well as the level of expression of the putative parasitism genes in the esophageal gland cells of *M. incognita* were detected by the in situ mRNA hybridizations (Fig. 1; Table 1). Observable mRNA abundance of specific parasitism genes in the gland cells ranged from low (Fig. 1A) to high (Fig. 1F and I) based on the relative intensity of the hybridization signal. Developmental expression patterns

Fig. 1. Hybridization of digoxigenin-labeled antisense cDNA probes (dark staining) of parasitism gene clones to transcripts expressed exclusively within the subventral or dorsal esophageal gland cells of *Meloidogyne incognita* at different life stages. Clone 8D05: **A**, Preparasitic second-stage juvenile and **B**, late parasitic second-stage juvenile; clone 16D10: **C**, migratory parasitic second-stage juvenile and **D**, late parasitic second-stage juvenile; clone 11A01: **E**, early parasitic second-stage juvenile and **F**, young adult female; clone 9H10: **G**, parasitic second-stage juvenile and **H**, late parasitic second-stage juvenile; clone 7A01: **I**, young adult female. DG = dorsal gland cell, M = metacarpus, SvG = subventral gland cells.

of the parasitism genes also varied greatly throughout the *M. incognita* life cycle (Fig. 1; Table 1). Although mRNA of most parasitism genes expressed in the subventral gland cells usually was detectable only in the nonfeeding preparasitic and migratory parasitic second-stage juveniles (Fig. 1A and C), transcripts of a few subventral gland cell-expressed parasitism genes also were detected in feeding sedentary second-stage juveniles and later parasitic stages (Fig. 1B and D). On the other hand, parasitism gene expression in the dorsal gland cell usually was restricted to the feeding sedentary parasitic stages (Fig. 1E, F, G, H, and I).

DISCUSSION

Products of parasitism genes expressed in nematodes' esophageal gland cells and secreted through their stylet (feeding spear) are thought to control the process of plant parasitism (Williamson and Hussey 1996). The production of esophageal gland proteins is developmentally regulated during a root-knot nematode's life cycle (Davis et al. 1994); therefore, obtaining a comprehensive profile of the parasitism genes requires analyzing gene expression in the esophageal gland cells throughout the parasitic cycle. By combining EST analysis of a gland-cell LD-RT-PCR cDNA library with high-throughput in situ screening of the clones encoding a signal peptide for secretion, we obtained a profile of 37 cDNA sequences encoding putative parasitism genes expressed exclusively within the secretory esophageal gland cells of *M. incognita* throughout the parasitic cycle.

The most surprising and interesting result of our study was the large number of the putative parasitism genes encoding novel proteins. Indeed, 27 sequences had no homology with functionally annotated genes in the databases (homologues of six other clones were hypothetical proteins). These "pioneer" genes will provide a significant challenge to determine their roles in *Meloidogyne* spp. parasitism of plants. Nevertheless, these pioneering sequences are intriguing candidates for functional assays. For example, clone 16D10, which was expressed in the subventral gland cells, encodes a 13 amino acid secretory peptide that possibly may function as a peptide signal secreted by *M. incognita* during the parasitic process. Small peptides represent a new class of signaling molecules now being identified in plants with functions in cell division control, defense responses, and reproductive mechanism (Lindsey et al. 2002). Recently, it was observed that a low molecular weight peptide or peptides secreted by *Globodera rostochiensis* enhanced plant cell proliferation (Goverse et al. 1999). The coiled-coil domains in predicted proteins of six candidate parasitism genes suggests a possible function in a structural protein (e.g., in formation of the *M. incognita* feeding tube wall) (Hussey and Mims 1991), or in binding to host molecules, because coiled-coil domains in proteins fulfill a variety of functions (Lupas 1996).

The pectate lyase encoded by clone 34C04 was the second category, after cellulases, of cell wall-degrading enzymes found to be encoded by parasitism genes. Like the cellulases, the pectate lyases are expressed in the subventral esophageal gland cells of plant-parasitic nematodes with a presumed function in nematode migration in plant roots (Davis et al. 2000; de Boer et al. 2002; Doyle and Lambert 2002; Popeijus et al. 2000). Furthermore, clone 34C04 was the only candidate parasitism gene *trans*-spliced with a leader sequence found on a high percentage of nematode transcripts (Blumenthal and Steward 1997). In *C. elegans*, the *trans*-splice site tends to be very close to the translation initiation codon (Blumenthal and Steward 1997) and, in the 34C04 cDNA, the leader sequence was spliced 9 bp from the initiating methionine codon. The spliced-leader (SL) sequence of 34C04, previously described

from *M. incognita* (Ray et al. 1994) and designated SL1M (Koltai et al. 1997), differs from the nematode conserved canonical SL1 only by a single nucleotide at position 19. Interestingly, the transcript of a pectate lyase gene cloned from *M. javanica*, which shares 96% sequence identity at the amino acid level with the *M. incognita* gene identified here, also contains the SL1M (Doyle and Lambert 2002). The absence of a SL sequence on 36 putative parasitism gene cDNAs, as well as the limited number of parasitism gene homologues in *C. elegans*, suggests a substantial divergence in evolution of nematodes for parasitism and the potential limitation of *C. elegans* as a model for parasitic nematodes.

Clone 30G11 encoded an acid phosphatase preceded by a signal peptide. Although acid phosphatase is the signature enzyme for lysosomes, which function in intracellular digestion, the expression of the clone only in the subventral gland cells may indicate a digestive role for the enzyme if it is secreted into host cells. Acid phosphatase was previously localized cytochemically in secretory granules in the subventral esophageal gland cells of *M. incognita* (Sundermann and Hussey 1988). The cellulose-binding protein encoded by clone 42G06 previously was cloned from *M. incognita* (Ding et al. 1998). Expression of a recombinant cellulose-binding domain in plant tissue modulates the elongation of different plant cells in vitro (Shpigel et al. 1995), indicating the possibility of the novel cellulose binding protein secreted by *M. incognita* having a role in the development of the feeding cells (giant-cells) in susceptible host roots (Ding et al. 1998).

Over 30% of the candidate parasitism genes were expressed in the subventral gland cells, which are the most active esophageal glands in the preparasitic and migratory parasitic second-stage juveniles. However, the morphology of the secretory granules and the subventral gland cells noticeably change once *M. incognita* establishes a feeding relationship with its host (Hussey and Mims 1990). The single dorsal gland cell becomes the predominate source of secretions during the rest of the parasitic cycle. In addition, nine of the candidate parasitism genes expressed in subventral gland cells have no functional homologues in other organisms. Although cell wall-degrading enzymes (i.e., cellulases and pectate lyases) represent important secretions released from the subventral esophageal gland cells of plant-parasitic nematodes (Davis et al. 2000), the high percentage of novel parasitism genes expressed in these glands in *M. incognita* indicates that the role or roles of secretions originating in the subventral gland cells may extend beyond simply being involved in nematode migration in host roots. The expression of calreticulin and chorismate mutase genes in the subventral gland cells of root-knot nematodes also supports this hypothesis (Jaubert et al. 2002; Lambert et al. 1999).

Efficient characterization of stylet secretions originating in the esophageal gland cells will accelerate our understanding of the molecular genetics of *Meloidogyne* spp.-plant interactions. Interspecific and intraspecific comparison of the structure of parasitism genes encoding stylet secretions that induce giant-cell formation will provide the knowledge that should lead to establishing a genetic basis for host range specificity among *Meloidogyne* spp. or races. The procedure outlined herein uses a novel strategy for rapidly cloning parasitism genes encoding these secretory proteins. Functional assays will be required to confirm the role of the putative parasitism genes in the *M. incognita*-host interactions.

MATERIAL AND METHODS

Microaspiration of gland cell cytoplasm.

M. incognita was cultured on roots of greenhouse-grown tomato (*Lycopersicon esculentum* cv. Marion). Various parasitic

stages of *M. incognita* were hand dissected from infected tomato roots, surface disinfested, and embedded in 0.7% agarose. The cytoplasm of the esophageal gland cells of 43 different viable nematodes covering the full range of parasitic stages were aspirated into glass micropipettes containing 10 μ l of mRNA extraction buffer to minimize nuclease degradation of the mRNA and transferred into separate microcentrifuge tubes for storage at -80°C until used (Gao et al. 2001).

Gland cell cDNA amplification by LD-PCR.

Poly(A)⁺ RNA was purified from the cytoplasm aspirated from the esophageal gland cells using Dynabeads Oligo (dT)₂₅ magnetic beads (Dyna, Lake Success, NY, U.S.A.) and eluted with 5 μ l of diethyl pyrocarbonate (DEPC)-treated d₂H₂O as previously described (Gao et al. 2001). Gland cell first-strand cDNA synthesis was carried out in 0.5-ml reaction tubes in a total 10- μ l volume of 4 μ l of mRNA sample, 0.5 μ l of 10 μ M 3'-RACE cDNA Synthesis Primer (Clontech Laboratories, Palo Alto, CA, U.S.A.), 0.5 μ l of 10 μ M SMART II oligonucleotide (Clontech), 2.0 μ l of 5 $\times$ first strand buffer, 1.0 μ l of 20 mM DTT, 1.0 μ l of 10 mM 50 $\times$ dNTP, and 1.0 μ l of Superscript II (200 units/ μ l; GIBCO-BRL, Grand Island, NY, U.S.A.). The SMART oligonucleotide system was used to enrich for full-length cDNA for subsequent signal peptide analyses. The tubes were incubated at 42°C for 1.5 h and 90 μ l of TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA) was added. Diluted (10 $\times$) first-strand reaction solution (10 μ l), 2 μ l of 10 mM dNTP mix, 10 μ l of TaqPlus Long 10 $\times$ low salt buffer, 1 μ l of TaqPlus Long polymerase (Stratagene, La Jolla, CA, U.S.A.), and 2 μ l of Nested Universal Primer (Clontech) were used in a total 100- μ l volume of LD-PCR reaction. LD-PCR was performed with hot start followed by 24 cycles at 94°C (20 s), 65°C (30 s), and 72°C (6 min). Negative controls of DEPC water were performed at each reaction step above.

Construction of gland-cell LD-PCR cDNA library.

A gland-cell LD-PCR cDNA library was created from the LD-PCR amplification product derived from first-strand gland-cell cDNA using the pGEM-T Easy (Promega, Madison, WI, U.S.A.) vector (Gao et al. 2001). The cDNA was purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA, U.S.A.) prior to ligation. Ligations were carried out at a mass ratio 3:1 (plasmid:cDNA) at 4°C overnight. Ligation products were precipitated with 10 mM glycogen and 95% ethanol followed by a wash with 70% ethanol. The purified ligation products were heat-shock transformed into *Escherichia coli* XL10-GOLD ultracompetent cells (Stratagene). The efficiency of the transformation was 10⁹ clones/ μ g of vector. *EcoRI* was used to check the insert size range in the pGEM-T Easy vector. White colonies (on blue-white selection) of the gland-cell LD-PCR cDNA library were hand picked at random (6,144 total) and transferred to 96-well MICROTTEST III Tissue Culture plates (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) containing 200 μ l of 10% glycerol Luria-Bertani medium with ampicillin and incubated overnight at 37°C prior to macroarraying onto sterile Hybond-XL nylon membranes (70 by 105 mm; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.).

cDNA sequencing and data analysis.

The macroarrayed gland-cell LD-PCR cDNA library was hybridized with cDNA probes from a LD-PCR cDNA library generated from the intestinal cells of 10 parasitic *M. incognita* as described above to remove common expressed housekeeping and structural genes (Gao et al. 2001). Intestinal cDNAs were purified with QIAquick PCR Purification Kit (Qiagen) and 20 ng of cDNAs were labeled with [$\alpha^{32}\text{P}$]dCTP in one labeling reaction. The mixed probes were hybridized to a macro-

array membrane of the gland-cell cDNA library, and clones not hybridizing to the intestinal cDNA (i.e., clones expressed specifically in the esophageal gland cells) were randomly selected for 5'-end single pass cDNA sequencing using the primer 5'-GGTAACAACGCAGAGTACGCG-3'. Sequencing reactions were prepared using an Applied Biosystems cycle sequencing kit. Sequences were collected on an ABI 3700 autosequencer (Applied Biosystems, Foster City, CA, U.S.A.). The average sequence read for the single pass sequencing was 528 bp.

Base calls and quality scores from the raw chromatograph files from the ABI 3700 Sequencer were generated with Phred (Ewing and Green 1998; Ewing et al. 1998). Sequencher (GeneCodes Corporation, Ann Arbor, MI, U.S.A.) was used to process (remove low-quality sequence from the ends of each read and vector sequence) the individual sequence files using the Phred base calls and quality scores. Contigs of the high-quality sequences (2,452) were assembled using Sequencher with a criteria of an 85% minimum match in at least a 50-bp overlap. The high quality sequences in fasta format were batch-BLASTed using the BLAST client software, blastcl3, at the NCBI website. A BLASTX search was performed to determine sequence identity at the protein level and the results were compiled into a Microsoft Excel-based database, with the aid of MuSeqBox (Xing and Brendel 2000). Analysis for an N-terminal signal peptide sequence for secretion was conducted on all translated processed sequences using SignalP from the Center for Biological Analysis website. Subcellular localization sites of the deduced proteins were predicted by PSORT II computer analysis. The predicted proteins encoded by cDNAs expressed in the gland cells were analyzed again with NCBI's PSI-BLASTP with novel sequences (*E* value < 0.005) designated as "pioneers". The SMART database was used for domain analysis of the predicted proteins.

The 3'-RACE was used to obtain full-length cDNAs for putative parasitism genes with truncated 3' ends. Mixed parasitic stages (150 μ l) of *M. incognita* were frozen in 1.5-ml microcentrifuge tubes with liquid nitrogen and ground with a smooth-end metal bar. mRNA was purified with Oligo (dT)₂₅ magnetic beads and eluted with 20 μ l of DEPC-treated water. Reverse transcription and 3'-RACE were carried out with SMART RACE cDNA Amplification Kit (Clontech) according to the manufacturer's instructions. Universal Primer Mix A and gene-specific primers were used for PCR amplification. The amplified PCR products were cloned into pGEM-T Easy Vector (Promega) for sequencing.

Developmental expression of candidate parasitism genes.

The 185 cDNA clones with predicted signal peptides were screened by high-throughput in situ mRNA hybridization in *M. incognita* specimens. Gene-specific forward and reverse primers for each gene were used to synthesize digoxigenin (DIG)-labeled antisense cDNA probes by asymmetric PCR amplification. The PCR reactions were performed in a 20- μ l reaction mixture with PCR DIG-labeling mix (Boehringer Mannheim, Mannheim, Germany) in the asymmetric PCR instead of dNTP in the normal PCR. In situ hybridization was performed as described by de Boer and associates (1998), but modified for high-throughput in situ screening (Gao et al. 2001). Parasitic second-stage juveniles were collected by hatching eggs on sieves with 25- μ m openings suspended over deionized water in plastic bowls (Ding et al. 1998). Mixed parasitic stages of *M. incognita* were collected 13 to 15 days after inoculation of plants by root maceration and sieving (de Boer et al. 1999). Nematodes, fixed in 10% phosphate-buffered (pH 7.0) formalin, were cut randomly on glass slides with a razor blade and then partially digested with proteinase-K (Boehringer Mannheim) at 500 ng/ml at 37°C for 1 h. After prehybridization,

nematode sections in hybridization solution were aliquoted to wells of 96-well MultiScreen plate (Millipore, Bedford, MA, U.S.A.). Denatured PCR DIG-labeled DNA probes (2 µl) were added to each well and solutions were changed by aspiration using the Millipore MultiScreen vacuum manifold (Gao et al. 2001). Substrate for alkaline phosphatase-conjugated anti-DIG was used to visualize hybridized cDNA probes within nematode specimens with a compound light microscope (de Boer et al. 1998).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

- SMART (Simple modular architecture research tool) database: smart.embl-heidelberg.de.
- NCBI BLAST server website: www.ncbi.nlm.nih.gov/blast/index.html.
- The Center for Biological Analysis website: www.cbs.dtu.dk/services/SignalP/index.html.
- PSORT WWW server website: psort.nibb.ac.jp.