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Isolation of a cDNA Encoding a β-1,4-endoglucanase in the Root-Knot Nematode *Meloidogyne incognita* and Expression Analysis During Plant Parasitism

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A β-1,4-endoglucanase encoding cDNA (EGases, E.C. 3.2.1.4), named *Mi-eng-1*, was cloned from *Meloidogyne incognita* second-stage juveniles (J2). The deduced amino acid sequence contains a catalytic domain and a cellulose-binding domain separated by a linker. In *M. incognita*, the gene is transcribed in the migratory J2, in males, and in the sedentary adult females. In pre-parasitic J2, endoglucanase transcripts are located in the cytoplasm of the subventral esophageal glands. The presence of β-1,4-endoglucanase transcripts in adult females could be related to the expression of the gene in esophageal glands at this stage. However, cellulase activity within the egg matrix of adult females suggests that the endoglucanase may also be synthesized in the rectal glands and involved in the extrusion of the eggs onto the root surface. The maximum identity of the predicted MI-ENG-1 catalytic domain with the recently cloned cyst nematode β-1,4-endoglucanases is 52.5%. In contrast to cyst nematodes, *M. incognita* pre-parasitic J2 were not found to express a β-1,4-endoglucanase devoid of a cellulose-binding domain.

Phytoparasitic root nematodes are major threats to agricultural production worldwide. Although their modes of parasitism are diverse they all have to weaken or break down plant tissue. Ectoparasitic nematodes have to perforate epidermal and subepidermal cell walls with their stylet to access plant cell cytoplasm for feeding. Endoparasitic nematodes feed in the cortical and vascular cell layers, and most of the sedentary genera have to access the pericycle to establish a feeding site close to the vascular cylinder (Sijmons et al. 1994). In the cyst nematodes (*Globodera* spp. and *Heterodera* spp.) and the root-knot nematodes (*Meloidogyne* spp.), only infective second-stage juveniles (J2) and males are migratory within the root tissues. Cyst J2 penetrate roots and migrate intracellularly toward the vascular cylinder (Wyss and Zunke 1986). Although root-knot nematodes may penetrate root epidermal cells directly, they migrate intercellularly to vascular tissues, separating adjacent cells without disrupting the protoplasts (Wyss et al. 1992). Cyst nematodes induce feeding sites called syncytia that result from cell wall degradation of adjacent cells and fusion of neighboring protoplasts without mitotic stimulation (Jones 1981). In contrast, giant cells induced for feeding by the root-knot nematodes develop by repeated mitosis without cytokinesis (Jones 1981). Swollen females of most cyst nematode species become filled with eggs, with most of the body protruding outside the root, while females of root-knot nematodes remain entirely in the root tissue and extrude eggs into a gelatinous matrix that they secrete onto the surface of the root.

For both the cyst and root-knot nematodes, stylet secretions have been assumed to play a key role in penetration and migration of the J2 within roots, and in the induction and maintenance of the feeding sites (Bird and Saurer 1967; Bird 1969; Veech et al. 1987; Hussey 1989). Stylet secretions are produced in three unicellular esophageal glands, two subventral and one dorsal. Cytoplasmic extensions of these glands are connected by elaborate valves to the esophageal lumen. The dorsal gland extension stretches to the base of the stylet. The subventral gland ducts reach the esophageal lumen just posterior to the metacorpal pump chamber. The release of dorsal gland secretions through the stylet has been proposed and observed for a long time, but the possibility for subventral gland products to cross the metacorpal bulb and be secreted out of the stylet has been controversial (Wyss and Zunke 1986). Secretion of subventral gland products through the stylet has recently been clearly demonstrated with esophageal secretion-specific antibodies (Davis et al. 1994; Goverse et al. 1994; Smant et al. 1997). Currently, clues to the nature and function of stylet secretions in parasitism are emerging.

Putative secretory enzymes that allow the nematode to pass through plant tissues at different developmental stages

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have been reported (Myers 1965). In particular, cellulase activity has been demonstrated in juveniles from several migratory and sedentary nematodes (Morgan and McAllan 1962; Myers 1965; Kojima et al. 1994). Increased cellulase activity has been reported in galls of tomato roots infested with *M. javanica* (Bird et al. 1975), but enzymatic tests on secretory granules of *M. incognita* J2 and females do not confirm the nematode origin of this activity (Sundermann and Hussey 1988). Endogenous production of cellulases by cyst nematodes has recently been unambiguously demonstrated by the isolation of β-1,4-endoglucanase cDNAs (EC 3.2.1.4.) from *G. rostochiensis* and *H. glycines* infective J2 (Smant et al. 1998). Moreover, it was demonstrated that secretion through the stylet of β-1,4-endoglucanases occurs at this stage in *G. rostochiensis* (Smant et al. 1997, 1998). A putative role of these enzymes is the softening of cell walls during penetration of the root epidermis and the intracellular migration of the juveniles. Cell-wall-degrading enzymes have been shown to act as pathogenicity factors in several intercellular plant pathogens including bacteria (Roberts et al. 1988; Beaulieu et al. 1993; Boccara et al. 1994) and fungi (Bodenmann et al. 1985; Mendgen and Deising 1993). While it might be hypothesized that root-knot nematodes would use pectinolytic enzymes for intercellular migration, it is unclear if cellulolytic enzymes may play a role in parasitism by these nematodes.

We report here the cloning of a β-1,4-endoglucanase cDNA (*Mi-eng1*) from the root-knot nematode *M. incognita.* Transcription of β-1,4-endoglucanase in the subventral esophageal glands of infective juveniles was demonstrated by mRNA in situ hybridization. Expression of β-1,4-endoglucanases occurred in egg, J2, adult male, and adult female stages of *M. incognita*. Differences in structure and temporal expression of *M. incognita* endoglucanases, compared with endoglucanases produced by cyst nematodes, suggest either that different enzyme systems are involved in the process of cell wall softening during parasitism or that the regulation of these systems differs.

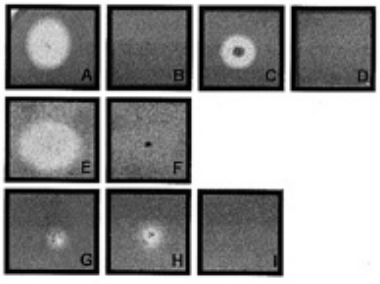

Fig. 1. Cellulase activity assay on carboxymethylcellulose (CMC) agar plate tests in **(A)** *Meloidogyne incognita* second-stage juvenile (J2) total homogenate, **(B)** *Cænorhabditis elegans* total homogenate, and **(C)** *M. incognita* J2 stylet secretions. For each sample, 6.5 µg of protein was loaded in a 2-µl spot. The same volume was spotted for the **(D)** secretion negative control. *Escherchia coli* clones obtained from the J2 cDNA library **(E)** containing the *Mi-eng1* cDNA and **(F)** not carrying an endoglucanase cDNA, grown on Luria-Bertani (LB) medium, 0.% CMC, supplemented with ampicillin and isopropyl-β-D-thiogalactopyranoside. **G,** Eggs embedded in their gelatinous matrix directly loaded on the CMC plate. **H,** Gelatinous matrix devoid of eggs. **I,** Whole eggs cleaned from gelatinous matrix.

RESULTS

Isolation and characterization of a cellulase gene from *Meloidogyne* spp.

Cellulase activity was observed in total homogenate and stylet secretions of *M. incognita* J2 in carboxymethylcellulose (CMC) degradation assays (Fig. 1A–D). To clone the corresponding gene, peptidic regions conserved in β-1,4-endoglucanases from cyst nematodes and plant-parasitic bacteria and fungi were used to design degenerate nucleotide primers. Polymerase chain reaction (PCR) was performed on genomic DNA of *M. incognita* J2 with the ENG1 and ENG2 primers encoding, respectively, the YVIVDWH and FVTEYGT regions located in the catalytic domain. An amplification product of 650 bp was obtained that after cloning and sequencing showed sequence similarity with endoglucanases from *Erwinia chrysanthemi* (P(N) 1.5e-31), *Pseudomonas fluorescens* (P(N) 5.1e-30), *Cellvibrio mixtus* (P(N) 6.7e-25), *G. rostochiensis* (P(N) 7.0e-25), and *H. glycines* (P(N) 2.4e-17) according to the BLASTX program (Altschul et al. 1990; Gish and States 1993). Comparison of the predicted protein sequence with those of other β-1,4-endoglucanases revealed the presence of two introns of 192 and 41 bp in the amplified product from *M. incognita* (data not shown). Southern blots of root-knot nematode DNA probed with the PCR product confirmed the nematode origin of this endoglucanase genomic fragment. Multiple DNA fragments hybridized with the PCR product in *M. incognita, M. arenaria,* and *M. javanica,* suggesting the presence of multiple endoglucanase homologues in their genome. In contrast, only one *M. hapla* DNA fragment hybridized to the PCR product (Fig. 2). No hybridization signal was observed with genomic DNA from other nematode genera tested, including *Bursaphelencus* spp., *Heterorhabditis* spp., and *Cænorhabditis* spp. (data not shown).

The genomic fragment of *M. incognita* endoglucanase was used to screen a cDNA library constructed from *M. incognita* pre-parasitic juveniles. The high number of positive clones in a first round of screening (4.8%) suggested a very high expression level of endoglucanase in pre-parasitic J2. A cDNA, named *Mi-eng-1,* that encodes a family 5 β-1,4-endoglucanase

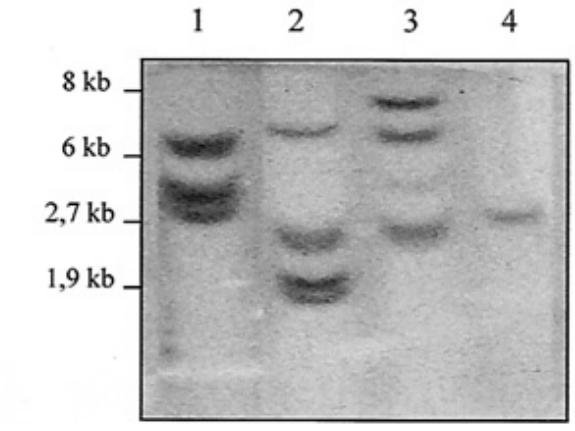

Fig. 2. Southern blot probed at 55°C with the radiolabeled β-1,4-endoglucanase 650-bp genomic amplification product from *Meloidogyne incognita*. Genomic DNA (10 µg each) from (lane 1) *M. incognita,* (lane 2) *M. javanica,* (lane 3) *M. arenaria,* and (lane 4) *M. hapla* was digested with *Eco*RI and *Hin*dIII prior to separation for Southern analyses.

(Henrissat 1991) was isolated. This cDNA is 1,668 nucleotides long with an open reading frame (ORF) of 1,518 bp (Fig. 3). No spliced leader sequence was observed.

The predicted protein is 506 amino acids long. An N-terminal secretion signal sequence of 23 amino acids could clearly be identified with the SignalP V1.1 World Wide Web Server (Nielsen et al. 1997). The enzyme is composed of a catalytic domain separated from a C-terminal cellulose-binding domain (CBD) by a peptide linker. The catalytic domain extends from residues 24 to 323. Based on similarity to the family 5 β-1,4-endoglucanases, we propose that the catalytic site consists of the glutamic acid residue at position 160 as the proton donor and the glutamic acid residue at position 246 as the nucleophile (Ohmiya et al. 1997). The linker region is rich in serine, glycine, asparagine, and threonine and carries a potential N-glycosylation site. The CBD is 90 amino acids long and belongs to CBD family II (Prosite: PDOC00485). At the peptide level, the identity between MI-ENG-1 and the cyst nematode endoglucanase catalytic domains ranges from 49.0% with HG-ENG-1 to 52.5% with GR-ENG-1. The CBDs are more divergent between the nematode genera (up to 46.0% identity with the GR-ENG-1 CBD) even though they all belong to CBD family II. Cellulase activity of the cDNA translation product

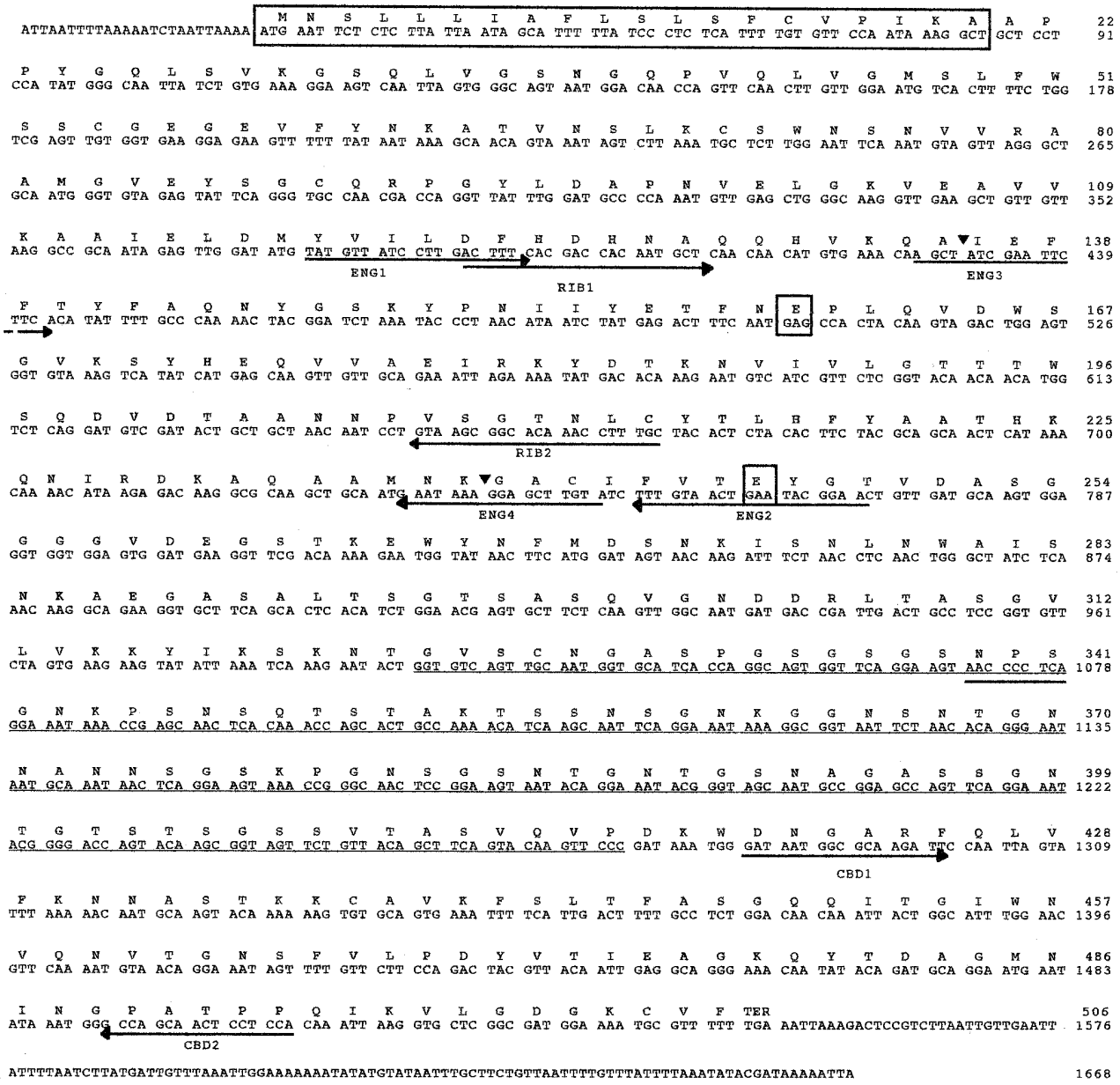

Fig. 3. *Meloidogyne incognita Mi-eng-1* cDNA sequence and predicted amino acid sequence. Predicted secretion signal sequence and amino acids from the catalytic site are indicated in boxes. The linker separating the N-terminal catalytic domain from the C-terminal cellulose-binding domain is underlined. The potential N-glycosylation site is double underlined. Predicted locations of the two intronic sequences identified are indicated by darkened triangles. Primers used for genomic amplification and reverse transcription–polymerase chain reaction are indicated by arrows. The nucleotide sequence is available in the GenBank data base under the accession number AF100549.

was demonstrated by growing bacteria containing the clone on CMC test plates (Fig. 1E,F).

In cyst nematode J2, two types of endoglucanase transcripts could be identified, one with a CBD and another without (Smant et al. 1998). To check whether endoglucanases homologous to *Mi-eng-1* but without CBD were expressed in *M. incognita* J2, a differential screening of the J2 cDNA library was performed with a catalytic-domain-specific probe and a CBD-specific probe. Of 12,000 clones tested, about 500 hybridized with the catalytic domain and the CBD probe (data not shown), suggesting that all endoglucanase transcripts homologous to *Mi-eng-1* in pre-parasitic *M. incognita* J2 bear a CBD.

Expression of cellulase during nematode development.

Transcription of the β-1,4-endoglucanase in different developmental stages was analyzed by reverse transcription (RT)-PCR on mRNA and total RNA from eggs, pre-parasitic J2, adult females, and males. As a control, RNA from uninfected tomato roots was used following the same procedure. Amplification products of the expected 324-bp size within the *Mi-eng1* catalytic domain were obtained from all nematode stages, but not from the tomato root control, in four separate experiments (Fig. 4).

As females are sedentary, we checked whether expression in females could be from a different origin, other than the esophageal glands. Gelatinous matrix and whole eggs were assayed separately for cellulase activity on CMC test plates (Fig. 1G–I). Although one cannot exclude possible contamination of the sample by soil organism or root cellulases, strong cellulase activity was observed in the gelatinous matrix separated from eggs.

In situ localization of cellulase transcripts in J2.

In situ localization of β-1,4-endoglucanase transcripts was performed on freshly hatched J2. An antisense riboprobe from the catalytic domain of *Mi-eng-1* was used as a probe. The same experiment was conducted with a complementary sense riboprobe as a control. The presence of cellulase mRNA could be demonstrated in all freshly hatched juveniles examined. A high local accumulation of cellulase transcripts was observed in the cytoplasm of the subventral, esophageal gland cells. No transcript was observed in the gland extensions (Fig. 5). No staining was observed with the sense control.

DISCUSSION

Because root-knot nematode J2 migrate intercellularly, one could hypothesize that mechanical forces and pectinolytic enzymes would be sufficient for the nematode to separate adjacent cells. However, softening of the cell wall during J2 migration was suggested by the change of the cell shape to conform to the contour of the nematode, as was observed with *M. incognita* and *M. hapla* (Smith and Mai 1965; Endo and Wergin 1973). Our results confirm the presence and expression of a functional β-1,4-endoglucanase in *M. incognita*. Therefore, root-knot nematodes appear to share some common invasion processes with other pathogens that invade plant tissues intercellularly, such as bacteria or biotrophic fungi (Mendgen and Deising 1993; Barras 1994).

The MI-ENG-1 predicted protein has low similarity to cyst nematode endoglucanases (up to 52.5% identity with GR-ENG-2 in the catalytic domain). However, suitable primers for genomic amplification could be designed by taking into account protein motifs conserved among cyst nematodes and plant-pathogenic bacteria and fungi. The endogenous origin of this *M. incognita* endoglucanase is supported by the absence of microorganisms associated with the nematode (Cardin and Dalmasso 1985), and the presence of β-1,4-endoglucanase transcripts in the cytoplasm of the two subventral glands of infective juveniles. The high percentage (4.8%) of positive clones observed in a J2 cDNA library may reflect a high endoglucanase transcription level at this stage.

β-1,4-endoglucanase transcripts could also be detected in eggs, females, and males. The presence of transcripts in eggs

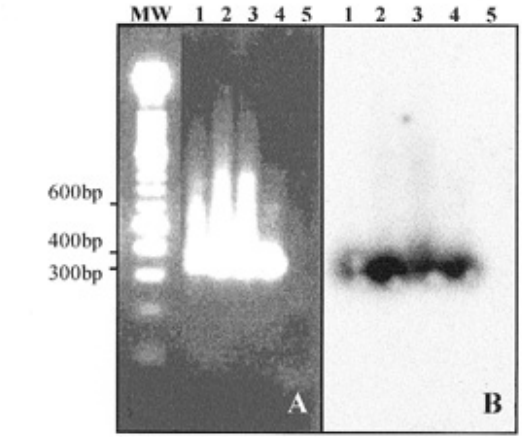

Fig. 4. Detection of *Mi-eng-1* transcripts in (lane 1) eggs, (lane 2) pre-parasitic second-stage juveniles (J2), (lane 3) females, (lane 4) males, and (lane 5) uninfected tomato roots by reverse transcription–polymerase chain reaction with endoglucanase primers. Equivalent amounts of RNA were used for synthesis of first-strand cDNAs. Amplification of a 324-bp fragment in the catalytic domain visualized on **(A)** agarose gel and **(B)** transferred onto Hybond N^+ membrane and probed with *Mi-eng-1* catalytic domain. MW: molecular weight marker.

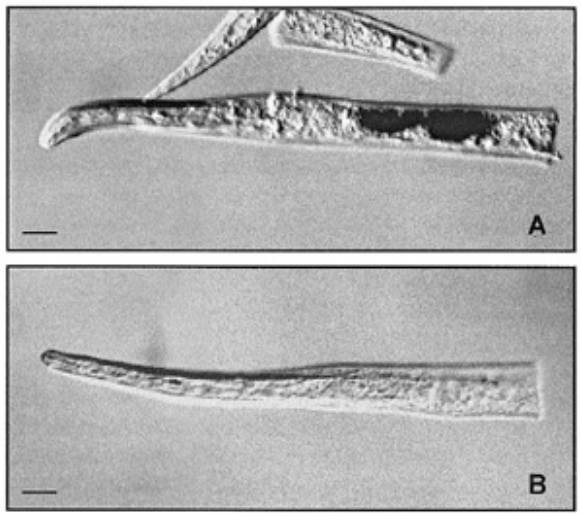

Fig. 5. Localization of β-1,4-endoglucanase transcripts in the subventral glands of *Meloidogyne incognita* second-stage juveniles. Fixed nematodes were cut in two to three sections and incubated with **(A)** antisense or **(B)** sense riboprobes from *Mi-eng-1* catalytic domain. Horizontal bars equal 0.01 mm.

is likely due to the large quantity of eggs containing coiled juveniles about to hatch. Indeed, the accumulation of secretory granules has been observed in the subventral glands of J2 just before hatching (Bird 1968). The finding of cellulase transcripts in pre-parasitic J2 may be correlated to the need for the juveniles to penetrate root epidermal cells directly. By hydrolyzing β-1,4 glycosidic bonds of the cellulose, the enzyme may participate in the weakening of the cell walls, allowing J2 and males to more easily separate adjacent cells during migration within roots. From the cDNA J2 library no endoglucanase gene homologous to *Mi-eng-1* and lacking the CBD sequence was found. In contrast, β-1,4-endoglucanases from the same glycosyl hydrolase family as *Mi-eng-1*, one with a CBD and one lacking the CBD, have been isolated from both *G. rostochiensis* and *H. glycines* J2 (Smant et al. 1998). The CBD is hypothesized to improve digestion of crystalline cellulose and, in several cases, improves enzymatic activity (Linder and Teeri 1997). In *M. incognita* pre-parasitic and parasitic J2, a secretory cellulose-binding protein containing a CBD but no identifiable catalytic domain was discovered, but its potential function in parasitism is unclear (Ding et al. 1998). The β-1,4-endoglucanases from cyst and root-knot nematodes have not yet been biochemically characterized and the role of their CBD in enzymatic activity is not established. However, the observed difference in β-1,4-endoglucanase structure between pre-parasitic juveniles of cyst and root-knot nematodes may reflect different enzymatic processes at this stage, which could involve other types of cellulases and cell-wall-degrading enzymes and be correlated to the different modes of migration in the root tissue.

The observation of β-1,4-endoglucanase transcripts in *M. incognita* adult females was more intriguing since the nematode is sedentary at this stage, and no endoglucanase was found to be synthesized in the esophageal glands of *G. rostochiensis* females (Smant et al. 1997, 1998). This difference in cellulase expression pattern during development could be related to a difference in feeding behavior. While cyst nematode females may feed from the same initial syncytial cell, root-knot nematodes feed from multiple giant cells at multiple times. Consequently, root-knot nematode females may need to produce cellulolytic enzymes for induction of new giant cells. The presence of β-1,4-endoglucanase transcripts in *M. incognita* females may also be related to the cellulase activity we observed in the *M. incognita* gelatinous matrix into which the female deposits its eggs. The gelatinous matrix is produced by six rectal glands and is excreted through a canal ending at the root surface (Orion and Franck 1990). Cell-wall-degrading enzymes such as cellulase are assumed to be involved in the formation of this canal (Orion et al. 1987). A rectal origin for the β-1,4-endoglucanase transcripts is in accordance with the absence of this enzyme in *G. rostochiensis* adult females, since *G. rostochiensis* females accumulate eggs inside their body at the exterior of the root. In situ hybridization or immunolabeling would be necessary to confirm whether endoglucanase transcripts in adult females are of esophageal and/or rectal gland origin.

Two different genes encode the β-1,4-endoglucanase isozymes characterized in *H. glycines* and *G. rostochiensis* (Yan et al. 1998), and we observed several putative homologues of *Mi-eng-1* in the genome of three *Meloidogyne* spp. on Southern blots. A complex regulation of cell-wall-degrading isozymes has been reported in plant-pathogenic bacteria of *Erwinia* (Barras et al. 1994), in which some cell-wall-degrading enzymes are induced only when the bacteria enter the plant tissue. Similarly, in *Meloidogyne* spp. and cyst nematodes, several cellulase genes could be differentially expressed during parasitism. The biochemical and molecular characterization of nematode β-1,4-endoglucanases should lead to a better understanding of the role of each enzyme in parasitism and the molecular events leading to plant tissue alterations specific to root-knot and cyst nematodes.

MATERIAL AND METHODS

Nematode and stylet secretion extraction.

M. incognita, M. javanica, M. arenaria, and *M. hapla* were grown on tomato roots (cv. St. Pierre) in a greenhouse. Eggs were collected after treatment of the egg masses in 0.5% NaOCl and were rinsed carefully with water. J2 were hatched in vitro on 10-µm sieves in 0.7% streptomycin sulfate. Adult females were extracted by maceration of root tissues in 5% pectinase (NovoNordisk, Bagsvaerd, Denmark). *Cænorhabditis elegans* and *Bursaphelenchus xylophilus* nematodes were multiplied in vitro on *Escherichia coli* OP50 and *Botrytis cinerea,* respectively (Brenner 1974; De Guiran and Bruguier 1989). Entomopathogenetic nematodes *Steinernema carpocapsae, Heterorhabditis indicus,* and *H. bacteriophora* were multiplied on *Galleria melonella* larvae (Woodring and Kaya 1988). Nematodes were collected from roots, nutritive media, or insect hosts and concentrated by centrifugation at 3,500 × *g* for 5 min in a 20% sucrose solution at 4°C. Nematodes concentrated at the surface were rinsed carefully with sterile water and stored at –80°C until further required.

Production of stylet secretions was induced by incubating freshly hatched J2 in 0.4% resorcinol for 4 h (McClure and Von Mende 1987; Davis et al. 1994). Secretions were solubilized in 0.1 M Tris pH 11.0 and concentrated on 3-kDa cutoff membranes (Amicon, Beverly, MA). As a control, the same procedure was conducted without resorcinol.

PCR amplifications and RT.

For the extraction of total genomic DNA and RNA from nematodes or uninfected tomato roots, tissues were frozen in liquid nitrogen and ground by mortar and pestle. Total genomic DNA was extracted from the resulting powder by a phenol/chloroform procedure, as described by Sambrook et al. (1989).

Amplification of genomic DNA was conducted on 10 ng of *M. incognita* DNA with degenerate primers. Protein sequences of β-1,4-endoglucanases from plant-parasitic bacteria and fungi were aligned with the β-1,4-endoglucanase sequences from *H. glycines* HG-ENG-1 and HG-ENG-2 (GenBank accession nos. AF006052 and AF006053, respectively) and *G. rostochiensis* GR-ENG-1 and GR-ENG-2 (GenBank accession nos. AF004523 and AF004716, respectively). Nucleotide primers ENG1 (5′TA(T/C)GTIAT(T/C/A)GTIGA(T/C)TG GCA3′), and ENG2 (5′GTICC(A/G)TA(T/C)TCIGTIAC (A/G)AA3′), were designed from the conserved peptidic regions YVIVDWH and FVTEYGT located in the catalytic domain.

RT-PCRs were conducted both on total RNA and on mRNA isolated from *M. incognita* eggs, J2, females, males, and tomato roots. Total RNA was purified according to Chomczyn-

ski and Sacchi (1987) and mRNAs were purified with the Quick Prep Micro mRNA Purification kit (Pharmacia, Uppsala, Sweden). First-strand cDNAs were synthesized with the CBD-specific primer CBD2 (5′TGTGGAGGAGTTGCT GGC3′) with the AMV Reverse Transcriptase (Promega, Madison, MI). To avoid amplification due to genomic DNA contamination, subsequent amplifications were performed with primers ENG3 (5′GGCGATCAATTTCTTCA C3′) and ENG4 (5′TCCAAACTCCCTTGTTTA3′), specific to the catalytic domain, and known to overlap intron sequences, as deduced from the genomic amplification product sequence. PCR was performed with *Taq* polymerase (Appligene Oncor, Gaithersburg, MD). DNA was first denatured at 94°C for 3 min. Amplification was then performed by a series of cycles with denaturation at 94°C for 1 min, annealing at decreasing temperatures for 1 min 30 s, and elongation at 72°C for 2 min. The annealing temperatures were 65°C for 5 cycles, 60°C for 5 cycles, and 55°C for 35 cycles. Finally, an elongation step at 72°C for 10 min was performed. RT-PCR products were transferred onto Nylon N^+ membrane (Amersham, Buckinghamshire, England) and hybridized with a catalytic domain-specific probe as described below.

cDNA library screening.

The β-1,4-endoglucanase cDNA was isolated from a pcDNA II (Invitrogen, Carlsbad, CA) cDNA library from *M. incognita* J2. About 72,000 colonies were transferred onto Nylon N^+ membrane following standard procedures (Smith and Summers 1980). The genomic amplification product was used as a probe and was radioactively labeled with [^{32}P]dCTP with the Ready to Go kit (Pharmacia). Hybridization was performed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium acetate), 5× Denhardt's, 0.5% SDS (sodium dodecyl sulfate) buffer at 65°C. Membranes were washed at 65°C for 1 h in 2× SSC, 0.1% SDS, then a further 1 h in 1× SSC, 0.1% SDS. For differential screening of the library, the same procedure was performed and hybridization was conducted in parallel with a catalytic-domain-specific probe and a CBD-specific probe. The catalytic-domain-specific probe was obtained by PCR amplification on the *Mi-eng-1* clone with the oligonucleotides ENG3 and ENG4. The CBD-specific probe was obtained by the same procedure with the oligonucleotides CBD1 (5′GATAATGGCGCAAGATTC3′) and CBD2.

CMC plate tests.

Cellulase activity was assayed on plates containing 1.5% agar and 0.5% carboxymethylcellulose (Sigma, St. Louis, MO). Protein samples were loaded as spots containing 6.5 µg of total proteins onto the agar. Since minute amounts of protein were present in the stylet secretion negative control, a volume of this sample equivalent to that of stylet secretions was spotted on the plate. After 16 h of incubation the plates were stained with 0.1% Congo red and washed with 1 M NaCl. In order to test cellulase activity in the gelatinous matrix, eggs were extracted from egg masses under a binocular microscope and the matrices were carefully rinsed in sterile water for assay. Cellulase activity in bacterial clones from the J2 library was demonstrated following the same procedure after growing the bacteria on 0.5% CMC LB medium (Sambrook et al. 1989) with the addition of ampicillin and isopropyl-β-D-thiogalactopyranoside. As a negative control, bacteria containing a cDNA clone not related to the endoglucanase cDNA were grown.

In situ hybridization.

For in situ hybridization, riboprobes were synthesized from the catalytic domain. Oligonucleotides RIB1 (5′AGGAATTC ACTTTCACGACCACAATGCTC3′) and RIB2 (5′ATGG ATCCGCAAAGGTTTGTGCCGCTTAC3′) were used that amplified a 278-bp fragment bearing the *Eco*RI and *Bam*HI restriction sites. This fragment was cloned into the pBluescript II transcription vector (Stratagene, La Jolla, CA). Digoxigenin-11-UTP-labeled RNA probes were transcribed in sense and antisense directions. In situ hybridizations were performed essentially as described previously (De Boer et al. 1998). Briefly, freshly hatched J2 were fixed in 2% paraformaldehyde for 21 h at 5°C followed by a second incubation for 6 h at room temperature. Nematodes were cut into sections and permeabilized with proteinase K, acetone, and methanol. The sections were then hybridized at 50°C with the sense or antisense riboprobe. Following digestion with RNase A, bound riboprobe was detected by alkaline phosphatase immunostaining. The stained J2 were examined with differential interference contrast microscopy.

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