A Secretory Cellulose-Binding Protein cDNA Cloned from the Root-Knot Nematode (*Meloidogyne incognita*)

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A cDNA encoding a secretory cellulose-binding protein was cloned from the root-knot nematode (*Meloidogyne incognita*) with RNA fingerprinting. The putative full-length cDNA, named *Mi-cbp-1*, encoded a 203 amino acid protein containing an N-terminal secretion signal peptide. The C-terminal sequence of the putative MI-CBP-1 was similar to a bacterial-type cellulose-binding domain, whereas the N-terminal sequence did not show significant similarity to any proteins in data bases. Recombinant MI-CBP-1 lacked cellulase activity, but bound to cellulose and plant cell walls. In Southern blot hybridization, *Mi-cbp-1* hybridized with genomic DNA from *M. incognita*, *M. arena-ria*, and *M. javanica*, but not *M. hapla*, *Heterodera glycines*, or *Caenorhabditis elegans*. Polyclonal antibodies raised against recombinant MI-CBP-1 strongly labeled secretory granules in subventral gland cells of second-stage juveniles in indirect immunofluorescence microscopy. Enzyme-linked immunosorbent assay detection of MI-CBP-1 in stylet secretions of second-stage juveniles with the polyclonal antibodies indicated MI-CBP-1 could be secreted through the nematodes' stylet, suggesting that the cellulose-binding protein may have a role in pathogenesis.

Additional keywords: feeding, giant-cell.

The sedentary, endoparasitic, root-knot nematode (*Meloidogyne* spp.) is a major contributor to annual yield losses (US\$77 billion) caused by plant-parasitic nematodes to world crops (Sasser and Freckman 1987). This obligate, biotrophic parasite has evolved a very specialized and complex feeding relationship with susceptible host plants. Pathogenesis by root-knot nematodes has been extensively studied (Wyss et al. 1992; Jones and Payne 1978) and reviewed (Hussey et al. 1994; Hussey 1985; Williamson and Hussey 1996). However, knowledge about the mechanism of pathogenesis is still fragmentary, especially at the molecular level. The infective second-stage juvenile (J2) penetrates roots near the zone of elongation by destroying the epidermal cells through either mechanical force with its protrusible stylet, enzymatic digestion of cell walls by esophageal gland cell secretions emitted through the stylet, or both. The J2 then moves toward the root tip intercellularly along the middle lamellae in the cortical tis-

sue to the meristematic zone, where it turns around to enter the center of the root and migrates to the differentiating vascular cylinder (Wyss et al. 1992). When differentiating vascular tissue is reached, the J2 transforms five to seven root cells into permanent specialized feeding cells, called giant-cells, by modifying and regulating cell phenotype, function, and metabolism. The nematode is entirely dependent on the giant-cells for nourishment for development and reproduction.

Giant-cells are enlarged, multinucleate cells with elaborate wall ingrowths and an expanded and dense cytoplasm that is metabolically active. The specialized giant-cells are similar to transfer cells in phenotype (Jones and Payne 1978), but their induction and maintenance depend entirely on the feeding activities of the root-knot nematode (Hussey et al. 1994). Although the induction mechanism is still unknown, several lines of evidence suggest that bioactive molecules synthesized in the nematode's esophageal gland cells and secreted through its stylet may be either directly or indirectly involved in giant-cell induction and maintenance in addition to having other roles in the infection process (Williamson and Hussey 1996). In fact, cellulases, which have a putative role in the infection process, have recently been shown to be synthesized in the subventral gland cells of cyst nematodes and the corresponding cDNAs have been cloned (Smant et al. 1998). Root-knot nematode infective J2 and adult females contain one dorsal and two subventral gland cells where secretory proteins are synthesized and packaged in Golgi-derived, membrane-bound, secretory granules (Hussey et al. 1994; Hussey and Mims 1990). Monoclonal antibody studies showed that proteins from both subventral and dorsal gland cells are components of stylet secretions (Davis et al. 1994). Therefore, characterization of stylet secretions is critical for elucidating the mechanism of pathogenesis.

In this study, RNA fingerprinting was used to identify RNAs present in the parasitic J2 of *M. incognita* but absent or reduced in pre-parasitic J2. A cDNA encoding a secretory cellulose-binding protein was cloned from *M. incognita*.

RESULTS

RNA fingerprinting and cloning of the cDNA.

A total of 65 different primer combinations were used in the RNA fingerprinting reactions. Fifty-four cDNA fragments that were specific or more strongly expressed for parasitic J2 were cloned and sequenced after selecting against pre-parasitic J2. The predicted protein sequence of one fragment, F43, was similar to a bacterial-type cellulose-binding domain (CBD)

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Nucleotide and/or amino acid sequence data have been submitted to the GenBank data base as accession number AF049139.

based on a BLAST search (Altschul et al. 1990) against the GenBank/EMBL data base. In Northern (RNA) blot analysis, an approximately 1-kb transcript was detected in pre-parasitic and parasitic J2 but not in adult females when the F43 fragment was used as a probe (data not shown). Therefore, the same probe was used to screen an *M. incognita* J2 cDNA library. Six positive clones were identified and digestion of plasmids isolated from these clones with *XhoI/BstXII* released 0.8- to 1.0-kb inserts. The clone with the longest cDNA insert, named *Mi-cbp-1*, was selected for further characterization.

Predicted amino acid sequence of the *Mi-cbp-1* clone.

Mi-cbp-1 contained 998 nucleotides with a putative open reading frame (ORF) of 609 bp (Fig. 1). Sequence analysis of *Mi-cbp-1* based on the GCG program (Genetics Computer Group, Madison, WI) revealed that the ORF encoded a putative protein of 203 amino acids, with an eukaryotic secretion signal sequence (Nielsen et al. 1997) terminating at amino acid residue 20 (Fig. 1).

Computer-assisted protein sequence similarity search (Altschul et al. 1990; Smith and Smith 1992) revealed that the C-terminal 95 amino acids of MI-CBP-1 had 21 to 24% identity with type II CBD from different bacterial origins and 24 to 25% identity with CBDs in two β -1,4-endoglucanases from cyst nematodes (Fig. 2) (Smant et al. 1998). The sequence contained all the characteristic CBD structural features (Gilkes et al. 1991), such as high numbers of hydroxy amino acids, a deficiency in charged amino acids, and conserved tryptophan, asparagine, and glycine residues. The N-terminal 71 amino acid sequence of MI-CBP-1 did not show similarity to any proteins in the data base. The N- and C-terminal sequences were connected by a serine-rich 16 amino acid Q-type linker (Gilkes et al. 1991), where an N-glycosylation site (NGSS) was detected (Bairoch et al. 1995) (Fig. 1).

Southern blot hybridization.

To determine if genes analogous to *Mi-cbp-1* were present in other nematode species, *Mi-cbp-1* was radiolabeled and used to probe Southern blots containing *EcoRI*-digested total DNA from five plant-parasitic nematodes, the free-living nematode *Caenorhabditis elegans*, and soybean. The *Mi-cbp-1* probe detected analogous sequences in three *Meloidogyne* spp.: *M. incognita*, *M. arenaria*, and *M. javanica*. No hybridization at high stringency was observed with genomic DNA from *M. hapla*, *Heterodera glycines*, *C. elegans*, or the host plant, soybean (Fig. 3). Identical hybridization patterns also were observed under low stringency conditions.

Immunological analyses.

A polyclonal antiserum was raised against recombinant MI-CBP-1 for immunological analyses. To determine the location of the MI-CBP-1 in the nematode, sections of pre-parasitic J2 of *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, and *H. glycines* and mixed stages of *C. elegans* were treated with the polyclonal antiserum in immunofluorescence assays. For the pre-parasitic J2 of *M. incognita*, *M. arenaria*, *M. javanica*, and *M. hapla*, the antibodies reacted strongly and specifically with secretory granules in the subventral gland cells and their cytoplasmic extension and expanded ampullae, which are located posterior to the pump chamber in the metacarpus (Fig. 4A). The labeling pattern for the *M. incognita* parasitic J2 was

identical to that observed for the pre-parasitic J2. No labeling was observed with the adult females of *M. incognita* or the pre-parasitic J2 of *H. glycines* and mixed stages of *C. elegans*. To establish that MI-CBP-1 can be secreted through the stylet of pre-parasitic *M. incognita* J2, stylet secretions were induced in vitro and concentrated for use in an enzyme-linked immunosorbent assay (ELISA) with the MI-CBP-1 polyclonal antibody. MI-CBP-1 was detected in the stylet secretions and total extracts of J2 by the polyclonal antibodies (Fig. 5).

The putative cellulose-binding function of the CBD of MI-CBP-1 was confirmed by cellulose-binding assays and recombinant MI-CBP-1 binding to tobacco cell walls. The 29-kDa recombinant MI-CBP-1 was eluted from a cellulose resin column as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining, indicating the ability of the CBD to bind to cellulose (Fig. 6). Another *M. incognita* recombinant protein, MI-AMP-1,

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AGTTTGAGCGTCAGCAATTTTAAATTAAAAAAGACAACTATAA 45
AATCTCTCTTATTTTAAAAAAGCAGTATACCCTTCAATCTATCCA 90
CAATCCAATAAACTTTTCTAATAAAAAATCCTCCACTAAAATGGCA 135
                                     M A 2
TCCTTTTTTTTATTCTTATTATTCTGTAGTCTTTTGATTCTA 180
S F F Y F L F I S V S L L I L 17
GCTAATGCTGATGATGCTGGTAGATATCCTTCAGGAGATGATTAA 225
A N A D D A G R Y P S G D D L 32
GTTGAAGGTACTACTGCTGCTCGGCTTCATTCGTCTCTGACCTA 270
V E G T T A A R L H S S S D L 47
CCAGACGATGATGAAGAAGATGCGAGTGCAGAGATGACGACGAG 315
P D D D E E E C E C E D D D E 62
ACAACATCGCAACTCACATTTCTACACGACGATGGTTACCCCT 360
T T V A T H I S T R S N G Y P 77
TCTAATAACGGAGCCCCCACTAGCACTAAACGCCCTTCAAACAAC 405
S N N G A P T S T K R P S N N 92
                                     .
GGAAGCTCAAACAATGGAGGCTCAAGCTCTGTACAGGATCTGTT 450
G S S N N G G S S S V T G S V 107
. . .
ATATTGAGAGATAAATGGGTAAATGGCGCAATTGTATTTTAGCT 495
I L R D K W V N G A N C I L A 122
TTCAGAAGATAATGGAACGCTAGAGCATGTGGTGTCAAGTTCGAG 540
F K N N G N A R A C G V K F E 137
CTGACTCTCGGTGATAATCAAAGAATCCAAGCATTTGGAACGTT 585
L T L G D N O R I O S I W N V 152
GAGAAAGTCGGAGATAAAGTTTACAGAATTCCGGACTACATCCAA 630
E K V G D K V Y R I P D Y I Q 167
CTTGGTCCAGGAGTCGAAAACAGAGATATTGGAGTTGTTTACAAT 675
L G P G V E N R D I G V V Y N 182
GATGTGCCAGAACTCTTCCACAATCAAGGTCCTGGACAAGAAGAA 720
D V P E L F H N O G L G O E E 197
GGATGCAACATTATTGAATAAAAAATATGGATATAAAAAATATTTA 765
G C N I I E * 203
AAAAAGATTAAATAAGTATTATTAAAGCTTGTGAATATAAACTTT 810
TTCGAAAATTAAAAATAATGGCAGAAAAAAGCGCGCGC 855
TTCAACATGCTCTAAAGGGCCAATTCCCTATATTGAATCTTTTA 900
CAATTCCTGGGCGTCTTTTACACCTCCTGAATGGGAAAAACCGGC 945
GTTCCCACTTATCCCTGCACCCATTCCCTTCCCCCTGGGTTA 990
TCC 993

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Fig. 1. cDNA and predicted amino acid sequence of *Mi-cbp-1* from *Meloidogyne incognita*. Putative secretion signal peptide indicated in bold. Linker separating N-terminal sequence from cellulose-binding domain is double underlined. Putative N-glycosylation site indicated by solid circles. Cellulose-binding domain sequence marked by a single underline. TAA translation termination signal marked with an asterisk. Nucleotide sequence has been submitted to GenBank data base as accession number AF049139.

without a CBD was used as a control. MI-AMP-1 did not bind to the cellulose resin and subsequently was not detected in the polyacrylamide gel. Tobacco BY-2 cells were used to assess the ability of the recombinant MI-CBP-1 to bind to plant cell walls in an indirect immunofluorescence assay with the MI-CBP-1 polyclonal antiserum. These assays showed that the recombinant MI-CBP-1 strongly bound to walls of tobacco cells (Fig. 4B). A polyclonal antibody to MI-AMP-1 and the secondary antibody alone did not bind to the cell walls (data not shown).

To exclude the possibility that the MI-CBP-1 polyclonal antiserum recognized other CBD-containing proteins in the immunoassays, the 228-bp fragment containing the CBD sequence of *Mi-cbp-1* was subcloned and overexpressed to obtain recombinant CBD. The 17-kDa recombinant CBD and the recombinant MI-CBP-1 were detected in immunoblots with the MI-CBP-1 polyclonal antiserum (Fig. 7). In immunoblots, the MI-CBP-1 polyclonal antiserum recognized a diffuse band (approximately 43 kDa) in extracts of *M. incognita* J2 that had a higher M_r than the recombinant MI-CBP-1, possibly because of glycosylation (Fig. 7). CBD-specific antibodies in the MI-CBP-1 polyclonal antiserum were removed by absorption to recombinant CBD. In all immunoassays when the CBD-absorbed MI-CBP-1 polyclonal antiserum was substituted for the original MI-CBP-1 polyclonal antiserum, the results were identical to those derived from use of the nonabsorbed MI-CBP-1 polyclonal antibodies (data not shown).

β -1,4-glucanase assay.

A β -1,4-glucanase assay with agarose plates was conducted to test for cellulase activity by the recombinant MI-CBP-1. In this assay, the recombinant MI-CBP-1 did not show any β -1,4-glucanase activity, whereas the positive control, cellulase from *Aspergillus niger*, produced a well-defined zone of activity (Fig. 8).

DISCUSSION

Esophageal gland cell secretory proteins delivered through the root-knot nematode's stylet are thought to have a critical role in pathogenesis (Williamson and Hussey 1996). The *Mi-cbp-1* cDNA cloned from *M. incognita* parasitic J2 encoded a subventral gland cell secretory protein that was present in pre-parasitic and parasitic J2 and was a component of stylet secretions. Synthesis of MI-CBP-1 in the esophageal gland cells was confirmed by immunolocalization of the MI-CBP-1 in subventral gland cells of J2. The presence of an N-terminal signal peptide indicated that MI-CBP-1 was a secretory protein.

Fig. 3. Southern blot hybridization of genomic DNA from five nematode species (*Meloidogyne incognita*, *M. hapla*, *M. arenaria*, *M. javanica*, *Heterodera glycines*, *Caenorhabditis elegans*) and soybean (*Glycines max*). DNA (10 μ g) was digested with *Eco*RI, fractionated in 1% agarose, transferred onto a nylon membrane, and probed with the 32 P-labeled *Mi-cbp-1*.

Fig. 2. Sequence alignment between deduced amino acid sequence of cellulose-binding domain of MI-CBP-1 from *Meloidogyne incognita* with cellulose-binding domains from other organisms. HG-ENG-1, β -1,4-endoglucanase from *Heterodera glycines* (GenBank accession no. AF006052); GR-ENG-1, β -1,4-endoglucanase from *Globodera rostochiensis* (AF004523); MI-CBP-1, cellulose-binding protein from *M. incognita* (AF049139); EGA, endoglucanase A precursor from *Pseudomonas fluorescens* (P10476); EGE, cellulase E1 from *Acidothermus cellulolyticus* (P54583); EGD, endoglucanase D precursor from *Clostridium cellulovorans* (P28623). Residues identical to those of MI-CBP-1 are shaded, residues conserved in all molecules are marked with black shadow and asterisks.

Mi-cbp-1 was *Meloidogyne*-specific and not detected in *H. glycines*, which has a different mechanism of pathogenesis, or *C. elegans*, which feeds on bacteria. Interestingly, *Mi-cbp-1* did not hybridize under high and low stringency with genomic DNA from *M. hapla* in the Southern blot but the polyclonal antisera detected MI-CBP-1 in the subventral gland cells of pre-parasitic J2 of this species. The detection of MI-CBP-1 in the subventral gland cells of pre-parasitic and parasitic J2 and not in adult females in the immunofluorescence assays suggests that MI-CBP-1 may only be involved in the early stages of root infection by the *M. incognita* J2. During development of the parasitic *M. incognita* J2, the size of the subventral gland cells and the number of the secretory granules they contain gradually decrease, while the dorsal gland cell enlarges and forms large quantities of secretory granules to become the principal esophageal gland cell in the female (Hussey and Mims 1990).

A putative role of MI-CBP-1 in pathogenesis was suggested by the detection of the cellulose-binding protein in J2 stylet secretions. The synthesis of β -1,4-endoglucanases in subventral gland cells of cyst nematodes and their secretion through the nematode's stylet have established the importance of subventral gland cell secretions in pathogenesis (Smant et al.

1998). The high similarity of the C-terminal sequence of MI-CBP-1 to CBDs suggested that the protein might bind to cellulose, which was confirmed in cellulose-binding assays as well as binding to tobacco cell walls. The binding to cellulose suggests that the MI-CBP-1 might be involved indirectly in cellulose degradation or might be used as an anchor to support other (as yet unknown) function(s).

Cellulose is one of the major components of plant cell walls. Many plant pathogens use a complex of cellulases and other hydrolytic enzymes during pathogenesis. That endoparasitic nematodes might use a similar strategy during penetration and migration in roots is supported by the cloning of β -1,4-endoglucanase cDNAs from cyst nematodes (Smant et al. 1998) and from root-knot nematodes (Marie-Noell Rosso, *personal communication*). MI-CBP-1 might be a member of the complex cellulase system (Gilkes et al. 1991) and assist other CBD-lacking cellulases to bind to cellulose since neither cellulase activity nor a catalytic domain were detected in the sequence for MI-CBP-1. Nevertheless, the CBD of MI-CBP-1 only has 43.5% identity with CBDs of cellulases from root-knot nematodes (Marie-Noell Rosso, *personal communication*) even though they have the same common structural features conserved in all cellulase CBDs from different bacteria. Although CBD has a critical role in hydrolyzing insoluble cellulose, some cellulases entirely lack this domain (Gilkes et

Fig. 4. Indirect immunofluorescence microscopy with polyclonal antiserum to MI-CBP-1. **A**, Labeling of subventral esophageal gland cells (SG) of pre-parasitic, second-stage juveniles of *Meloidogyne incognita* with MI-CBP-1 polyclonal antiserum. M = metacarpus. **B**, Labeling of recombinant MI-CBP-1 bound to walls of tobacco cells. Intense labeling occurs at crosswalls (arrow).

Fig. 5. Enzyme-linked immunosorbent assay of concentrated stylet secretions (SS) and total extracts (J2) of *Meloidogyne incognita* second-stage juveniles and recombinant MI-CBP-1 with polyclonal antiserum raised against recombinant MI-CBP-1. Control coated only with bovine serum albumin.

Fig. 6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of recombinant MI-CBP-1 eluted from a CBIND column. Recombinant MI-AMP-1 was used as a negative control. MM = molecular mass markers.

al. 1991). In fact, one of the two β -1,4-endoglucanase cDNAs cloned from each of two species of cyst nematodes lacked a CBD (Smant et al. 1998). Furthermore, although some catalytic domains still possess cellulase activity when they are separated from the CBD by proteolytic cleavage, the ability to hydrolyze insoluble cellulose is dramatically reduced in comparison with the intact enzyme (Gilkes et al. 1988).

Alternatively, MI-CBP-1 might have other functions mediated by an interaction of the N-terminal sequence (unknown function) and the CBD. Possibly, MI-CBP-1 might directly or indirectly have a role in early giant-cell induction by its CBD serving as an anchor domain to assist the N-terminal domain to function. An interaction of the N-terminal sequence and the

CBD is made possible by the Q-linker, which also occurs in cellulases, xylanases, and bacterial regulatory and sensory proteins (Gilkes et al. 1991). Furthermore, to assure connection between these two domains, N-glycosylation of the linker could protect MI-CBP-1 from proteolytic cleavage, as reported for bacterial cellulase (Langsford et al. 1987). Interestingly, some polysaccharide-binding proteins, e.g., the pilus-associated adhesion protein and the carbohydrate recognition domain of animal lectin, have the same conserved glycine residues as MI-CBP-1 (Gilkes et al. 1991). In addition, CBDs have been isolated from proteins that have no apparent hydrolytic activity (Goldstein et al. 1993; Morag et al. 1995) and a recombinant CBD enhanced elongation of different plant cells in vitro (Shpigel et al., *in press*). Giant-cell formation involves extensive cell elongation, although this is thought to occur from the general elongation of cells behind the root tip (Jones and Payne 1978). Further studies are needed to determine the actual function of the root-knot nematode secretory MI-CBP-1 in pathogenesis.

MATERIALS AND METHODS

Nematode cultures and collection of stylet secretions.

Meloidogyne incognita was cultured and collected from greenhouse-grown tomato plants (*Lycopersicon esculentum* cv. Rutgers) as described previously (Hussey and Barker 1973). *Heterodera glycines* was grown on greenhouse-grown soybean (*Glycine max*). Pre-parasitic J2 of both genera were collected by hatching eggs on sieves with 25- μ m openings suspended over deionized water in plastic bowls. Parasitic J2 and adult females of *M. incognita* were dissected from infected tomato roots. Mixed stages of *C. elegans* were maintained as monoxenic cultures on *Escherichia coli* (OP50) and collected by the method of Brenner (1974).

Stylet secretions from *M. incognita* J2 were produced and collected in vitro as described by Davis et al. (1994). Pre-parasitic J2 were incubated in 0.4% resorcinol (Sigma, St. Louis, MO) for 4 h at room temperature in a humid chamber. Stylet secretions were solubilized by adding an equal volume of 20 mM Tris buffer at pH 12 or pH 8.5. Solubilized stylet secretions were concentrated with 10,000 MW cutoff Centricons (Amicon, Beverly, MA), resuspended in 300 μ l of Tris buffer, transferred to 0.5- μ l microcentrifuge tubes, and stored at -80°C until needed.

Collection of infected root tissue.

Soybean seeds were placed on water-saturated germination paper and germinated in the dark for 3 days at room temperature. The seedlings were placed on moist sand and the root tips were inoculated with *M. incognita* J2 and covered with sand. The inoculated soybeans were incubated in the dark at room temperature for 24 h to allow the J2 to invade the roots. The seedlings were then washed with sterile water, placed on wet germination paper, and incubated in the dark for another 24 h at room temperature. Infected root tips were excised and stored in centrifuge tubes at -80°C for further use.

Nucleic acid isolation.

Genomic DNA was isolated from 1-month-old soybean leaves by a procedure described by Rogers and Bendich (1985). Genomic DNA was isolated from *M. incognita* J2,

Fig. 7. Immunoblot analysis of extracts of *Meloidogyne incognita* second-stage juveniles (J2), recombinant MI-CBP-1, and recombinant cellulose-binding domain (CBD) derived from *Mi-cbp-1* with polyclonal antiserum raised against recombinant MI-CBP-1. MM = molecular mass markers.

Fig. 8. Agarose plate assay for cellulase activity with carboxymethyl-cellulose (CMC) as substrate. **A**, Cellulase from *Aspergillus niger*. **B**, Extraction buffer alone. **C**, Recombinant MI-CBP-1 from *Meloidogyne incognita*. Gel containing CMC was stained with Congo red to detect activity indicated by the clear zone.

H. glycines J2, and *C. elegans* mixed stages as described by Ray et al. (1994). Total RNA was isolated from soybean roots and *M. incognita* J2 with TRIzol reagent (Life Technologies, Grand Island, NY). Poly(A)⁺ RNA (mRNA) was isolated from *M. incognita* pre-parasitic and parasitic J2 and adult females with Dynabeads Oligo(dT)²⁵ magnetic beads (DynaL, Lake Success, NY) according to the manufacturer's instruction.

RNA fingerprinting and nucleotide sequencing.

RNA fingerprinting (McClelland et al. 1993) was performed with the Delta RNA Fingerprinting Kit (Clontech Laboratories, Palo Alto, CA) following the manufacturer's instruction with minor modifications. Briefly, 2 µg of total RNA isolated from pre-parasitic *M. incognita* J2 (PPJ2), parasitic *M. incognita* J2 (PJ2), and *M. incognita* J2-infected soybean root tip tissues (R) was reverse-transcribed into cDNAs. Polymerase chain reaction (PCR) fingerprinting reactions were accomplished with the synthesized cDNAs as templates, kit-provided T/P primers as 3' and 5' primers, and [α^{32} P]dATP as an isotope. Each PCR was performed twice to eliminate false positives. At the same time, negative controls were performed with the same PCR conditions as the above reactions, with water and total RNAs from PPJ2, PJ2, and R as templates. All the PCR products were fractionated on 5% polyacrylamide/8 M urea gels. DNA fragments that were specific or more strongly expressed for PJ2 were excised from the gel, re-amplified by PCR with the same primers, and cloned into pGEM-T vector (Promega, Madison, WI) for further sequencing, and Southern blot analyses.

DNA sequencing was done by the University of Georgia Molecular Genetics Instrumentation Facility by the fluorescent dye terminator method with *Taq* DNA polymerase.

cDNA library construction and screening.

Poly(A)⁺ RNAs (5 µg) isolated from *M. incognita* J2 were used for cDNA library construction (Sambrook et al. 1989). First-strand DNA was synthesized with oligo-dT as a primer. Following second-strand synthesis, the cDNAs were inserted into *Bst*XI/*Not*I sites of pcDNA II (Invitrogen, Carlsbad, CA). The cDNA library contained 4.2×10^6 primary recombinants. cDNA library screening (Sambrook et al. 1989) was performed with the ³²P-labeled (Feinberg and Vogelstein 1987), fingerprinting-derived F43 fragment as a probe.

Southern blot hybridization.

Southern blot hybridizations were done as previously described by Ray et al. (1994) with minor modifications. Genomic DNA (10 µg) from *M. incognita*, *M. hapla*, *M. arenaria*, *M. javanica*, *H. glycines*, *C. elegans*, and soybean (*Glycines max*) was digested with *Eco*RI, fractionated in a 1% agarose gel, and transferred onto a Nytran filter (Schleicher & Schuell, Keene, NH). High-stringency hybridization was performed at 62°C in hybridization buffer (7% SDS, 1% bovine serum albumin [BSA], 1 mM Na₂EDTA, 0.5 M Na₂HPO₄) overnight, and washed with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS at 62°C. Low stringency hybridization involved hybridization overnight at 55°C in the hybridization buffer and washing with 0.5× SSC, 0.1% SDS at 50°C. ³²P-labeled *Mi-cbp-1* was used as the probe.

Expression and purification of recombinant MI-CBP-1, MI-AMP-1, and CBD.

A 549-bp fragment derived from the ORF of the *Mi-cbp-1* was amplified by PCR (Sambrook et al. 1989) with the synthesized primers (5'-CGGGATCCGATGATGCTGGTAGATATC-3', 5'-GCGTCGACATATCCATATTTTTTATTCAAT-3') and *Mi-cbp-1* sequence as a template. A 683-bp fragment derived from the ORF of *Mi-amp-1* (GenBank accession no. AF013289) from *M. incognita* was amplified by PCR with the synthesized primers 5'-CGGGATCCTCTTTGACTGTTCC TGAACAA-3' and 5'-GCGTCGACCAGCCCTCAAATAAA TTT-3' and *Mi-amp-1* sequence as a template. Highest expression in *E. coli* was obtained when the leader sequence was deleted from each ORF (data not shown). The 228-bp fragment containing the CBD sequence from the *Mi-cbp-1* clone was amplified by PCR with the synthesized primers (5'-CGGGATCCATATTGAGAGATAAATGGGTAAATGGC-3', 5'-GCGTCGACATATCCATATTTTTTATTCAAT-3') and *Mi-cbp-1* sequence of the *Mi-cbp-1* clone as a template. The amplified fragments were inserted into *Bam*HI/*Sal*I sites of expression vector pET 28A (Novagen, Madison, WI). The recombinant MI-CBP-1 or MI-AMP-1 were purified with Ni-NTA resin (Qiagen, Santa Clarita, CA) following the manufacturer's instruction.

Antiserum production.

Polyclonal antibodies to MI-CBP-1 were produced by injecting a New Zealand rabbit intradermally twice with purified recombinant MI-CBP-1. Purity was confirmed by SDS-PAGE. Complete Freund's adjuvant was used for the initial injection and incomplete Freund's adjuvant for the 4-week boost injection.

Protein extraction and immunoblotting.

Proteins were extracted by grinding *M. incognita* pre-parasitic J2 in 200 µl of extraction buffer (100 mM Tris, pH 7.0, 150 mM NaCl) in microcentrifuge tubes in liquid nitrogen. The homogenates were centrifuged at $20,000 \times g$ for 10 min. Protein concentration was estimated with a protein quantitation kit (Sigma Chemical, St. Louis, MO) with BSA as a standard. Proteins (200 µg) were separated in SDS-PAGE and transferred to nitrocellulose-1 membranes (Life Technologies, Grand Island, NY) (Winston et al. 1987). The membrane was incubated in a blocking solution (1% nonfat milk, 1× Tris-borate-sulfate [TBS: 10 mM Tris, pH 7.2, 0.9% NaCl]) for 3 h at 37°C and then treated with the MI-CBP-1 polyclonal antibodies, followed by anti-rabbit IgG (whole molecule) alkaline phosphatase conjugate (Sigma). The membrane was washed three times in 1× TBS buffer at room temperature and incubated in the substrate solution (44 µl of nitroblue tetrazolium [NBT] solution and 33 µl of 5-bromo-4-chloro-3-indolyl-phosphate toluidinium [BCIP] solution [5 Prime → 3 Prime, Boulder, CO] in 10 ml of AP buffer [100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM MgCl]).

MI-CBP-1 N-terminal-specific antibody production.

Nitrocellulose membranes were first soaked in purified recombinant CBD dissolved in 1× TBS buffer for 2 h at room temperature, washed three times in 1× TBS buffer at room temperature, incubated in a blocking solution (1% nonfat milk, 1× TBS) for 3 h at 37°C, and then treated with the MI-

CBP-1 polyclonal antibodies. This process was repeated until the CBD-specific antibodies were completely removed from the MI-CBP-1 polyclonal antiserum as determined by immunoblot analysis.

β -1,4-glucanase assay.

A β -1,4-glucanase assay was performed as previously described by Mateos et al. (1992). Purified, recombinant MI-CBP-1 (1 μ g), cellulase from *Penicillium funiculosum* (Sigma #C-0901, 0.08 units) as a positive control, and protein extraction buffer as a negative control were loaded into small holes in CMC agarose plates (0.5% agarose, 0.2% carboxymethyl cellulose [Sigma], in phosphate citric acid buffer [0.05 M K_2HPO_4 , brought to pH 5.2 with 1 M citric acid]) and incubated at 30°C overnight. The agarose was stained with 0.1% Congo red in deionized water (Fisher Scientific, Pittsburgh, PA) at 30°C for 30 min and washed with 1 M NaCl solution.

Immunofluorescence assay.

The polyclonal antiserum (absorbed and nonabsorbed) was used to localize MI-CBP-1 in sections of pre-parasitic J2, parasitic J2, and adult females *M. incognita*, pre-parasitic J2 of *M. arenaria*, *M. javanica*, *M. hapla*, and *H. glycines*, and mixed stages of *C. elegans* with indirect immunofluorescence as described previously by Davis et al. (1994). Following fixation in freshly prepared 4% paraformaldehyde in M9 buffer (22 mM KH_2PO_4 , 42.3 mM Na_2HPO_4 , 85.6 mM NaCl, 1 mM $MgSO_4$, pH 7.4) for 4 h at room temperature, the nematodes were washed three times in M9 buffer and once in deionized water. The fixed nematodes were cut into sections and incubated in 2 mg of proteinase K (Sigma) per ml in enzyme buffer (100 mM Tris-HCl, 1 mM $CaCl_2$, 0.1% Triton X-100, pH 7.4) at room temperature for 20 min (J2 and *C. elegans*) or 40 min (adult females). The enzyme solution was removed and sections were placed in a -80°C freezer for 20 min, incubated in ice-cold methanol for 30 s, then incubated in ice-cold acetone for 90 s. The sections were quickly brought to near dryness with a stream of air and rehydrated by slowly adding 500 μ l of deionized water. The water was removed and sections were resuspended in blocking solution (10% goat serum, 0.02% NaN_3 , 1 mM phenylmethylsulfonyl fluoride, phosphate buffered saline [PBS]), incubated for 3 h and then used immediately for immunofluorescence or stored at -80°C until needed.

Plant cell binding assay.

Purified recombinant MI-CBP-1 solution (100 μ g of MI-CBP-1, 30% glycerol, 1 $\times$ TBS), recombinant MI-AMP-1 (100 μ g of MI-AMP-1, 30% glycerol, 1 $\times$ TBS), secondary antibody alone or buffer only as a negative control (30% glycerol, 1 $\times$ TBS) were added to 200-ml culture flasks containing 50 ml of fresh tobacco (*Nicotiana tabacum* var. BY2) cell suspension culture (4.3 g of Murashige & Skoog salt mixture, 30 g of sucrose, 0.1 mg of myoinositol, 0.1 mg of thiamin HCl, and 0.1 mg of 2,4-D (2,4-dichlorophenoxyacetic acid) per liter, 1,000 cells per ml) (Nagata et al. 1992). The culture was kept in the dark and incubated at 28°C for 3 days in a shaker running at 200 rpm (Gyrotory Shaker, model G2; New Brunswick Scientific, Edison, NJ). The cells were fixed and labeled according to the procedure described above for the immunofluorescence

assay, but the post-fixation steps of proteinase K, acetone, and methanol treatments were omitted.

Cellulose-binding assay.

Binding of the recombinant MI-CBP-1 to cellulose was performed with CBIND 100 resin (Novagen, Madison, WI) according to manufacturer's instruction. Twenty microliters of purified recombinant MI-CBP-1 solution (20 μ g of MI-CBP-1, 20 mM Tris-HCl, pH 7.5) was mixed with 2 mg of CBIND resin. The protein was eluted with water and then separated on SDS-PAGE. Under the same conditions, purified recombinant MI-AMP1 was used as a negative control.

ELISA.

ELISA was performed according to Pratt et al. (1986). Briefly, Immulon (Dynatech, Fisher) plate wells were coated overnight at 4°C with 10 μ g of total protein diluted in borate saline (0.2 M sodium borate, 75 mM NaCl, pH 8.5) from the following sources: extracts of *M. incognita* pre-parasitic J2 and solubilized stylet secretions of *M. incognita* J2 or BSA (Sigma) as a negative control. As a positive control, wells were coated with 10 μ g of recombinant MI-CBP-1. Wells were rinsed three times with wash buffer (10 mM Tris, 0.5 M NaCl, pH 8.0) and blocked with 1% BSA in PBS (32.9 mM Na_2HPO_4 , 1.77 mM NaH_2PO_4 , 0.14 M NaCl, pH 7.4) for 30 min at room temperature. After being rinsed once with wash buffer, each coated well was incubated with MI-CBP-1 polyclonal antisera diluted 1:1,000 with 0.5% BSA in PBS for 1 h at room temperature. Negative controls included omitting incubation with the primary polyclonal antibody. The wells were washed three times, incubated with alkaline phosphatase-conjugated goat anti-rabbit antibody (Sigma) at 1:5,000 dilution for 1 h at room temperature, and washed three times before phosphate colorimetric substrate was added. The substrate, p-nitrophenyl phosphate (Sigma 104), was prepared according to manufacturer's directions in alkaline phosphatase buffer (1 M diethanolamine, 0.5 mM $MgCl_2$, pH 9.8) and incubated in treated wells 30 min at room temperature before the reaction was stopped with 3 N NaOH. Absorbance was measured at 405 nm on an ELISA reader (Titertek Multiskan MCC/340, EF Labs, Finland).

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