

Research note

Molecular cloning and characterisation of a venom allergen AG5-like cDNA from *Meloidogyne incognita*[☆]

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Abstract

RNA fingerprinting was used to identify RNAs that were expressed in parasitic second-stage juveniles of *Meloidogyne incognita*, but absent from or reduced in preparasitic second-stage juveniles. A cDNA encoding a putative secretory protein was cloned from a *M. incognita* second-stage juvenile cDNA library by probing with a 0.5 kb fragment derived from fingerprinting that was more strongly expressed in parasitic second-stage juveniles. The cDNA, named *Mi-msp-1*, contained an open reading frame encoding 231 amino acids, with the first 21 amino acids being a putative secretory signal. In Southern blot analysis the *Mi-msp-1* hybridised with genomic DNA from *M. incognita*, *Meloidogyne arenaria*, *Meloidogyne javanica*, but not *Meloidogyne hapla*, *Heterodera glycines* or *Caenorhabditis elegans*. In Northern blot analysis a 1 kb transcript was detected in both preparasitic and parasitic second-stage juveniles, but not in adult females of *M. incognita*. Comparing the predicted amino acid sequence with protein databases revealed significant similarity to the venom allergen antigen 5 family of proteins in hymenoptera insects and homologues found in several other nematode species. © 2000 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

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Nematodes are important parasites of plants and animals, and cause widespread crop losses and diseases of animals and humans worldwide. Central to our understanding of disease development in either plants or animals is the investigation of secretions released into tissues of the hosts by the nematodes.

Of the plant-parasitic nematodes, the sedentary endoparasitic root-knot nematode (*Meloidogyne* spp.) is a major contributor to annual losses (US \$77 billion) to world crops [1]. This biotrophic parasite has evolved a very specialised and complex feeding relationship with susceptible host plants. Although parasitism by root-knot nematodes has been extensively studied,

knowledge about the mechanism of parasitism is still fragmentary, especially at the molecular level [2].

Identifying secretory protein genes that are specific for, or more strongly expressed in, root-knot nematode parasitic stages is critical for understanding the molecular basis of parasitism. In this paper, we present results of cloning a cDNA encoding a venom allergen-like secretory protein from root-knot nematode based on RNA fingerprinting.

Adult females, parasitic second-stage juveniles (J2), and preparasitic J2 of *Meloidogyne* species and *Heterodera glycines* were cultured and isolated as described previously [3]. Propagation of *Caenorhabditis elegans*, strain N2, was based on the method of Brenner [4].

Soybean (*Glycine max*) seeds were germinated in the dark for 3 days at room temperature on water-saturated germination paper. The seedlings were placed on moist sand and the root tips were inoculated with freshly hatched *Meloidogyne incognita* J2 and covered

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with sand. The inoculated seedlings were incubated in the dark at room temperature for 24 h. 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. Root tips infected with parasitic J2 were excised and stored in centrifuge tubes at -80°C for further use.

Genomic DNA was isolated from 1-month-old soybean leaves [5] and from J2 of *M. incognita*, *Meloidogyne hapla*, *Meloidogyne arenaria*, *Meloidogyne javanica*, and *H. glycines* and mix stages of *C. elegans* as previously described [3]. Total RNA was isolated

from soybean roots, nematode-infected soybean roots, and *M. incognita* J2 using TRIzol reagent (Life Technologies). For Northern blots, poly(A)⁺ RNA (mRNA) was isolated from preparasitic and parasitic *M. incognita* J2 by using Dynabeads Oligo(dT)₂₅ magnetic beads (Dyna) according to the manufacturer's instruction.

RNA fingerprinting [6] was performed using the Delta RNA Fingerprinting Kit (Clontech Laboratories) following the manufacturer's instructions with minor modifications. Briefly, total RNA (2 μg) isolated from *M. incognita* preparasitic J2 and parasitic

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AATCAATAAAAGTTTTTTCTTTTAAAAATAATTAAATAATTAGT 45
AAAAATGTCAAATAAAATTAATTATTTCTATTTTAATATTGACAATT 90
  M S N K L I I S I L I L T I 14
ATTTATACTGTTGTAAATTCCTTGACTGTTCCCTGAACAAAATGCC 135
I Y T V V N S L T V P E Q N A 29
GTCGTTGATTGTATCAATAAAATACCGTTCTCAACTTGCCAATGGA 180
V V D C I N K Y R S Q L A N G 44
AAAAC TAAAAATAAAATGGGGGGAATTTCCCTCTGGAAAGGAT 225
K T K N K N G G N F P S G K D 59
ATTTTGGAAGTTTCTTATAGTAAAGACTTAGAGAAATCTGCTCAA 270
I L E V S Y S K D L E K S A Q 74
AGATGGGCTAATAAAATGCATATTTGATCATAATGGAACAGATTTA 315
R W A N ' K C I F D H N G T D L 89
TATTCTGGAGGAAAATTTTATGGAGAAAATCTTTATTTGGATGGA 360
Y S G G K F Y G E N L Y L D G 104
GACTTTGAGCATAAAAAACATAACCCAATTGATGATTGATGCCTGT 405
D F E H K N I T Q L M I D A C 119
AATGCATGGTGGGGAGAAAGTACTACAGATGGAGTGCCCCCAAGT 450
N A W W G E S T T D G V P P S 134
TGGATAAATAACTTTTTGCCTACTGATAATAAGGAGAATGATGAA 495
W I N N F L P T D N K E N D E 149
AAATTCGAAGCGGTTGGACACTGGACACAAATGGCGTGGGCTAAA 540
K F E A V G H W T Q M A W A K 164
ACTTACCAAATTGGTTGTGCGCTTAAAGTTTGCCACAAACCAGAC 585
T Y Q I G C A L K V C H K P D 179
TGTAACGGGAATTTGATTGATTGTCGTTATTATCCAGGTGGAAT 630
C N G N L I D C R Y Y P G G N 194
GGTATGGGTTCCGCCGATTTATCAACAAGGAAAACCAGCTTCAGGA 675
G M G S P I Y Q Q G K P A S G 209
TGTGGAAGCTGGACCTAGTACGAAATATAGCGGACTATGCAAG 720
C G K A G P S T K Y S G L C K 224
CCTGATCCACACCAAAATAATTAATAAATTATTTGAGGGCTGAAA 765
P D P H Q N N * 231
TATAACTGAAATATGCAAAATTAATTAAGTAATTGAAAATTTGA 810
AAAATATATTTAATTGAATTAACCTTTAAATTTGTAAATTTAAAT 853
GAAATTAGAATAAACAATATTATGGGAAAAAAAAAAAAAAAAAGCG 898
G 899

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Fig. 1. Nucleotide and predicted amino acid sequence of the *M. incognita* *Mi-msp-1* cDNA clone. A putative signal peptide is indicated in bold. The TAA translation termination signal is marked with an asterisk. The nucleotide sequence has been submitted to GenBank database as accession number AF013289.

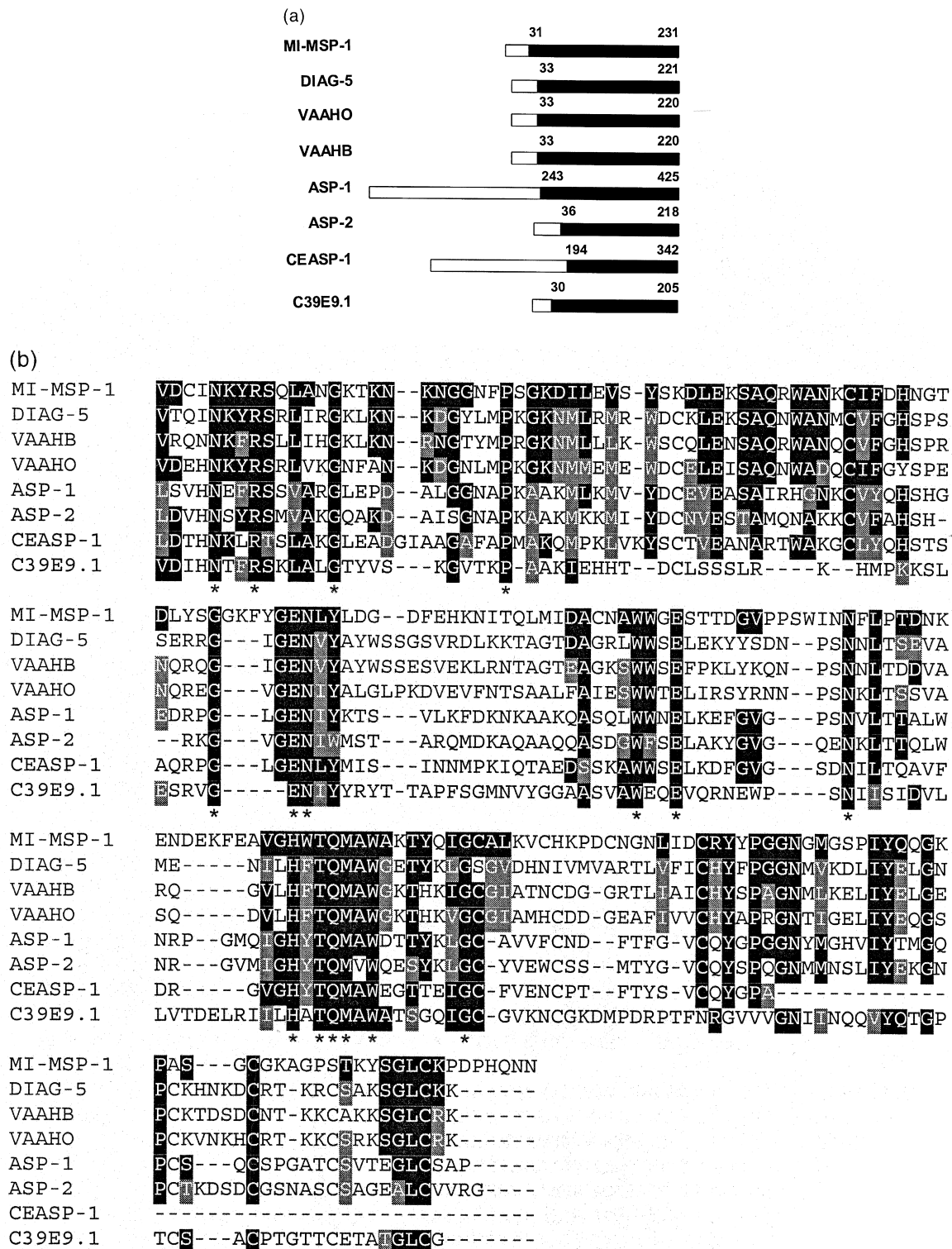


Fig. 2. Sequence alignment among predicted amino acid sequence of MI-MSP-1 from *M. incognita* with proteins from other nematodes. (A) Schematic of the alignment of the entire molecules. (B) Alignment of the amino acid residues between the numbers in (A) MI-MSP-1, from *M. incognita* (GenBank accession No. AF013289); DIAG-5, venom allergen antigen 5-like protein from *Dirofilaria immitis* (AF001100); VAAHO, vespid allergen antigen homologue from *Onchocerca volvulus* (AF042087); VAAHB, vespid allergen antigen homologue from *Brugia malayi* (AF042088); ASP-1, *Ancylostoma*-secreted protein precursor from *Ancylostoma caninum* (Q16937); ASP-2, secreted protein ASP-2 precursor from *A. caninum* (AF089728); CEASP-1, a protein from *Caenorhabditis elegans* similar to *Ancylostoma*-secreted protein (U42839); C39E9.1, protein from *C. elegans* (Z70307). Residues identical to those of MI-MSP-1 and those that are similar are shaded. Residues conserved in all molecules are marked with a black shadow and asterisk.

J2 isolated from soybean roots or parasitic J2-infected soybean root tips was reverse-transcribed into cDNAs [7]. Polymerase chain fingerprinting reactions were accomplished by using the synthesised cDNAs as templates, kit-provided T/P primers and [^{32} P]dATP as isotope. Each PCR reaction was repeated twice to eliminate false positives. At the same time, negative controls were processed under the same PCR conditions using water and total RNAs of the J2s as templates. All the PCR products were fractionated in 5% polyacrylamide/8 M urea gel. Amplified cDNA fragments that were specific or more strongly expressed for parasitic J2 were excised from the gel, re-amplified by PCR, and cloned into pGEM-T vector (Promega) for sequencing and Southern and Northern blot analyses. Automatic DNA sequencing was done at the University of Georgia Molecular Genetics Instrumentation Facility using the fluorescent dye terminator method and *Taq* DNA polymerase. One fingerprinting-derived fragment, F29, more strongly expressed in parasitic J2 was ^{32} P-labelled [8] and used as a probe to screen a *M. incognita* J2 cDNA library [7,9]. A cDNA, named *Mi-msp-1*, was isolated from the library.

Northern and Southern blot hybridisations were done as previously described [3], with minor modifications. In Northern blots, Poly(A)⁺ RNA (6 μ g isolated from pre-J2, adult female and soybean, and 1.5 μ g from par-J2) was fractionated in formaldehyde-denatured 1.2% agarose gel, and transferred to Nytran filters. In Southern blots, genomic DNA (10 μ g) isolated from preparasitic J2 of *M. incognita*, *M. arenaria*, *M. javanica*, *M. hapla*, and *H. glycines*, and mixed stages of *C. elegans*, and soybean (*G. max*) was digested with *Eco*R1, fractionated on a 1% agarose gel, and transferred onto Nytran filters. High-stringency hybridisation for both Southern and Northern blots was performed at 62°C in hybridisation buffer (7% SDS, 1% BSA, 1 mM Na₂EDTA, 0.5 M Na₂HPO₄) overnight and washed with 0.1×SSC, 0.1% SDS at 62°C. Low-stringency hybridisation was done overnight at 55°C in the hybridisation buffer and washing with 0.5×SSC, 0.1% SDS at 50°C. ^{32} P-Labelled *Mi-msp-1* was used as the probe.

Fifty-four cDNA fragments that were specific or more strongly expressed for parasitic J2 were cloned and sequenced from 65 different primer recombinations used in the PCR fingerprinting reactions. The predicted protein sequence of one 450 bp fragment, F29, more strongly expressed in the PJ2, was similar to a venom allergen AG5-like protein based on BLAST search [10] against GenBank/EMBL database. Five positive clones were identified when the *M. incognita* J2 cDNA library was screened with the F29 fragment. Inserts, 0.7–0.9 kb, were obtained from the plasmids isolated from these clones by digestion with

*Xho*I/*Bst*EXII. The clone with the longest cDNA insert was selected for further characterisation and named *Mi-msp-1*, for *Meloidogyne* secretory protein.

Mi-msp-1 contained 896 nucleotides with a putative open reading frame (ORF) of 693 bp (Fig. 1). The cDNA sequence analysis based on GCG program (Genetics Computer Group, Madison, Wisconsin) revealed that the ORF encoded a putative protein of 231 amino acid with *M_r* of 25.55 kDa. A putative signal sequence [11] terminated at aa residue 21 immediately upstream of a protease cleavage site (Fig. 1).

Computer-assisted protein sequence similarity search [10,12] revealed that the predicted MI-MSP-1 aa sequence had high similarity to the allergen antigen 5 (AG-5) family of extracellular proteins from hymenopteran venom [13,14]. Interestingly, members of the AG-5 family of proteins are found in several animal parasitic nematodes, *Ancylostoma caninum*, *Dirofilaria immitis*, *Onchocerca volvulus*, and *Brugia malayi* and the free-living nematode, *C. elegans* (Fig. 2A). Local alignments revealed that MI-MSP-1 shared the same conserved sequences with other members of this family (Fig. 2B). The predicted MI-MSP-1 protein sequence had high similarity with ASP-2 and with the carboxy-terminal region of ASP-1, which are the major E/S proteins released by infective L3 of *A. caninum* stimulated to feed in vitro [15,16]. The secretion of ASP-1 and ASP-2 is coupled with the invasion of the host by the larvae and is thought to have a central role in the transition from the external environment to parasitism. A putative role for the E/S AG5-like proteins from the other parasitic nematodes is unknown, although they are also associated with parasitic stages. Microdissection of adults of *Haemonchus contortus* showed that an E/S AG5-like protein may be produced in the oesophagus, suggesting that this protein may be

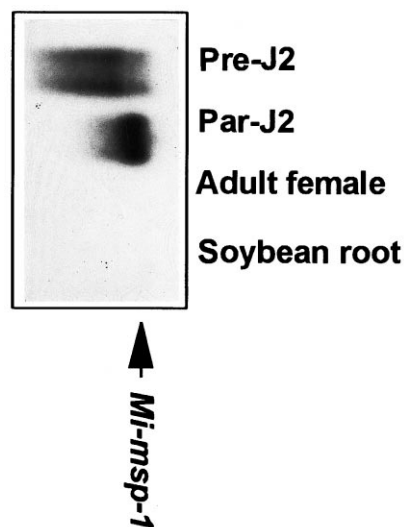


Fig. 3. Northern blot analysis of *Mi-msp-1* transcription.

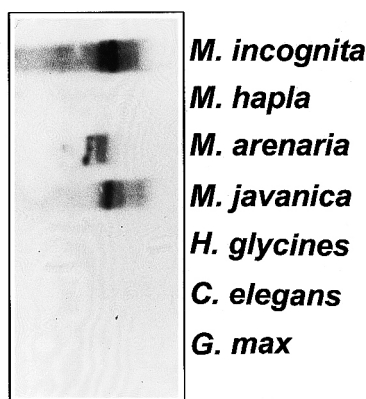


Fig. 4. Southern blot hybridisation of genomic DNA from six nematode species *Meloidogyne incognita*, *Meloidogyne hapla*, *Meloidogyne arenaria*, *Meloidogyne javanica*, *Heterodera glycines*, *Caenorhabditis elegans* and soybean (*Glycine max*).

secreted during feeding [17]. No evidence was obtained for the presence of MI-MSP-1 in E/S products of *M. incognita*.

To determine the *Mi-msp-1* transcript profile in different developmental stages of *M. incognita*, mRNA was prepared from different stages and analysed by Northern blot. Transcripts (1 kb) of *Mi-msp-1* were detected in both preparasitic and parasitic J2 of *M. incognita*, but not in adult females (Fig. 3). *Elongation factor 1- α* (*EF1- α*) *M. incognita* was used as an internal standard to normalise nematode RNA [18]. The expression of *Mi-msp-1* was 10 times greater in parasitic than in preparasitic J2 based on their ratio with the internal standard *EF1- α* (data not shown). *Mi-msp-1* was radiolabelled and used to probe genomic Southern blots containing *Eco*R1-digested DNA from five plant-parasitic nematodes, the free-living nematode *C. elegans*, and soybean. *Mi-msp-1* hybridised with genomic DNA from three of the four *Meloidogyne* species, *M. incognita*, *M. arenaria*, and *M. javanica* (Fig. 4). No hybridisation was observed with genomic DNA from *M. hapla*, *H. glycines*, *C. elegans*, or the host plant, soybean, probably due to divergent sequences not being detected with the hybridisation conditions used or lack of similar sequences. The differences in the *Mi-msp-1* hybridisation for the *Meloidogyne* species are consistent with what is known about the phylogenetic relationships of these species [19].

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