

Cloning, Sequence, and Expression Analysis of a New MnSOD-Encoding Gene from the Root-Knot Nematode *Meloidogyne incognita*

LAURA C. ROSSO

Abstract: A gene encoding a manganese superoxide dismutase (MnSOD) enzyme (*Mi-mnsod*) was identified and characterized in second-stage juveniles of the root-knot nematode *Meloidogyne incognita*. The *Mi-mnsod* gene was found to possess five exons and four introns with (GT/AG) consensus splice-site junctions. The deduced amino acid sequence of *Mi-mnsod* encodes a putative 25 kDa protein, with conserved amino acid residues of the MnSOD family, including the Parker-Blake signature and four metal-binding sites. The derived amino acid sequence showed high similarity to other eukaryotic MnSODs, including a 23 amino acid N-terminal putative mitochondrial transit peptide. Gene expression was observed throughout the posterior nematode body region with elevated signal intensities at the anterior portion of the intestine. DNA blot analysis and sequencing data showed the occurrence of three putative copies of the *MnSOD* gene with nucleotide polymorphisms found at the fourth exon and the 3' un-translated region.

Key words: *Meloidogyne incognita*; root knot nematode; manganese superoxide dismutase; anti-oxidant defense.

Root-knot nematodes (*Meloidogyne spp.*) are root endoparasites of most cultivated crops that cause significant economic losses worldwide (Sasser, 1980). The infective second-stage juveniles (J2) penetrate the roots at the elongation zone and then migrate intercellularly, establishing feeding sites in the developing vascular tissue (Wyss et al., 1992). Although tomato (*Solanum lycopersicon*) is an excellent host for these nematodes, some varieties are resistant to *M. incognita* because of the presence of the dominant gene *Mi*. Resistance conferred by *Mi*-gene is exerted through hypersensitive response (HR) by the cells surrounding the feeding site of the nematode, which ultimately leads to a localized necrosis of the challenged host tissues (Droopkin et al., 1969; Paulson et al., 1972).

Hypersensitive response is triggered by a recognition event and consists of a complex response network which results in a programmed cell death, preventing further nourishment of the parasite and the establishment of a feeding site. One key feature of HR is the generation of reactive oxygen species (ROS), the oxidative burst. There are several proposed functions for ROS in the plant-pathogen interaction, such as signaling for local and systemic defense reactions, antimicrobial activity and cell-wall strengthening (Mehdy, 1994; Baker and Orlandi, 1995).

Toxic oxygen species can damage nucleic acids, proteins, and membrane lipids and therefore can be lethal (Fridovich et al., 1986; Hunter et al., 1997). To protect against these potentially damaging oxygen species, aerobic organisms evolved a multi-layered anti-oxidant defense system in which the anti-oxidant enzymes superoxide dismutase (SOD), glutathione peroxidase, and catalase, as well as non-enzymatic anti-oxidants, play a pivotal role (McCord and Fridovich, 1969; Henkle-Dührsen et al., 1995).

Anti-oxidant enzymes have been postulated to protect parasitic nematodes from host-induced oxidative damage in addition to the cellular scavenging functions of their own metabolism (Callahan et al., 1988). For instance, it has been found that manganese superoxide dismutase (MnSOD) activity was markedly higher in J2 extracts from virulent nematodes, selected from avirulent populations by repeated inoculations on resistant tomato, than in avirulent counterparts. This observation suggested a possible relationship between enhanced MnSOD activity and the ability of nematodes to grow on resistant tomato plants (Molinari et al., 2005).

In eukaryotes, free radicals have directly and indirectly been implicated in the aging process (Hartman et al., 1995) and cellular activities that eliminate free radicals have been associated with longer lifespan. For example, a transgenic strain of *Drosophila melanogaster* over-expressing both SOD and catalase exhibited a life-extension of up to one-third that of the wild-type (Orr and Sohal, 1994). Long-lived genetic variants of *Caenorhabditis elegans* also provided evidence for a positive correlation between SOD cellular levels and extended lifespans (Honda and Honda, 1999).

As originally formulated, the superoxide theory of oxygen toxicity ascribed the damaging effects of high oxygen tension to the formation of the superoxide radical ($O_2^{\cdot-}$), although much of the cellular damage now appears to be the consequence of more reactive species (Halliwell and Gutteridge, 1989). SODs protect cells by catalyzing the dismutation of $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Three major classes of SODs have been described on the basis of the metal composition in the active site, i.e., Fe-Mn, and Cu-Zn SODs. FeSOD is found primarily in prokaryotes, MnSOD is found in both prokaryotes and eukaryotes, while the presence of Cu-Zn isoenzymes is restricted to eukaryotes (Halliwell and Gutteridge, 1989).

An increase in MnSOD activity was observed in virulent compared to avirulent populations of *M. incognita* which led us to investigate the sequence and expression of MnSOD in *M. incognita* (Molinari et al., 2005). Available genomic data for *C. elegans* SOD and partial

Received for publication July 1, 2008.

Istituto per la Protezione delle Piante, CNR, Bari, Italy.

The author thanks S. Molinari and A. Ciancio for helpful comments on the manuscript. D. Catalano is gratefully acknowledged for assistance with bioinformatic analysis. This work was supported in part by Regione Puglia PE040.

E-mail: l.rosso@ba.ipp.cnr.it

This paper was edited by Isgouhi Kaloshian.

SOD EST sequences from *M. incognita* were integrated with data from the *M. incognita* genomic project which revealed the existence of three SOD genes in the *M. incognita* genome strain Morelos, two copies encoding the Cu-Zn enzyme and one copy encoding the Fe-Mn enzyme (Abad et al, 2008). In this study using available genomic data, it was possible to identify and characterize a gene, herein indicated as *Mi-mnsod*, encoding a MnSOD enzyme from J2 of a population of *M. incognita*.

MATERIALS AND METHODS

Nematode population: To obtain *M. incognita* J2, the nematode population (MILEV-L4) originally from Leverano (Italy) was maintained in a greenhouse on susceptible tomato (cv. UC82) under controlled conditions (24-26°C). Galled roots were removed from soil and rinsed, then egg masses were collected with a scalpel. Harvested eggs were incubated at 26°C for 5 days on moist filter papers. The hatched J2, which migrated through the paper, were collected in a water-filled Petri dish. Afterwards, J2 were counted under a stereoscope before collection with a sterile glass pipette tip.

Nucleic acids isolation: For DNA extraction, J2 were placed in a 1.5 ml tube and incubated at -80°C for 10 minutes. Subsequently, 100 µl of extraction buffer and 50 mg of acid-washed glass beads (425-600 µm diameter, Sigma, St. Louis MO) were added and tissues disrupted by vortex for 5 minutes. The lysate was mixed with 50 µl phenol and incubated for 10 minutes at 60°C; then, 50 µl chloroform/isoamyl alcohol (24:1) were added and the suspension mixed by inversion. The aqueous layer was separated by centrifugation for 10 minutes at 11,000 rpm. DNA was precipitated by addition of 4 µl 5M NaCl and 200 µl 100% ethanol at -20°C for one hour. After centrifugation at 12,000 rpm for 10 minutes, the pellet was washed twice with 70% ethanol and dissolved in sterile distilled water (SDW).

Total RNA was extracted from approximately 3000 J2 nematodes. Extraction was carried out by improving the single-step RNA isolation method with a monophasic solution of phenol and guanidine isothiocyanate (TRIzol, Invitrogen Carlsbad, CA USA), according to the manufacturer's instructions. For nematode disruption, 0.1 g of glass beads was added to 250 µl J2 suspension in TRIzol and tubes vortexed for 5 minutes. The RNA pellet was dissolved in 15 µl nuclease-free water and treated with DNase (Roche Applied Science, Indianapolis, IN, USA) to remove possible contaminating genomic DNA. The RNA pellet was dissolved in 10 µl of nuclease-free water and stored at -70°C.

Amplification and sequencing analysis: cDNA synthesis was performed with 1 µg of total RNA in two steps with Enhanced avian HS RT-PCR Kit (Sigma, St. Louis MO, USA). For first strand synthesis, an anchored-oligo dT primer and AMV reverse transcriptase was used according to the manufacturer's instructions. *Meloido-*

gyne incognita ESTs CF980963 (Mc Carter J. P.) and BI773441 (Bird-Rao library, Mc Carter, J. P.) showing a high degree of similarity to the *C. elegans* Mn-SOD (CAEEL P31161) were used as a starting point for primer design. Specific internal primers were used in PCR on reverse-transcribed *M. incognita* poly (A)+ RNA. The 5' end of the *Mi-mnsod* cDNA was obtained using forward random primers and specific SODR reverse, whereas the 3' end was obtained using specific SODF forward and reverse anchored primer (Table 1). For PCR amplification 2 µl cDNA template were used directly in a 25 µl reaction mixture consisting of 1x PCR Buffer (Roche, Applied Science, Indianapolis, IN, USA), 100 µM each dNTPs, 1 unit *Taq* polymerase, and 500 nM primers. Amplification was carried out in a Thermocycler (iCycler, BioRad, Hercules CA, USA) programmed with an initial denaturation at 94°C for 4 minutes, 35 cycles at 94°C for 30 seconds, 50°C for 30 seconds, and 72°C for 30 seconds, with a last step at 72°C for 5 minutes with a 4°C hold. The PCR products were analyzed by agarose gel electrophoresis and cloned into a pGemT easy vector (Promega, Madison WI, USA).

Using partial sequences generated by cDNA amplification, specific primers were designed to obtain the full-length genomic DNA sequences for *M. incognita* *Mi-mnsod*. PCR, cloning, and sequencing were performed using 40 ng of DNA and forward SODF2 and reverse SODR5 primers (Table 1) following amplification conditions described for the cDNA amplification.

The cloned PCR products from cDNA and genomic DNA were sequenced by the MWG Biotech (Ebersberg, Germany) sequencing service and deposited in GenBank (Table 2). The SOD encoded protein sequence was analyzed for an N-terminal mitochondrial target domain by the MitoProt (Claros and Vincens, 1996) software. To compare MnSOD across species, related sequences were obtained from public databases (EMBL and NCBI). Where protein sequences were not available, homology searches were performed on genomic databases using the tblastn search program and the predicted proteins were assembled from identified translated exons. Sequences were aligned with Clustal W (Thompson et al., 1994).

DNA blot analysis: Genomic DNA from approximately 100,000 J2 was isolated and 8 µg of DNA were digested with *Eco* RI and *Hind* III restriction enzymes at 37°C for

TABLE 1. Acronyms and sequences of the oligonucleotides used in this study.

Oligonucleotide	Sequence (5'-3')
SODF forward	GGCACATCAATCATAGTA
SODR reverse	TATATTGCAAATAGTAGGC
β -ActF forward	GTCAACACGCCTGCCATGTAT
β -ActR reverse	TGAGGATCTTCATCAAGTAGTCG
SODF2 forward	TTCTTAAATGGCCCGATACG
SODR5 reverse	GCATTAATATATCCTCGAAACCG

TABLE 2. Identification of sequences mentioned in this study.

Accession	Organism - Strain	Molecule type	Author
CF980963	<i>Meloidogyne incognita</i>	cDNA	Mc Carter J. P.
BI773441	<i>M. incognita</i>	cDNA	Mc Carter J. P.
ABLG01001331	<i>Meloidogyne hapla</i> -VW9	genomic DNA	Opperman, C.H. and Bird, D. M.
CABB01007004	<i>M. incognita</i> - Morelos	genomic DNA	Abad, P. et al.
AM749994	<i>M. incognita</i> - MILEV-L4	genomic DNA	Rosso, L.C.
FM865859	<i>M. incognita</i> - MILEV-L4	cDNA	Rosso, L.C.
FM865861	<i>M. incognita</i> - MILEV-L4	cDNA	Rosso, L.C.
FM865865	<i>M. incognita</i> - MILEV-L4	genomic DNA	Rosso, L.C.
FM865868	<i>M. incognita</i> - MILEV-L4	genomic DNA	Rosso, L.C.

18 hours and resolved on 0.7% agarose gel. After denaturation and neutralization, the DNA was capillary transferred onto a nylon membrane (Roche). *Mi-mnsod* chemiluminescent probe, produced by PCR using the PCR Dig probe synthesis kit (Roche, Applied Science, Indianapolis, IN, USA) was synthesized using, as a template, a plasmid DNA containing a fragment of the *Mi-mnsod* gene and SODR and SODF primers (Table 1). The *Mi-mnsod* probe sequence was confirmed by local BLAST against contigs resulting from the *M. hapla* strain VW9 available on line (<http://www.hapla.org>) and *M. incognita* strain Morelos genome assembly, deposited in the EMBL GenBank databases (Table 2). An 88% and a 97% similarity was observed with *M. hapla* ABLG01001331 (nucleotides 13873-14098) and *M. incognita* CABB01007004 (nucleotides 902-1246), respectively (Table 2). The hybridization temperature (45°C) was 2°C higher than the optimal temperature calculated according to both GC content and percent homology of probe to target indicated on the DIG Easy Hyb Granules (Roche) information sheet. Membranes were pre-hybridized at 45°C for 1 hour and hybridized at the same temperature overnight in DIG Easy Hyb Granules solution. After hybridization, the nylon membrane was washed twice at room temperature in 2 × SSC (NaCl, 3M; sodium citrate 0.3M; pH 7), 0.1% (w/v) sodium dodecyl sulfate (SDS) solution, and twice for 15 minutes with 1 × SSC, 0.1% (w/v) SDS at 65°C. Hybrid molecules were detected by the Dig luminescent detection kit (Roche) according to manufacturer's instructions. Hybridization events were detected by exposure to an x-ray film for 4 and 16 hours.

MnSOD gene induction assay: Paraquat dichloride (N,N-dimethyl-4,4-bipyridium dichloride, C₁₂H₁₄Cl₂N₂ · xH₂O Sigma St. Louis MO, USA) solution was used to induce *Mi-mnsod* gene expression. A test to determine the threshold concentration of paraquat tolerated by J2 was performed by placing nematodes in 1.5 ml tubes containing paraquat solutions at 10, 50, and 100 mM final concentrations. The J2 were incubated in darkness at room temperature for 1, 3, 7, and 12 hours and viability checked under a stereoscope. Nematodes in 10 and 50 mM concentrations survived after 12 hours, whereas the J2 exposed at 100 mM died. The 50 mM concentration was selected for gene expression analysis.

Approximately 3,000 *M. incognita* J2 were placed in 1.5 ml tubes containing 50 mM paraquat and incubated in darkness at room temperature for 1, 3, 7, and 12 hours. After incubation, J2 were harvested by centrifugation at 2600 rpm for 3 minutes and washed twice in cold 0.1 M NaCl. The samples were frozen as a pellet in liquid nitrogen and stored at -80°C for subsequent RNA extraction. Sterile distilled water was added in control tubes. Transcript levels were measured by real-time PCR quantification using SYBR Green I Master Mix (Roche). Specific primers pairs SODF/SODR and β ActF/ β ActR were used in the PCR reactions. β -actin primers were designed from *M. incognita* β -actin sequence, accession number BE225475 (amplicon 198 bp). The quantitative PCR reactions (20 μ l) consisted of 2 μ l first strand reverse transcriptase product, gene specific primers (500 nM), and fluorescent dye SYBR Green I (1×). The thermal profile was performed in a Mx3000P (Stratagene) 1 cycle 94°C for 4 minutes and 40 cycles at 94°C for 30 seconds, 49°C for 30 seconds, and 72°C for 20 seconds. Melting curve analysis was performed after the PCR reaction, to confirm that the signal was the result of a single product amplification. Cycle threshold (Ct) values were determined for three biological assays, with two or three replicates each. Amplification efficiencies were 101% for MnSOD and 109% for β -actin products. The corresponding Ct values were applied to estimate the ratio of starting amounts DNA according to the modified Pfaffl equation (Pfaffl, 2001): normalized treated (with paraquat)/normalized control (without paraquat) = $(2^{-\Delta C_{t \text{ target}}}) / (2^{-\Delta C_{t \text{ norm}}})$. Data were statistically analyzed by unpaired Student's *t*-test.

In situ hybridization: Primer SODR and SODF were used to synthesize digoxigenin-labeled sense and anti-sense single-stranded DNA by asymmetric PCR from the *M. incognita* *Mi-mnsod* cDNA. In situ hybridization was performed as described by De Boer et al. (1998). Approx 40,000 J2 of *M. incognita* were treated with 50 mM paraquat for 7 hours and washed three times with 1 ml SDW. Juveniles incubated in SDW were used as control. After treatment, J2 were fixed in 2% formalin buffered in M9 (pH 7.0) for 18 hours at 4°C and 4 hours at room temperature. Nematodes were randomly cut on glass slides with a razor blade. Partial digestion was performed with 500 ng/ml proteinase-K (Roche) at room

temperature for 40 minutes. Denatured PCR digoxigenin-labeled DNA probes (approximately 20 ng) were added to each tube and hybridization was performed overnight at 45°C. cDNA was detected by alkaline phosphatase immunostaining at 4°C for 16 hours and sections were examined by light microscopy. Images were captured using a Nikon digital camera.

RESULTS

cDNA sequence analysis: Using *M. incognita* cDNA as a template and the primers pairs SODF forward and anchored oligo dT, or random primers and SODR reverse, yielded amplicons that were 98% identical to CF980963. Assembling these amplicons resulted in a putative full-length open reading frame (ORF) of 660 bp within a total of 820 bp cDNA (Fig. 1). This cDNA sequence also contained a partial 5' untranslated region (UTR) and a 3' UTR of 30 bp and 119 bp, respectively. Six adenylate-uridylylate-rich elements (ARE) were observed in the sequence, three of which were found in the transit peptide coding region, two were positioned in the mature peptide coding region, and one in the 3' UTR. The 3' UTR included a poly-(A) tail and the polyadenylation signal sequence 5'-AATAAA-3' at position 702. *Mi-mnsod* mRNA contained also two polypyrimidine tracts at the 3' UTR (Fig. 1).

```

-30 ttcttaaatggccgatacgtttcttaaata 0
ATGTTCTACAAAAATTTAAACAAACTATCCTATGGgtatttatttatttttttttctactaa 60
M F Y K N L N K L S Y G 12
taaatttaattttctagGATTCGGCAGCTTCATTTATCAAAACGATTTAAGCATGCTTTG 120
I R Q L H L S K R F K H A L 26
CCTGATCTCCCTATGACTATAATGCTCTTGAGCCAGTAATTTCTGCTGAAATTTAGAAA 180
P D L P Y D Y N A L E P V I S A E I M K 46
ATTCATCATCAAAACATCATGgtttgtctgttgatttaattcttttttttatattt 54
I H* H Q K H H 23
tcagCAACTTATGTAACAATTTAAATGACTGAAGAAAAAATTCAAGAGGCTCTTGCC 300
A T Y V N N L N M T E E K I Q E A L A 72
AAAGgtttatttttttttttaattttacagttttatttttagGAGATATTCGTTCCGGTT 360
K G D I R S V 79
ATTCAATTGCAATCCGCTCTAAAATTTAATGGTGGCGGGCACATCAATCATAGTATTTTC 420
I Q L Q S A L K F N G G G H I N H* S I F 99
TGGACAAATCTTTGTAAGGATGGAGGTGAACCATCTGGCAAACCTCTGCAAGCGATTAAT 480
W T N L C K D G G E P S G K L L Q A I N 119
CGTGATTTGGTCTCTCCAAGTATTGCAAGCCCGTTTAAATGCAATTCGCAATAGCAGTT 540
R D F G S L Q V L Q A R L N A I A I A V 139
CAAGGATCTGGTGGGGATGGCTGGCTACAACAAAATTGACAAAACGTTTGGAAAGTTGCT 600
Q G S G W G W L G Y N K I D K R L E V A 159
TGTTGTCGAATCAGGACCCGCTGGAACCTACCACAGgtttttttaaataaataaaaaat 660
C C P N Q D P L E P T T 171
atgtaagaataaatttaatttgagGACTTGTGCTCTCTTTTGAATTCAGCTTTGGGAG 720
G L V P L F G I D* V W E 183
CATGCCTACTATTTGCAATATAAAAATGTGCGTCTGATTTATGTAATGCTATTGGAAAG 780
H* A Y Y L Q Y K N V R A D Y V N A I W K 203
ATTGCCGATTGGAAGAATATTGAGGAGCGGTATGAAAGTGCTCAGACTGATTAaaagag 840
I A D W K N I E E R Y E S A Q T D End 220
gacaagtaaatatagataagcttaaaataaataattttttctctcggttttcca 900
ggatataatttaagtcaacgttgaaggttagtaaatattttttttttttctctcggtt 960
tcaggatattttaaagtc

```

FIG. 1. Nucleotide and deduced amino acid sequence of *M. incognita* *Mi-mnsod* sequence, AM749994. Numbering is given for the gene (including introns) and amino acid sequences. Primers sequences (SODF2 forward and SODR5 reverse) are indicated in bold. Introns and flanking regions are shown in lowercase letters and exon in uppercase letters. Underlined motifs represent potential regulatory elements (see text). The putative mitochondrial transit leader sequence of 22 amino acids in length is shown in italics. The Parker and Blake signature amino acids of MnSODs are marked with grey boxes; conserved metal-binding residues are identified by asterisks.

The translational start site was a typical ATG codon of eukaryotic mRNAs (Kozak, 1991). BlastP (Altschul et al., 1997) of the translated sequence, consisting of 220 amino acids with a predicted molecular mass of 25 kDa, revealed a high degree of similarity to the SOD family member (*sod-2*) of *C. elegans* (amino acid identities 74%, positives 83%), *C. briggsae* (amino acid identities 73%, positives 82%), and the MnSOD from the giant freshwater prawn *Macrobrachium rosenbergii* (amino acid identities 73%, positives 85%).

DNA Sequence analysis: Sequences of the entire *Mi-mnsod* gene, as well as 30 bp of 5' flanking sequence (not shown in Fig. 1) and 86 bp of 3' sequence were determined by amplification and sequencing from *M. incognita* genomic DNA with primers SODF2 forward and SODR5 reverse, located in the 5' and 3' UTR, respectively (Fig. 1). The genomic sequence was deposited in GenBank (EMBL) as accession number AM749994. The *Mi-mnsod* genomic sequence showed 96% similarity to *M. incognita* strain Morelos CABB01007004 (nucleotides 474-1417) and 82% with *M. hapla* ABLG01001331 (nucleotides 13431-14364).

Sequence comparisons of the cDNA and genomic clones indicated that *Mi-mnsod* gene is composed of five exons encoding 12, 41, 20, 98, and 49 amino acids and four introns 42, 42, 39, and 48 bp long each. The intron/exon boundaries of the *Mi-mnsod* gene had a conserved 5'-GT-AG-3' intron splice-site junctions (Mount, 1982) (Fig. 1).

Analyzing partial sequences obtained from cDNA (accession number FM865859 and 61) and genomic DNA (accession number FM865865 and 68) of *M. incognita* MILEV-L4, nucleotide polymorphisms were observed. AM749994 sequence showed a 97% similarity with FM865865, CABB01007004 and FM865868 in the exon 4 and intron 4 regions (Fig. 2). The AM749994 3' UTR region showed 93% similarity with FM865859 and CABB01007004 and 79% with FM865861 (Fig. 3).

Amino acid sequence analysis: The deduced amino acid sequence of AM749994 (CAO78623) displayed the consensus region DXWEH[STA][FY]. In addition, it presented the four residues known to be responsible for the Mn binding (His-48, His-96, Asp-180 and His-184) and the Parker and Blake signature MnSOD (Gly-91, Gly-92, Phe-99, Gln-164 and Asp-165) (Parker and Blake, 1988) (Fig. 1). The MitoProt analysis identified a putative mitochondrial localization signal encoded by amino acids 1-22. Removal of this 2.7 kDa mitochondrial transit peptide resulted in a 22.3 kDa mature enzyme. The transit peptide amino acid sequence was rich in positively charged groups (Arg-14, Arg-21, Lys-4, Lys-8 and Lys-20) and lacked acidic amino acids. The putative transit peptide of *Mi-MnSOD* also possessed an arginine residue in the penultimate position, present in all eukaryotic MnSODs. The N-terminal LPD region, which is conserved in all eukaryotic MnSODs, was also present in *M. incognita* (Fig. 4). When considering the

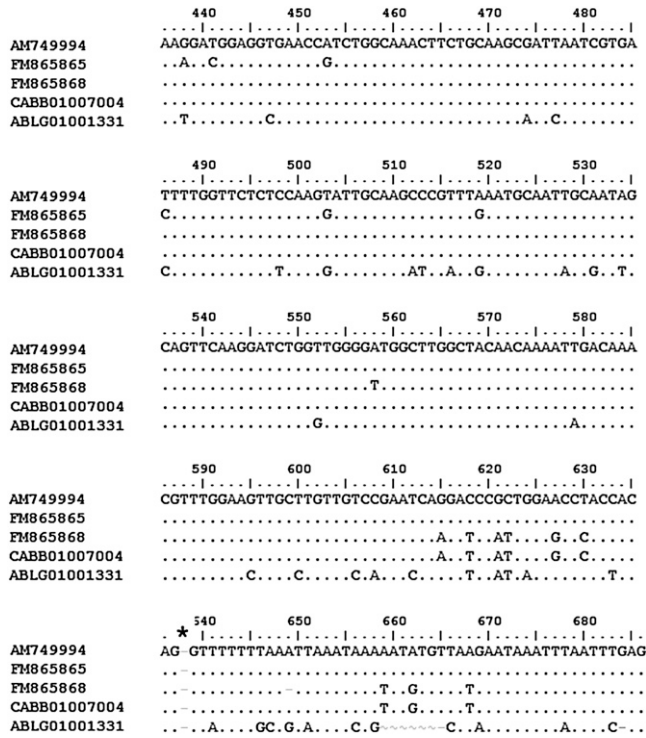


FIG. 2. Comparison of *Mi-mnsod* sequences of exon and intron number four from *M. incognita* strain MiLEV-L4 (AM749997, FM865865, FM865868), strain Morelos (CABB01007004), and *M. hapla* (ABLG01001331). Identity between sequences is indicated by a dot, asterisk indicates the start of the intron site.

nucleotide polymorphisms observed in coding regions, only one present at nucleotide 503 introduced an amino acid substitution at position 27, giving rise to glycine-to-valine substitution (Fig. 1 and 2).

The DNA blot analyses of the digested DNA with *Eco* RI or *Hind* III, hybridized with the Dig-labeled *Mi-mnsod* probe, showed three hybridization signals for both restriction enzymes (Fig. 5). Cleavage sites for *Eco* RI or *Hind* III are not present in the sequence of the *Mi-mnsod* probe.

Expression analysis: Higher levels of antioxidant enzyme activity in response to paraquat have been demonstrated in several organisms, including *Drosophila* (Orr and Sohal, 1994) and *C. elegans* (Vanfleteren, 1993). Therefore, paraquat was used for SOD gene

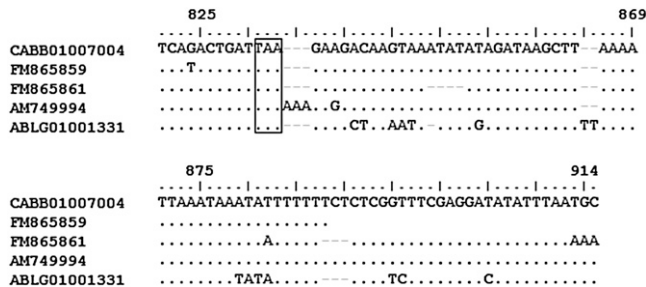


FIG. 3. Comparison of *Mi-mnsod* 3'UTR sequences from *M. incognita* strain Morelos (CABB01007004), strain MiLEV-L4 (AM749997, FM865859, FM865860), and *M. hapla* (ABLG01001331). The stop codon is indicated in a box.

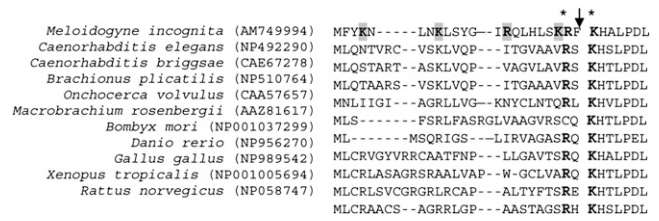


FIG. 4. Alignment of the translated AM749994 Mi-MnSOD mitochondrial transit peptide and the first seven amino acids of mature Mi-MnSOD protein with characterized MnSOD from other species. The predicted cleavage site is indicated by an arrow. Positively charged amino acid residues are shown in bold. The asterisks indicate conserved lysine and arginine residues at the beginning of the mature proteins and penultimate to the maturation cleavage site, respectively.

expression analyses in *M. incognita*. Exposures of *M. incognita* J2 to 50 mM paraquat was carried out successfully and no mortality was observed at any of the exposure times. *Mi-mnsod* expression was significantly higher ($P < 0.05$), than in untreated controls, at 1 (1.61-fold) and 7 hours (2.09-fold) exposures. After 12 hours, the *Mi-mnsod* mRNA quantity decreased ($P < 0.05$) 1.3-fold when compared to the 7 hours after exposure (Fig. 6).

In situ hybridization: The digoxigenin labeled anti-sense probe for *M. incognita Mi-mnsod* showed a signal present throughout the posterior portion of the nematode body (Fig. 7A and B), with higher intensity at the anterior portion of the intestine (Fig. 7A). No signal was observed in controls hybridized with the sense strand probe (Fig. 7C) and a background level signal was observed in the anterior portion of the nematode (Fig. 7D).

DISCUSSION

This study allowed the identification and characterization of *Mi-mnsod*, a gene encoding a MnSOD enzyme

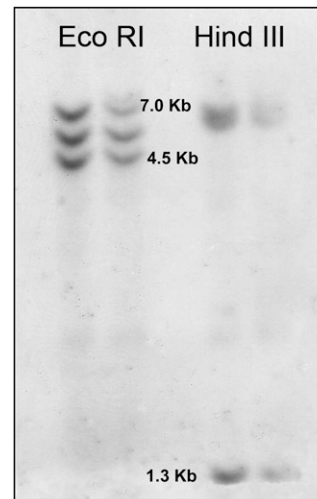


FIG. 5. DNA blot analysis of *MnSOD* genes. *Meloidogyne incognita* genomic DNA was digested with *Eco* RI or *Hind* III and hybridized with the *Mi-mnsod* probe in two replicates.

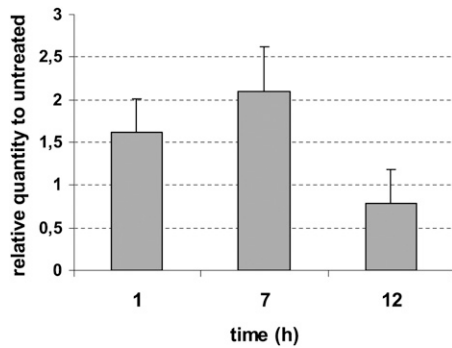


FIG. 6. Effect of paraquat on transcript levels of *Mi-mnsod* at different exposure times. Transcript levels were monitored with RT-PCR in three replicates and normalized against β -actin expression. Bars represent standard errors.

from *M. incognita*. The complete nucleotide sequences of MnSOD genes have been previously characterized in *C. elegans* (Hunter et al., 1997) and *Onchocerca volvulus* (Henkle-Dührsen et al., 1995). Whereas the *C. elegans* genome encodes a 1.3 kb and 1.5 kb genes for *Sod-2* and *Sod-3* respectively, and the *Ov* MnSOD from *O. volvulus* is 1.9 kb in length, the *Mi-mnsod* is only 1 kb in length. However, there is a striking similarity in the overall gene organization and structure, with all genes being organized with five exons and four introns. In addition, the deduced *M. incognita* Mi-MnSOD mature enzyme molecular mass of 22.3 kDa is similar to that of other eukaryotic species, including *O. volvulus* 22.3 kDa, *C. elegans* 21.9 - 22.1 kDa, and *Bombyx mori* 24 kDa.

In the eukaryotic cells, MnSOD is encoded in the nucleus, synthesized in the cytosol, and imported post-

translationally into the mitochondria matrix where 90% of the oxygen in the cells is consumed (Halliwell and Gutteridge, 1989). The amino acid sequence of Mi-MnSOD indicates the presence of a leader sequence at the amino-end of the deduced precursor protein, suggesting that the Mi-MnSOD might also be synthesized as a precursor in the cytosol and then transferred into the mitochondria (Fig. 4).

The conservation of the four residues responsible for the Mn binding present in all eukaryotic MnSODs, (amino acids His-48, His-96, Asp-180 and His-184 in the Mi-MnSOD) suggests the important role played by these residues in determining the catalytic activity of the enzyme and, consequently, redox balance in the cell. Regulation of the cell's redox state is critically linked to the levels of mitochondrial MnSOD, whose activity is regulated both at the transcriptional and post-translational levels (Davis et al., 2001). The genomic DNA sequence of *Mi-mnsod* revealed some features that also suggest a possible post-transcriptional control mechanism associated with mRNA stability, namely presence of ARE elements and polypyrimidine tracts.

The *Mi-mnsod* (-AUUUA-) ARE elements appear to belong to class I, one of the most ubiquitous and destabilizing elements found within rapidly degraded mRNA (Zhang et al., 2002). These elements are characterized by the presence of one to three pentamers that are distributed within the 3' UTR, coupled with a nearby U-rich region (Xu et al., 1997). At least three classes of AREs have been identified based on their composition, number of pentamer repeats, and subsequent effects of RNA deadenylation and decay (Xu et al., 1997). Even if *M. incognita* AREs were observed mainly in the coding region (only one is present at 3' UTR), it is interesting to note the high frequency of these elements within a confined genomic region, which are not present (or occur in a low frequency) in nematode mRNA population (Vassella et al., 1994; De Giorgi et al., 1997). The polypyrimidine tracts found in the 3' UTR of *Mi-mnsod* have been described in the 5' and 3' UTR of procyclin-associated genes (PAGs) of *Trypanosoma brucei* (Vassella et al., 1994) and within a gene encoding a cuticlin protein of *Meloidogyne artiellia* (De Giorgi et al., 1997). This region binds proteins involved in the processing of primary transcripts to mature mRNAs (Singh et al., 1995). Further studies will be needed to test the role of these elements in any post-transcriptional regulatory processes.

The presence of three fragments in the DNA blot analysis, together with the occurrence of three different amplified products characterized by replicated nucleotide polymorphisms, suggest the occurrence of three putative copies of the MnSOD gene in *M. incognita*. This is similar to what has been found for *C. elegans* (Hunter et al., 1997) but not for *O. volvulus*, in which a single-copy MnSOD gene was observed (Henkle-Dührsen et al., 1995).

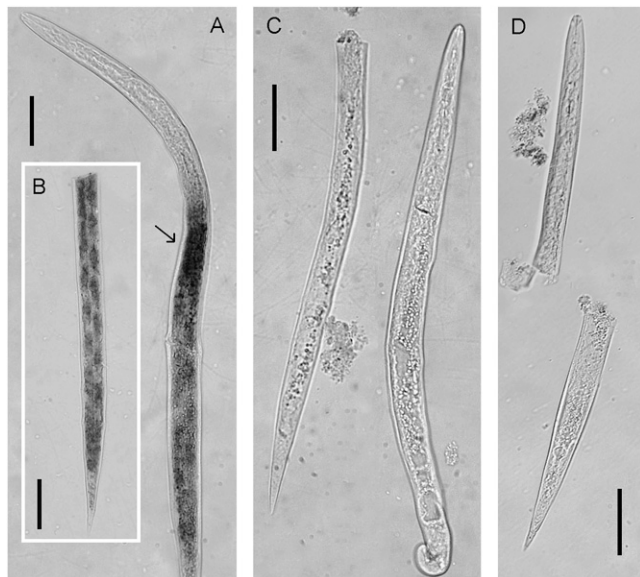


FIG. 7. *In situ* hybridization of digoxigenin labeled cDNA probe of *Meloidogyne incognita* *Mi-mnsod*. Juveniles were treated with 50mM paraquat for 7 hours. *MnSOD* expression is detected throughout the posterior body region A and inset B. Arrow A, anterior region of intestine. C, sense strand hybridization control. D, untreated control. Scale bars = 20 μ m.

The *Mi-mnsod* transcript levels are significantly higher (1.61-fold) after 1 hour of paraquat treatment than in untreated control, suggesting a rapid response to oxidative stress. The decrease in the gene expression after 12 hours could be related to an irreversible toxic condition caused by the continuous exposure to an oxidative environment. The increase in the amount, and spatial location of *Mnsod* mRNA as shown by the in situ hybridization, could suggest a reaction to the oxidative stress generated by paraquat, according to the role of this enzyme in the ROS detoxification process. The presence of *Mnsod* mRNA in the intestine also suggests a putative detoxification function, active in this body region, where plant products are present due to feeding.

The ability of J2 to quickly react to the oxidative stress could play an important role in establishing and maintaining the nematode inside the plant tissue. Biochemical observations showed that tomato plants react to root-knot nematodes by mounting defense responses, implicating ROS production (Melillo et al., 2006). The superoxide induction is an early event during nematode infection which determines different patterns of ROS production in compatible and incompatible nematode-tomato interactions. A high concentration of ROS was detected as a result of the incompatible interaction, which can be related to a rapid induction of HR (Melillo et al., 2006). Present data showed that *Mi-mnsod* may play a key role in nematode survival under oxidative stress conditions. MnSOD activity, which scavenges superoxide anions and catalyzes their dismutation into hydrogen peroxide and oxygen, may represent the first defense of the J2 against ROS overproduction, occurring as early as 1 hour after exposure to oxidative stress. This early defense system may be important in determining the nematode's capability to organize a complex response to counteract the plant defense mechanisms in incompatible reactions.

LITERATURE CITED

- Abad, P., Gouzy, J., Aury, J. M., Castagnone-Sereno, P., Danchin, E. G. J., Deleury, E., Perfus-Barbeoch, L., Anthouard, V., Artiguenave, F., Blok, V., Caillaud, M. C., Coutinho, P. M., Dasilva, C., De Luca, F., Deau, F., Esquibet, M., Flutre, T., Goldstone, J. V., Hamamouch, N., Hewezi, T., Jaillon, O., Jubin, C., Leonetti, P., Magliano, M., Maier, T., Markov, G. V., McVeigh, P., Pesole, P., Poulain, J., Robinson-Rechavi, M., Sallet, E., Ségurens, B., Steinbach, D., Tytgat, T., Ugarte, E., Van Ghelder, C., Veronico, P., Baum, T. J., Blaxter, M., Blevé-Zacheo, T., Davis, E. L., Ewbank, J. J., Favery, B., Grenier, E., Henrissat, B., Jones, J. T., Laudet, V., Maule, A. G., Quesneville, H., Rosso, M. N., Schiex, T., Smant, G., Weissenbach, J., and Wincker, P. 2008. Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology* 26:909–915.
- Altschul, S. F., Madden, T. L., Schäffer, A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25:3389–3402.
- Baker, C. J., and Orlandi, E. W. 1995. Active oxygen species in plant pathogenesis. *Annual Review of Phytopathology* 33:299–321.
- Callahan, L. H., Crouch, R. K., and James, E. R. 1988. Helminth anti-oxidant enzymes: a protective mechanism against host oxidants? *Parasitology Today* 4:218–25.
- Claros, M., and Vincens, G. P. 1996. Computational method to predict mitochondrially imported proteins and their targeting sequences. *European Journal of Biochemistry* 241:770–786.
- Davis, C. A., Monnier, J. M., and Nick, H. S. A. 2001. Coding region determinant of instability regulates levels of manganese Superoxide Dismutase mRNA. *Journal Biological Chemistry* 276:37317–37326.
- De Boer, J. M., Yan, Y., Smant, G., Davis, E. L., and Baum, T. J. 1998. In situ hybridization to messenger RNA in *Heterodera glycines*. *Journal of Nematology* 30:309–12.
- De Giorgi, C., De Luca, F., Di Vito, M., and Lamberti, F. 1997. Modulation of expression at the level of splicing of cut-1 RNA in the infective second-stage juvenile of the plant parasitic nematode *Meloidogyne artiellia*. *Molecular and General Genetics* 253:589–598.
- Droopkin, V. H., Helgeson, J. P., and Upper, C. D. 1969. The hypersensitivity reaction of tomatoes resistant to *Meloidogyne incognita*: reversal by cytokinins. *Journal of Nematology* 1:55–60.
- Fridovich, I. 1986. Biological effects of the superoxide radical. *Advances in Enzymology and Related Areas of Molecular Biology* 58:62–97.
- Halliwell, B., and Gutteridge, J. M. C. 1989. Free radicals in biology and medicine. 2nd ed. Oxford, UK: Oxford University Press.
- Hartman, P., Chidress, E., and Beyer, T. 1995. Nematode development is inhibited by methyl viologen and high oxygen concentrations at a rate inversely proportional to life span. *Journal of Gerontology* 50:B322–B326.
- Henkle-Dührsen, K., Tawe, W., Warnecke, C., and Walter, R. D. 1995. Characterization of the manganese superoxide dismutase cDNA and gene from the human parasite *Onchocerca volvulus*. *Biochemical Journal* 308:441–446.
- Honda, Y., and Honda, S. 1999. The *daf-2* gene network for longevity regulates oxidative stress resistance and Mn – superoxide dismutase gene expression in *Caenorhabditis elegans*. *FASEB Journal* 13:1385–1393.
- Hunter, T., Bannister, W. H., and Hunter, G. J. 1997. Cloning, expression and characterization of two Manganese Superoxide Dismutase from *Caenorhabditis elegans*. *The journal of Biological Chemistry* 272:28652–28659.
- Kozak, M. 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. *The journal of Biological Chemistry* 266:19867–70.
- McCord, J. M., and Fridovich, I. 1969. Superoxide dismutase. An enzymic function for erythrocyte (hemocuprein). *The journal of Biological Chemistry* 244:6049–6055.
- Mehdy, M. 1994. Active oxygen species in plant defense against pathogens. *Plant Physiology* 105:467–72.
- Melillo, M. T., Leonetti, P., Bongiovanni, M., Castagnone-Sereno, P., and Blevé Zacheo, T. 2006. Modulation of reactive oxygen species activities and H₂O₂ accumulation during compatible and incompatible tomato-root-knot nematode interactions. *New Phytologist* 170:501–512.
- Molinari, S., Rosso, L., and Ornat Longaron, C. 2005. The role of antioxidant enzymes in the virulence of root-knot nematodes on resistant tomato. *Nematropica* 35:88.
- Mount, S. A. 1982. Catalogue of splice junction sequences. *Nucleic Acids Research* 10:459–72.
- Orr, W. F., and Sohal, S. R. 1994. Extension of life-span by over-expression of superoxide dismutase and catalase in *Drosophila melanogaster*. *Science* 263:1128–1130.
- Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* 29:45.

- Parker, M. W., and Blake, C. F. 1988. Iron- and manganese-containing superoxide dismutases can be distinguished by analysis of their primary structures. *FEBS Letters* 229:377–382.
- Paulson, R. E., and Webster, J. M. 1972. Ultrastructure of the hypersensitivity reaction in roots of tomatoes, *Lycopersicon esculentum* L., to infection by the root-knot nematode, *Meloidogyne incognita*. *Physiological and Molecular Plant Pathology* 2:227–234.
- Roise, D., and Schatz, G. 1988. Mitochondrial presequences. *The journal of Biological Chemistry* 263:4509–4511.
- Sasser, J. M. 1980. Root-knot nematodes: a global menace to crop production. *Plant Disease* 64:36–41.
- Singh, R., Valcarcell, J., and Green, M. R. 1995. Distinct specificities and function of higher eukaryotic polypyrimidine tract – binding proteins. *Science* 268:1173–1176.
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research* 25:4876–82.
- Vanfleteren, J. R. 1993. Oxidative stress and ageing in *Caenorhabditis elegans*. *Biochemical Journal* 292:605–608.
- Vassella, E., Braun, R., and Roditi, I. 1994. Control of polyadenylation and alternative splicing of transcripts from adjacent genes in a prokaryotic expression site: dual role for polypyrimidine tracts in *Trypanosoma*. *Nucleic Acids Research* 22:1359–1364.
- Wyss, U., Grundler, F. M. W., and Munch, A. 1992. The parasitic behaviour of second-stage juveniles of *Meloidogyne incognita* in roots of *Arabidopsis thaliana*. *Nematologica* 38:98–111.
- Xu, N., Chen, C. Y., and Shyu, A. B. 1997. Modulation of the fate of cytoplasmic mRNA by AU-rich elements: key sequence features controlling mRNA deadenylation and decay. *Molecular Cell Biology* 17:4611–4621.
- Zhang, T., Kruys, V., Huez, G., and Gueydan, C. 2002. Au-rich element-mediated translational control: complexity and multiple activities of *trans*-activating factors. *Biochemical Society Transactions* 30:952–958.