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Meloidogyne javanica Chorismate Mutase Transcript Expression Profile Using Real-Time Quantitative RT-PCR

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Abstract: A developmental expression profile of the *Meloidodgyne javanica* esophageal gland gene *chorismate mutase-1* (*Mj-cm-1*) could suggest when in the lifecycle of the nematode the *Mj-cm-1* product is functional. This study used real-time quantitative RT-PCR to examine the variation in *Mj-cm-1* transcript levels over six timepoints in the nematode lifecycle: egg, infective second-stage juveniles (Inf-J2), 2-day post-inoculation (pi), 7-day pi, 14-day pi, and adult. The *Mj-cm-1* mRNA levels peaked at 2-day pi, about 100-fold above levels expressed at the egg and Inf-J2 stages. Some expression of *Mj-cm-1* remained during the 7-day pi, 14-day pi, and adult stages. High transcript levels of the beta-actin control gene *M. javanica Beta-actin-1* (*Mj-ba-1*) demonstrated the presence of cDNA at all timepoints. The peak in *Mj-cm-1* transcript expression at 2-day pi as well as the previously shown esophageal gland localization of *Mj-cm-1* mRNA suggest that the product of this gene may be involved early in the establishment of parasitism.

Key words: cDNA, chorismate mutase, developmental profile, *Meloidogyne javanica, Mj-ba-1, Mj-cm-1,* mRNA, nematode, parasitism gene, real-time quantitative RT-PCR, root-knot nematode, shikimate pathway, Taqman assay, transcript.

It is during the second juvenile (J2) stage that the root-knot nematodes (*Meloidogyne* spp.) penetrate a host plant root and establish a feeding site. The nematodes induce about six vascular parenchyma cells to replicate their nuclei without cell division, and each of these cells becomes a giant multinucleate feeding cell (Bird, 1967; Sasser, 1980; Trudgill and Blok, 2001). This elaborate manipulation of plant cell cycle and metabolism interests many biologists because it indicates that nematodes can alter basic plant developmental processes.

Secretions from nematode esophageal glands could play a role in this manipulation of plant processes (Bird, 1967; Trudgill and Blok, 2001). Not only do the esophageal glands increase in size during the establishment of parasitism (Bird, 1967), but also a number of cloned genes that could be involved in parasitism have shown expression in the esophageal glands during this point in the nematode lifecycle (Davis et al., 2000). These genes include cellulases (De Meutter et al., 2001; Ding et al., 1998; Goellner et al., 2000; Rosso et al., 1999), pectate lyases (de Boer et al., 2002; Doyle and Lambert, 2002; Popeijus et al., 2000b), chorismate mutases (Lambert et al., 1999a; Popeijus et al., 2000a), and a large assortment of others with little homology to known genes in the database (De Meutter et al., 2001; Gao et al., 2002; Wang et al., 2001).

Chorismate mutase gene family member *M. javanica Chorismate Mutase-1* (*Mj-cm-1*) was shown by Lambert et al. (1999a) to be expressed exclusively within the esophageal glands of *M. javanica* (Treub) Chitwood. This gene encodes an enzyme commonly found in the shikimic acid pathway. Plants, bacteria, fungi, and protists all have a shikimic acid pathway that produces aromatic amino acids and other metabolites. The shikimate pathway is considered absent in the heterotrophic animal kingdom (Roberts et al., 1998; Roberts et al., 2002; Romero et al., 1995), yet *M. javanica* has the *Mj-cm-1* esophageal gland gene that encodes a chorismate mutase (Lambert et al., 1999a). The fact that the nematode has a potentially secreted chorismate mutase in its esophageal glands suggests that this gene product may be used to alter the shikimate pathway in the plant (Lambert, 1999a).

One way to explore the function of *Mj-cm-1* is to establish a transcript expression profile over the lifecycle of the nematode. While the establishment of a transcript profile for *Mj-cm-1* mRNA may be useful, traditional methods of mRNA level measurement have proven impractical. Slot blots, RNA gel blots, and ribonuclease protection assays require high amounts of starting material difficult to achieve from a microscopic endoparasite like *M. javanica* (Ding et al., 2000; Hermsmeier et al., 2000; Reue, 1998; Zhong et al., 1998). More sensitive methods such as in situ hybridization (de Boer et al., 1999; Lambert et al., 1999a) and conventional reverse-transcriptase polymerase chain reaction (RT-PCR) assays (Goellner et al., 2001; Rosso et al., 1999) are not considered to be quantitative (Bustin, 2000; Schmittgen et al., 2000). While the localization data gathered for nematodes by in situ hybridization is valuable, this signal is difficult to quantify or accurately compare between nematodes collected at different timepoints (Bustin, 2000). Real-time quantitative RT-PCR yields precise and accurate results with the least amount of starting material, theoretically down to a single copy of a transcript (Gibson et al, 1996; Heid et al., 1996; Lockey et al., 1998; Reue, 1998). This sensivity and accuracy made real-time quantitative RT-PCR an ideal method to generate an expression profile for *Mj-cm-1* mRNA over the entire lifespan of *M. javanica*.

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Materials and Methods

Nematode harvesting: Meloidogyne javanica was cultured on *Lycopersicon esculentum* Mill. cv. UC82B tomato roots (Lambert et al., 1992). Eggs were NaOCl extracted as described by Hussey and Barker (1973), concentrated by sucrose flotation and sieving (Jenkins, 1964), and counted with a dissecting microscope. Hatched J2 nematodes were collected from hydroponic cultures of infected tomato plants as described in Lambert et al. (1992). The J2 were cleaned of plant debris and biotic contaminants by being allowed to crawl through layers of Kimwipes (Lambert et al., 1992) and a sterile sand column (Lambert, 1995) into sterile distilled water (dH_2O). The clean J2 were collected by filtration onto a 10.0 micron-opening polycarbonate membrane (Osmonics, Inc., Minnetonka, MN) and suspended in equal amounts of sterile dH_2O and 1% medium viscosity carboxymethylcellulose (CMC) (Sigma-Aldrich Co., St. Louis, MO) (Lambert et al., 1999b). This 0.5% CMC J2 nematode suspension was used either as the infective J2 (Inf-J2) timepoint or as tomato seedling root inoculum. The nematodes in 10 to 20-µl aliquots were counted from at least two samples. Independent collections of Inf-J2 nematodes were performed three times.

Plant infection and culture: Tomato seeds were surface-sterilized in NaOCl and germinated for 4 days as described in Ho et al. (1992). Those used for 2-day post inoculation (pi) and 7-day pi points were transferred to inoculation boxes and inoculated with 50 nematodes per root as previously described (Lambert et al., 1999b). Control roots ("mock-inoculated") were handled identically to 2-day pi but were treated with 0.5% CMC without J2. Those seedlings for 14-day pi infections were transplanted to individual 50-ml Corning tubes filled with sand. After growing for 2 weeks, the seedlings in sand were inoculated with 540 J2 in 200 µl 0.5% CMC suspension and grown for an additional 14 days at 25 °C in a growth room under fluorescent and incandescent lights. The adult nematodes developed in serially inoculated plants grown in sand culture as described by Lambert et al. (1992).

Parasitic nematode harvesting: The 2-day pi and 7-day pi nematode-infected roots were harvested as described in Lambert et al. (1999b). Galled tissue from each 14-day pi root was separated from non-galled tissue in dH_2O. The number of nematodes in the 2-day pi, 7-day pi, and 14-day pi tissues was counted manually with a dissecting microscope after acid fuchsin staining of subsample root tips or galls using the procedure outlined in Byrd et al. (1983). All nematode-infected plant tissue was frozen immediately in liquid nitrogen and stored at −80 °C until extraction. Adult nematodes were excised individually from tomato root galls with forceps using a dissecting microscope, collected into dH_2O, and then transferred into a 2-ml tube on dry ice. Independent inoculations and subsequent tissue collections were performed twice for each of these four timepoints.

Nucleic acid extraction and DNase treatment: Extraction of total nucleic acid from egg, Inf-J2, and adult nematodes proceeded as described for "preparasitic J2" nematodes in Doyle and Lambert (2002). However, nematodes within tomato root tissue from 2-day pi, 7-day pi, and 14-day pi were crushed with a stainless steel tissue pulverizer (Fisher Scientific, Pittsburgh, PA), phenol:chloroform extracted with buffer (0.028 M Triisopropylnaphthalenesulfanate, 0.342 M 4-aminosalycilinate, 0.1 M Tris-HCl [pH 8.0], 0.05 M EDTA, 0.1 M NaCl, 1% SDS, and 0.5 M 2-mercaptoethanol), and precipitated with ethanol. Nucleic acid samples with excessive salt were dissolved in 50 µl of diethyl pyrocarbonate (DEPC)-treated dH_2O and filtered through a DEPC-Chroma Spin^{-100} column according to manufacturer's instructions (BD Biosciences, Clontech Unit, Palo Alto, CA). Samples were treated with DNase I using the DNA-*free* kit and following manufacturer's instructions for high DNA-content preparations (Ambion, Inc., Austin, TX).

Reverse transcription: DNase I-treated samples were reverse transcribed with Oligo$(dT)_{12–18}$ primer and reverse transcriptase (RT) using the manufacturer's instructions for Superscript First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Invitrogen Corp., Carlsbad, CA). Controls (−RT controls) were processed identically to the RT reactions (+RT reactions), except +RT reactions contained reverse transcriptase, whereas −RT controls contained DEPC-dH_2O. Completed samples were then diluted 5-to-10-fold to allow more accurate pipeting.

DNA sequences used for Taqman assays: The *Mj-cm-1* chorismate mutase cDNA and genomic DNA sequences cloned by Lambert et al. (1999a) [Genbank Accession Nos. AF095949 and AF095950, respectively] functioned as design templates for this transcript. The control gene *M. javanica Beta-actin-1 (Mj-ba-1)* was amplified from *M. javanica* genomic DNA and cDNA using primers MjBa1 F: 5′-GGT CAA CAC GCC TGC CAT GTA TGT-3′ and MjBa1 R: 5′-TGC AAT CCA ACA GAG TAT TAC GCT-3′ designed from an *M. incognita* expressed sequence tag [Accession No. BE225475] (Dautova et al., 2001). The resulting PCR products were cloned into the pCRII Topo vector (Invitrogen) and sequenced by the University of Illinois W. M. Keck Center for Comparative and Functional Genomics (Urbana, IL) [Genbank Accession No. AF532604 for genomic DNA and No. AF532605 for cDNA].

Taqman primer and probe design: Primer and probe sequences were designed using Primer Express software (Applied Biosystems, Foster City, CA) and OLIGO Primer Analysis software (Molecular Biology Insights, Inc., Cascade, CO). Due to design constraints of the primer and probe sequences for the Taqman assay, only the *Mj-ba-1* primer set spans an intron to detect

only cDNA. The Taqman primer and probe sets hybridize close to the 3′-end of each sequence to help eliminate effects from possible variation in reverse transcription between mRNAs. *Mj-cm-1 primers:* Forward: 5′-TTA TGA GAG TTA ATC AAA TTG ATG AAC AAA-3′ Reverse: 5′-ACA TGC TGG GCA GAG GTC TT-3′ Probe: 5′-6FAM-TGA TGC TTT GAA AAT GGC TGT GAA AGG C-TAMRA-3′ *Mj-ba-1 primers:* Forward: 5′-GCG ACA TTG ACA TCC GTA AAG AC-3′ Reverse: 5′-CAA TGC CTG GAT ACA TGG TTG T-3′ Probe: 5′-VIC-TTT ACG CCA ACA CTG TCC TTT CTG GAG G-TAMRA-3′

Copy number standards: The standard primer set M13 (−35) Reverse and M13 (−30) Forward was used to PCR amplify *Mj-cm-1* (Lambert et al., 1999a) from its cloning vector. This yielded a 948-base pair (bp) fragment containing the Taqman primer and probe sequences. A 647-bp fragment for *Mj-ba-1* was generated using the MjBa1 F and MjBa R primers. The fragments for each gene were column-purified using Chroma Spin^{-100} columns and quantified using a Genesys 10 Series spectrophotometer (Spectronic Unicam, Rochester, NY). The molecular weight of each double-stranded DNA (dsDNA) PCR fragment sequence was calculated with OLIGO Primer Analysis Software and used to compute dsDNA molecules per microgram. Because twice the number of target cDNA copies are needed to produce the same fluorescence signal in real-time quantitative RT-PCR as molecules of dsDNA, each dsDNA molecule number was multiplied by 2 for use as copy number standards for single-stranded cDNA. Once the gene copy number for the standards was known, a 10-fold dilution series over a 10^7 range was established for each gene's standard. *Mj-ba-1* standards were diluted such that their gene copy numbers approximately matched those of the *Mj-cm-1* standard series.

Real-time quantitative RT-PCR: The Taqman assay for real-time quantitative RT-PCR was used in 96-well plate format with the ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Foster City, CA). The procedures described in Gibson et al. (1996), Heid et al. (1996), and the manufacturer's instructions were followed except 25-µl PCR reactions were used instead of 50-µl reactions. The +RT samples were run in triplicate PCR reactions for each gene for each run, while the −RT control samples were run in either triplicate or duplicate PCR reactions. A subset of the standard copy number 10-fold dilution series for each gene, spanning from 10^4 to 10^1 copies, was run in triplicate PCR reactions for each dilution during each machine assay. The dilution standards series subsets also were run on a Stratagene MX4000 (Stratagene, LaJolla, CA), an ABI Prism 7900HT, an ABI Prism 7000, and a Biorad iCycler (Bio-Rad Laboratories, Hercules, CA).

Copy number per nematode calculations: The threshold cycle (Ct) data graphed vs. the log copy numbers for the standard dilution sets became the standard curve from which gene transcript copy number per PCR reaction for each gene could be determined. This was calculated following the procedures outlined in the manufacturer's instructions.

The absolute number of nematodes per reaction was calculated using the known input number of nematodes into the initial total nucleic acid extractions. The number of nematodes per real-time quantitative RT-PCR reaction resulted from the multiplication of the nematode concentration in the RT treatment by the number of microliters of this RT treatment reaction used in the quantitative PCR reaction. The absolute transcript copy number per PCR reaction divided by this number of nematodes per PCR reaction thus yielded the copy number per nematode.

Data comparison: Tissues for the six timepoints used in generating the expression profiles were independently generated at least twice. Some aliquots of the same tissue were independently extracted, and multiple RT was conducted from the same extraction. These independent replications were compiled into the mean and standard error of copies per nematode for each gene at each timepoint for both +RT reactions and −RT control reactions. Transcript levels were "corrected" by subtracting the copies per nematode in −RT control reactions from the corresponding +RT reactions for the timepoint and gene of interest, and the standard error of the +RT reactions was used for each corrected timepoint.

Results

Nematode transcript expression profiles: Optimized *Mj-cm-1* and *Mj-ba-1* Taqman assays for real-time quantitative RT-PCR were used to quantify cDNA from the six different nematode lifecycle timepoints (egg, Inf-J2, 2-day pi, 7-day pi, 14-day pi, and adult), as well as mock-inoculated control roots. Figure 1 shows the transcript expression profile of *Mj-cm-1* corrected for the remaining genomic DNA levels in the −RT control reactions. Table 1 shows the calculated mean copy number per nematode for both the +RT PCR reactions and −RT control PCR reactions of the two genes included in this study. The +RT reactions contained cDNA, while the −RT controls contained only small amounts of genomic DNA (Table 1).

Mj-cm-1 mRNA was expressed at very low levels in the egg and Inf-J2 points (Fig. 1). Transcript levels of *Mj-cm-1* per nematode peaked at tens of thousands of copies at the 2-day pi point, with some measurable mRNA levels in the remaining three timepoints (Fig. 1). In the uncorrected data, the *Mj-cm-1* level appeared to increase again at the 14-day pi and adult timepoints (Table 1). While the +RT reactions showed increased levels, these increases actually were elevated artificially by higher levels of genomic DNA amplification at these timepoints, as evidenced by the −RT controls (Table 1).

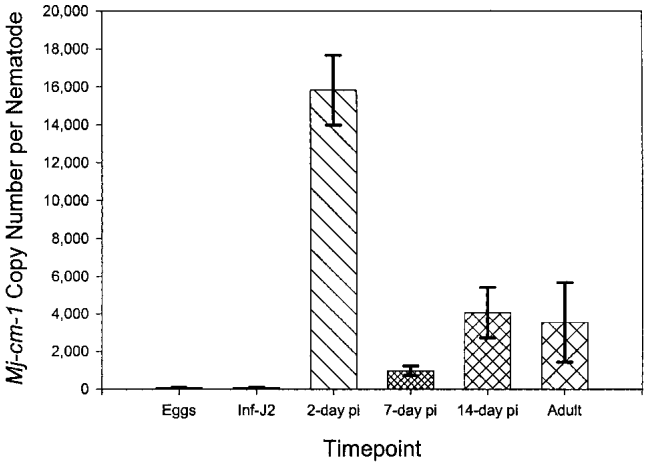

FIG. 1. *Meloidogyne javanica Chorismate Mutase-1 (Mj-cm-1)* transcript expression profile. The corrected per-nematode transcript copy number of *Mj-cm-1* is shown for six timepoints in the *M. javanica* lifecycle: egg, infective second-stage juveniles (Inf-J2), 2-day post inoculation (pi), 7-day pi, 14-day pi, and adult. Each bar is the mean of all +RT quantitative PCR reactions for that timepoint corrected for genomic DNA amplification by subtracting the −RT control mean for that timepoint. Error bars are the standard errors for the +RT reaction group.

Because some *Mj-cm-1* transcript levels were extremely low, the control gene *Mj-ba-1* was employed to demonstrate that all samples contained cDNA template. The same cDNA pools used for *Mj-cm-1* showed a different expression profile for *Mj-ba-1*. *Mj-ba-1* levels remained uniform at the tens of thousands of copies per nematode for the egg, Inf-J2, 2-day pi, and 7-day pi timepoints. The *Mj-ba-1* levels then increased 10-fold for 14-day pi and peaked in the millions for the adult timepoint (Fig. 2). *Mj-ba-1* in the −RT control PCR reactions was minor compared to that of the +RT PCR reactions because of the cDNA-specific nature of the primer and probe set for *Mj-ba-1* (Table 1). Neither *Mj-cm-1* nor *Mj-ba-1* expression was seen in mock-inoculated control roots (data not shown).

Precision of real-time quantitative RT-PCR: Each of the primer and probe sets reproducibly amplified precisely down to 10^2 copies per reaction and sometimes amplified precisely at 10^1 copies. This level of reproducibility was observed on five different quantitative PCR instruments (data not shown). Figure 3 illustrates that there was little variation in per-nematode transcript level between assays (shown only for the 2-day pi timepoint). There was good reproducibility not only between assays of the same cDNA pools but also between different inoculation events (Fig. 3).

TABLE 1. Absolute mean transcript or genomic DNA copy number per nematode of *Meloidogyne javanica chorismate mutase-1* gene (*Mj-cm-1*) and *M. javanica Beta-actin-1* (*Mj-ba-1*) detected using real-time quantitative RT-PCR.[a]

Developmental stage		Gene	
		Mj-cm-1[b]	*Mj-ba-1*[c]
Egg	+RT[d]	112 ± 19	23,334 ± 2,542
	−RT[e]	43 ± 9.17	0.002 ± 0.002
Inf-J2	+RT	126 ± 22	10,380 ± 865
	−RT	54 ± 12	12 ± 4.42
2-day	+RT	16,101 ± 1,841	23,265 ± 2,000
	−RT	282 ± 75	117 ± 42
7-day	+RT	1,106 ± 253	21,338 ± 2,680
	−RT	136 ± 53	41 ± 15
14-day	+RT	5,798 ± 1,347	139,511 ± 33,664
	−RT	1,738 ± 704	11 ± 6
Adult	+RT	10,124 ± 2,118	1,840,552 ± 179,332
	−RT	6,582 ± 1,682	103 ± 58

[a] Data for each reverse transcription (RT) type for each timepoint is a compilation of different independent inoculation events and nucleic acid extractions.
[b] Mean ± Standard Error.
[c] The *Mj-ba-1* primer set (positive control) spanned an intron; the *Mj-cm-1* set did not.
[d] +RT indicates the level of cDNA copies per nematode.
[e] −RT controls show genomic DNA remaining post-DNase I treatment and amplified in PCR reactions, especially *Meloidogyne javanica chorismate mutase-1* (*Mj-cm-1*).

DISCUSSION

Expression of *Mj-cm-1* mRNA changed over the lifecyle of the nematode *M. javanica*. *Mj-cm-1* expression peaked at the 2-day pi timepoint after showing minimal levels at the egg and Inf-J2 timepoints, increasing 100-fold at the 2-day pi point. Slightly elevated *Mj-cm-1* mRNA levels at the 7-day pi and 14-day pi points suggest expression above that of the pre-penetration timepoints (egg and Inf-J2), but not to the level achieved at 2-day pi. However, as the developmental stages of the parasitic nematodes at 7-day pi and 14-day pi were not completely synchronous, the measurable levels may be the result of high *Mj-cm-1* mRNA expression in a few remaining early-stage nematodes in the root. The rela-

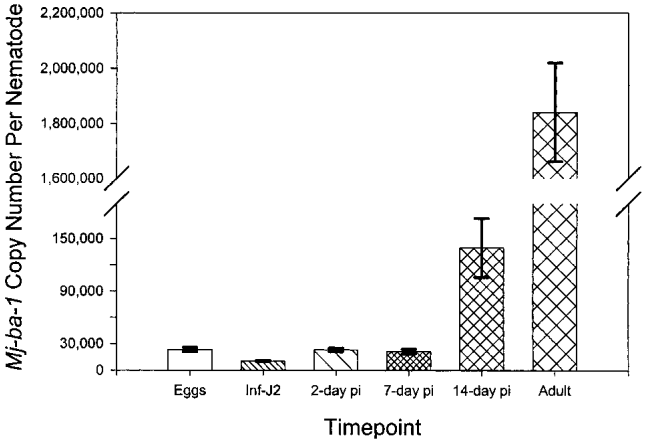

FIG. 2. *Meloidogyne javanica Beta-actin-1 (Mj-ba-1)* transcript expression profile. The corrected per-nematode transcript copy number of *Mj-ba-1* is shown for six timepoints in the *M. javanica* lifecycle: egg, infective second-stage juveniles (Inf-J2), 2-day post inoculation (pi), 7-day pi, 14-day pi, and adult. Each bar is the mean of all +RT quantitative PCR reactions for that timepoint corrected for genomic DNA amplification by subtracting the −RT control mean for that timepoint. Error bars are the standard errors for the +RT reaction group. The break in the graph represents the levels from 190,000 to 1,600,000 copies per nematode.

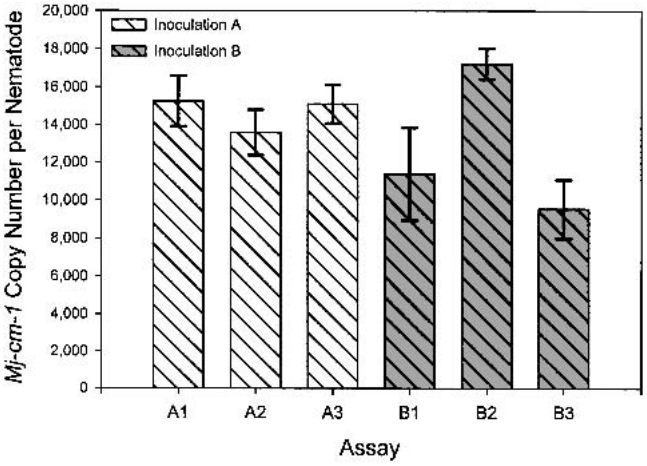

FIG. 3. Precision of real-time quantitative RT-PCR. The corrected per-nematode transcript copy numbers of *Meloidogyne javanica Chorismate Mutase-1* (*Mj-cm-1*) are shown at the 2-day post inoculation (pi) timepoint for three individual assays for each of two completely independent inoculation events A and B. Each bar is the mean of the three +RT PCR reactions during a single real-time quantitative PCR assay corrected for genomic DNA amplification by subtracting the mean of the –RT control reactions for the same sample. Error bars represent the standard error of the +RT reactions.

tively low-level expression of *Mj-cm-1* mRNA detectable above genomic DNA amplification in the adult may be a result of the vast increase in nematode size or cell number at this stage (Huang, 1985).

The variation seen in *Mj-cm-1* mRNA levels between timepoints was not due to a lack of cDNA because comparatively high levels of cDNA for control gene *Mj-ba-1* were detected at all six timepoints tested. *Mj-ba-1* per nematode cDNA levels rose dramatically at both the 14-day pi and adult timepoints, increasing 100-fold by the adult stage. This striking increase in *Mj-ba-1* mRNA expression by the adult stage may reflect the 1,000-fold increase in nematode biomass from the Inf-J2 stage to the adult stage (Huang, 1985). One possible method for normalizing *Mj-ba-1* expression at all timepoints would be to calculate the amount of message per cell; however, the exact changes in cell size and cell number that occur in the *M. javanica* lifecycle are unknown at this time.

There was still a need for a more direct method of data comparison between timepoints than absolute transcript level, because of differences in amounts of overall starting material. For example, free-living Inf-J2 nematodes were relatively easy to obtain in abundance from a hydroponic culture, whereas only a small number of adults were easily collected by hand extraction from root galls. By reporting the data as "copy number per nematode," we were able to compare changes occurring in mRNA levels between nematodes of different timepoints.

Accurately measuring relative mRNA levels in microscopic nematodes is made possible by real-time quantitative RT-PCR, even if those nematodes still remain in the plant root as they did for the 2-day pi, 7-day pi, and 14-day pi timepoints. The mRNA yields from *M. javanica* could be too small for other methods to detect, especially when overwhelmed by the host plant mRNA (Bustin, 2000; Hermsmeier et al, 2000; Reue, 1998). Real-time quantitative RT-PCR provides a precise, sensitive, and message-specific method to trace nematode mRNA levels throughout the developmental points of this plant endoparasite (Gibson et al., 1996; Heid et al., 1996; Lockey et al., 1998; Reue, 1998). Once the real-time quantitative RT-PCR assay was established, turn around time and labor intensity became minimal. With set-up time and run time each less than 2 hours, reliable data can be repeatedly generated in a matter of hours for different gene transcripts from the same small cDNA populations.

The peak of *Mj-cm-1* transcript in *M. javanica* at the 2-day pi timepoint and its esophogeal location (Lambert et al., 1999a) suggest that this nematode gene may be involved in the establishment of parasitism in the plant root. Measurable *Mj-cm-1* mRNA expression remaining at 7-day pi, 14-day pi, and adult timepoints could suggest *Mj-cm-1* also is involved in the maintenance of the nematode feeding sites. This gene encodes a chorismate mutase enzyme involved in the shikimate pathway. The endpoint of this pathway is chorismate, the precursor to aromatic amino acids phenlylalanine, tyrosine, and tryptophan as well as a host of other chorismate-derived compounds (Roberts et al., 2002; Romero et al., 1995). Interference in these plant pathways by a nematode chorismate mutase could potentially disrupt general plant defenses (Maher et al., 1994), alter levels of plant compounds such as salicylic acid (Wildermuth et al., 2001) and indole-3-acetic acid (Bartel, 1997), and even disturb the very fabric of plant cell walls (Whetten and Sederoff, 1995). If *Mj-cm-1* is indeed involved in the establishment of parasitism, the impediment of this nematode gene could serve as a future form of control over plant-parasitic nematodes.

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