The *8D05* Parasitism Gene of *Meloidogyne incognita* Is Required for Successful Infection of Host Roots

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ABSTRACT

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Parasitism genes encode effector proteins that are secreted through the stylet of root-knot nematodes to dramatically modify selected plant cells into giant-cells for feeding. The *Mi8D05* parasitism gene previously identified was confirmed to encode a novel protein of 382 amino acids that had only one database homolog identified on contig 2374 within the *Meloidogyne hapla* genome. *Mi8D05* expression peaked in *M. incognita* parasitic second-stage juveniles within host roots and its encoded protein was limited to the subventral esophageal gland cells that produce proteins

secreted from the stylet. Constitutive expression of *Mi8D05* in transformed *Arabidopsis thaliana* plants induced accelerated shoot growth and early flowering but had no visible effects on root growth. Independent lines of transgenic *Arabidopsis* that expressed a double-stranded RNA complementary to *Mi8D05* in host-derived RNA interference (RNAi) tests had up to 90% reduction in infection by *M. incognita* compared with wild-type control plants, suggesting that *Mi8D05* plays a critical role in parasitism by the root-knot nematode. Yeast two-hybrid experiments confirmed the specific interaction of the *Mi8D05* protein with plant aquaporin tonoplast intrinsic protein 2 (TIP2) and provided evidence that the *Mi8D05* effector may help regulate solute and water transport within giant-cells to promote the parasitic interaction.

The ability to infect the roots of over 1,700 different plant species has made the root-knot nematodes (*Meloidogyne* spp.) one of the most successful and devastating parasites of crops found worldwide (5,11,48). Infective second-stage juveniles (J2) completely penetrate plant roots and migrate intercellularly to the root vascular tissue where they induce the formation of localized feeding sites called giant-cells from immature plant vascular cells (33). The giant-cells become the essential, permanent food source for the invading nematode as it assumes a sedentary lifestyle through growth and successive molts towards the pyroform reproductive adult stage. Morphological and molecular studies of giant-cell ontogeny suggest that root-knot nematodes manipulate fundamental plant cell biology to achieve the phenotype of giant-cells in such a wide array of plant species (1,4,20,21,34). The nematodes feed from giant-cells using the hollow, protrusible stylet in their head, and they also use the stylet to secrete effector proteins into plant cells to initiate and maintain the feeding site (13,15,45). The secreted effector proteins are encoded by parasitism genes that are specifically and developmentally expressed in three large secretory gland cells within the nematode esophagus (14,15,31). The two subventral esophageal gland cells are active mainly during root penetration and migration by the nematode, and become less active as the single dorsal gland cell grows in size and activity during feeding site initiation and maintenance throughout the parasitic cycle of the biotroph. Investigations to date have identified multiple parasitism genes in the

sedentary root-knot and cyst endoparasitic nematodes through analyses of developmentally expressed genes (13,15,45) and secretome prediction from genomic analyses (2,39).

Functional analyses of predicted effector molecules encoded by different phytoparasitic nematode parasitism genes have remained a challenge because the nematodes are microscopic biotrophs embedded within plant roots and they are currently not amenable to molecular transformation. The adoption of the model plant species *Arabidopsis thaliana* (50) for studies of parasitism gene function has, however, provided a wealth of technical and genetic resources on the host side of the plant–nematode interaction. Constitutive expression of individual nematode parasitism genes in transformed plant roots has been used for initial assessments of expressed effector effects on plant phenotype and susceptibility to nematode infection (18,24,25,28,35,41,42,54,56). The utility of the RNA interference (RNAi) technology developed in *Caenorhabditis elegans* (19) has also provided a powerful tool to specifically silence target phytonematode parasitism genes (46) through assays that either soak infective J2 (3,10) in solutions of double-stranded RNA (dsRNA) or provide dsRNA complementary to target parasitism genes for nematode ingestion from within transgenic plants (27,41,42,51). Yeast two-hybrid screens using single expressed parasitism proteins as baits and total root proteins (expressed from root cDNA libraries) as prey (24,25,28,35, 41,44) have begun to identify specific interactions between nematode effectors and host proteins that appear to manipulate different cellular processes in plants (21,45).

Greater than 40 parasitism genes were identified in the root-knot nematode, *Meloidogyne incognita*, using esophageal gland cell microaspiration and transcript mining assays (29,30). While a number of those root-knot nematode parasitism genes encoded effector proteins like cell wall-modifying enzymes and a few

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other database orthologs, approximately 70% of the *M. incognita* parasitism genes encoded novel effector proteins. The 8D05 parasitism gene of *M. incognita* encodes one of those novel effector proteins and is somewhat unusual in that 8D05 is expressed in the nematode's subventral esophageal gland cells through the entire life cycle (30). Analyses presented here suggest that 8D05 is critical for parasitic success of *M. incognita* and that it interacts specifically with host plant tonoplast intrinsic protein 2 (TIP2).

MATERIALS AND METHODS

Nematodes and infection assays. The root-knot nematode, *M. incognita* race 4, was propagated in the greenhouse on roots of soil-grown tomato plants (*Solanum lycopersicum* 'Rutgers') and *M. incognita* eggs were extracted from egg masses on root galls using 0.5% sodium hypochlorite as previously described (32). Nematode eggs were hatched over water at 28°C on Baermann pans for 48 h and the hatched preparasitic second-stage juveniles (pre-J2s) were collected for use in root infection assays or frozen at -80°C for subsequent nucleic acid extractions. Mixed parasitic stages of *M. incognita* were collected for gene expression analysis from within roots of tomato plants by gentle root blending and sieving as described in Ding et al. (17). Intact *M. incognita* life stages extracted from tomato roots were separated into groups of parasitic J2 (para-J2), J3, J4, and adult female stages under a dissecting microscope and frozen at -80°C for subsequent use in the quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) experiments described below.

Arabidopsis thaliana ecotype Columbia (Col-0) was used as wild type to generate all transgenic plant lines described below and for infection assays with *M. incognita*. For nematode infection assays, all *Arabidopsis* seeds were surface sterilized with 10% sodium hypochlorite and 0.01% Tween 20 for 3 min followed by 1 min with 70% ethanol. Seeds were washed five times with sterile distilled water to remove residual sterilization solution. Five days after seed germination on MS medium (38), the *Arabidopsis* seedlings were transplanted into 3-inch plastic pots of soil (93.3% sand, 3.3% silt, and 3.4% clay), one plant per pot. One week after transplanting, 1,000 freshly hatched J2 of *M. incognita* were added to the soil at the base of each *Arabidopsis* test plant. All treated plants were fertilized with liquid Knops medium (50) once a week in the first 2 weeks after transplanting. At 30 days postinoculation, root systems were gently washed in water, blotted dry to assess root fresh weight, and the number of galls on each root system was counted under a dissecting microscope. The mean and standard error of 20 replicates per treatment were calculated and statistical differences in mean ($n = 20$) were determined by the paired *t* test with an alpha level of 0.05 using SAS software (SAS Institute, Cary, NC).

Analyses of the *M. incognita* 8D05 gene and protein. Genomic DNA was extracted from J2 of *M. incognita* as previously described (41). The genomic DNA sequence of *Mi8D05* was obtained from the PCR product using the gene-specific primers 8D05-FL-F1 (5'-TAGTCAGTCATTTAAATAATTTAAT-3') and 8D05-FL-R1 (5'-GTTTAATTCACATTTTTCACATCAT-3'), which were designed on the cDNA sequence deposited in GenBank (AF531169). The full-length *Mi8D05* genomic DNA sequence was used for nucleotide search of the genomes of *M. hapla*, *Heterodera glycines*, and *C. elegans* in NCBI. Software Vector NTI (Invitrogen, Grand Island, NY) was used to analyze the DNA and amino acid sequences. Predicted secretion signal peptide and protein structure were analyzed, respectively, by SignalP 3.0. (6) and SMART (49) software.

Polyclonal antiserum to Mi8D05 was produced by immunizing rabbits with synthetic peptides 8D05-1 (CYDDNEKADYGD LAAELAK) and 8D05-2 (CKRHGRPHRLNDWNNPFP) (Alpha Diagnostic Intl. Inc., San Antonio, TX). Peptide affinity-purified

8D05 polyclonal antisera were used to localize 8D05 expression within specimens of *M. incognita* using immunofluorescence microscopy (28).

Transgenic *Arabidopsis* that expressed 8D05 and complementary RNAi. For overexpression in plants, the open reading frame (ORF) of parasitism gene *Mi8D05* without signal peptide was amplified by primers 8D05-pBI-wosp-F1 (5'-CGGATCCAT GGTAACCGAGAATAAGAAT-3') and 8D05-pBI-wosp-R1 (5'-GAGCTCTTAGTTGTGATAAAAATGGATT-3'). The PCR product was digested with *Bam*H1 and *Sac*I and then cloned into vector pBI121 (9) under the control of CaMV 35S promoter. The identity, orientation, and junctions of the resulting 35S::8D05 construct were confirmed by PCR and DNA sequencing.

To express hairpin dsRNA for in planta RNAi assays, the sense and antisense ORF sequences of 8D05 were amplified by primers 8D05-RNAi-HindIII-Xho (5'-AAGCTTCTCGAGATGTTTTTAC AAAAAC-3'), which introduces sense into *Xho*I site and antisense into *Hind*III site, and 8D05-RNAi-Cla-Kpn (5'-ATCGA TGGTACCTTAGTTGTGATAAAAAT-3'), which introduces sense into *Kpn*I site and antisense into *Cla*I site of pHANNIBAL (57) behind the CaMV 35S promoter. The 8D05-RNAi construct made in pHANNIBAL was isolated by restriction digestion with *Not*I enzyme and cloned into the pART27 binary vector (22) for subsequent *Agrobacterium* and plant transformation, resulting in pART-8D05.

Plasmids pBI-8D05 and pART-8D05 were introduced into *Agrobacterium tumefaciens* C58C1 by electroporation, verified by PCR, and transformed into *Arabidopsis thaliana* wild-type Col-0 plants by floral dip (12). Transgenic seeds were selected on MS media supplemented with kanamycin at 50 mg/liter. Segregation analyses identified homozygous lines and the resultant homozygous plants were characterized for construct expression using RT-PCR. The homozygous transgenic lines were monitored for potential effects of pBI-8D05 and pART-8D05 expression on plant growth phenotype ($n = 12$) and also subjected to infection assays with *M. incognita* as described above.

Interaction of Mi8D05 with plant proteins. To identify potential plant proteins that could potentially interact with Mi8D05 within plant cells during infection, yeast two-hybrid screenings were performed as described in the BD Matchmaker Library Construction and Screening Kits user manual (Clontech Laboratories, Mountain View, CA). The cDNA encoding the mature peptide of Mi8D05 without signal peptide was cloned in-frame into the GAL4-binding domain of pGBKT7 by using primers containing *Eco*RI (5'-GAATTCGTAACCGAGAATAAGAATA-3') and *Bam*H1 (5'-GCGGATCCCATCTTTTTCACATCAT T-3') restriction sites, respectively to generate pGBKT7-8D05 and expressed as bait to screen tomato root cDNA prey libraries constructed in the GAL4 activation domain of the pGADT7-Rec vector. The root cDNA libraries were constructed from mRNAs extracted from noninfected tomato roots (28) and from tomato roots at 7 days after infection by *M. incognita* (to enhance for plant genes and products upregulated in feeding sites). Positive yeast two-hybrid matings were selected on a series of selective media as described in the protocol of the Matchmaker yeast two-hybrid system and subjected to subsequent co-transformation of isolated clones to validate positive protein-protein interactions (25).

Quantitative analyses of gene expression. qRT-PCR was used to analyze the developmental expression of 8D05 within *M. incognita* and the gene encoding the tomato tonoplast intrinsic protein 2 (*tip2*; AY731066) that was confirmed to interact with Mi8D05 in yeast two-hybrid screens. Total RNA was extracted from 50 mg each of nematode tissue from pre-J2, para-J2, J3, J4, and female *M. incognita* life stages using the RNeasy Mini Kit (Qiagen, Valencia, CA). The RNeasy Plant Mini Kit (Qiagen) was used to isolate total RNA from 100 mg of frozen, ground tomato root tissue isolated at 5, 14, and 24 days after infection by

M. incognita. For each sample, 150 ng of DNase-treated RNA was used for cDNA synthesis and the cDNAs were generated by PCR amplification using SuperScript II Reverse Transcriptase (Invitrogen).

Gene-specific primers for *8D05* (AF531169), *tip2* (AY731066), *M. incognita* actin (BE225475), and tomato actin (U60481) were designed for quantitative analyses. All qRT-PCR runs were performed in a DNA Engine Mx3000P instrument (Agilent Technologies, Santa Clara, CA). A single 20- μ l PCR reaction included 1 $\times$ Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA), 2 μ l of cDNA template, and 5 μ M each forward and reverse primers. The PCR cycling parameters were set at 95°C for 10 min followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s. The dissociation melt curve analysis in which all products generated during the PCR amplification reaction were melted at 95°C for 1 min, then annealed at 55°C for 30 s, and subjected to gradual increases in temperature was conducted to discount the effects of primer-dimer formation and contamination. The qRT-PCR reactions were performed in triplicate and the negative controls included water and mRNA extracted from the nematodes to check for DNA contamination in the analyzed samples. All qRT-PCR samples were normalized against the tomato actin gene (U60481) or the *M. incognita* nematode actin gene (BE225475) as appropriate. Three biological replicates (separate tissue samples) were analyzed per treatment and a composite result was derived for comparative analyses. The paired *t* test with an alpha level of 0.05 was used to compare relative transcript level means from normalized qRT-PCR measurements using the statistical software package of SAS. The fold-change relative to control plants was calculated from normalized qRT-PCR measurements according to the $2^{-\Delta\Delta CT}$ method (36).

RESULTS

Analyses of the *Mi8D05* sequence, expression, and its encoded protein. Nucleotide sequence of the original *Mi8D05* cDNA pGEM clone, *8D05* cDNA derived from mRNA expressed in *M. incognita* nematode specimens, and genomic sequence of *Mi8D05* in these studies indicated that the original GenBank submission (AF531169) was missing a nucleotide at position 1179 resulting in a false frame-shift and truncation of the c-terminal region of the predicted protein. Thus, the *Mi8D05* coding sequence identified in these studies was used for all corresponding assays and comparative analyses. The longest ORF of the *Mi8D05* cDNA (1,149 bp) encoded a deduced protein (Fig. 1) of 382 amino acids (aa) that included a 22-aa N-terminal signal peptide as predicted by Signal P (6). A 1,414-bp genomic DNA fragment of *Mi8D05* (HQ728271) was isolated by PCR using primers designed based on the cDNA sequence of *Mi8D05* (AF531169). Analysis of the 1,414-bp sequence identified one intron of 163 bp in the 5'-UTR but no intron sequences were identified within the predicted ORF.

BLAST of the 1,414-bp genomic sequence of *Mi8D05* against the complete genome sequences of *M. hapla*, *H. glycines*, and *C. elegans* identified a 1,602-bp fragment (designated as *Mh8D05*) on contig 2374 (ABLG01002375) of the *M. hapla* genome (39) that has 75% nucleotide identity with *Mi8D05*. The longest ORF of the *Mh8D05* (1,335 bp) encoded a deduced protein of 444 aa including a 23-aa N-terminal signal peptide as predicted by Signal P (6). Analysis of the 1,602-bp *Mh8D05* genomic sequence identified one intron of 160 bp that was upstream of the predicted ORF, but similar to *Mi8D05*, no intron sequences were identified within the *Mh8D05* ORF. Comparison of amino acid sequences between *Mi8D05* and *Mh8D05* indicated that they have 55% similarity (Fig. 1). SMART (49) analyses predicted two regions of 38 and 36 aa located between amino acids 53 to 90 and 159 to 194 that could form a coil-coil motif within *Mi8D05*. A similar coil-coil motif was predicted in *Mh8D05* between 58 to 99 and 178 to 206 aa. No significant similarity in *8D05* nucleotide or predicted peptide sequence was identified within the *H. glycines* or *C. elegans* genomes or through more comprehensive database searches.

The developmental expression of *8D05* in five *M. incognita* life stages was assessed (Fig. 2) using qRT-PCR. *Mi8D05* mRNA expression peaked in the parasitic J2 life stage (3 to 5 days postinfection) at about fourfold higher expression level than observed in preparasitic J2, J3, J4, and adult female life stages.

Mi8D05	MFLQKQL-LFLVLLAFSLVKGVTENKKNSEIKNETT---TKVIQTSTGGYDDNEKAD
Mh8D05	MSLQKQLFLVFLPILFSLIEGVNDKNNKNETKDETTTITTKVTQITGGYDDKETAD
	* **** * : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *
	Signal peptide
Mi8D05	YGDIAELAKLVEEDELNKKKNLSSSENGNKS--TGKPYIQKDKSKKYLEEDKGYEE
Mh8D05	YGALAEALAEADEENKLNKKEDLSSVDLKKEDLKENPSNLKDKSINYVGNGKEFEK
	** **** * : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *
	Coil-coil motif I
Mi8D05	RNSRNKYENS-----ET---HESESGSSDEDLDEDNLERLPGPSHPNEGISRR
Mh8D05	GTV-NKYGDNFEDVKLDEEDSGLSTESSSEGKNSDEDNAGLQGTGKPHNEGWPRR
	. *** : : : : : : : : : : : : * : * : * : * : * : * : * : * : * : * : *
Mi8D05	RVEKEKGGDEEEEEEKEQEN---SNDK---EERKKRNTKYNPKDESEEDISFDGQI
Mh8D05	RIEKEEDKKEVDKEDEEEDEEEEREQNPKHDPKHDEGRKKRYKNRKNKDEDELDQOI
	* : * : : : : : : : : : : : * : : : : : * : : : : : * : : : : : * : : : : *
	Coil-coil motif II
Mi8D05	PKSVRLKLLQLAAGGKNPVIIPLIINNINPNR-RED---ESEWNKRHRGRPH----R-
Mh8D05	PKSLRLKLLQLAANGKNPLIIPLIINNINILPNGREEDDEEYKRGKKGKHEGRNERL
	*** : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *
Mi8D05	---LNDWNNPFPFQSSMFQPPMFPPFPQPPFQPPFGPPTFAHNSLPGGGLGGGLA-
Mh8D05	GKPPLDWYNP--HNFQSPFFPFPFPPQMFPIFPQQQ-PPQLGSS-FGVSGPLGGQQQF
	** * : : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *
Mi8D05	-----GSL-PNTNPFSLNLRGVSPNQFPNPPSNHVP-----PFGQ
Mh8D05	GLNLGGVPPSGNPP-TFTNPFPPNPPFPPTQNGPFGQPNQIPYLNKNINQPSHFGP
	* : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *
Mi8D05	QNQFYPPQQQQQNVNPGAGDGNVKKVN-
Mh8D05	PNQFPSPQQQPNQINPGQINGDGALKKTN
	** * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : * : *

Fig. 1. Alignment of the predicted amino acid sequences derived using Vector NTI Suite (Invitrogen) from the nucleic acid sequence of the novel *8D05* parasitism gene of the root-knot nematodes *Meloidogyne incognita* (Mi) and *M. hapla* (Mh). Included are amino acid sequences predicted from a 1,414-bp *Mi8D05* genomic clone (HQ728271) and from a 1,602-bp *Mh8D05* genomic DNA sequence identified on contig 2374 (ABLG01002375) of the *M. hapla* genome (39). A 55% predicted amino acid similarity was observed between *Mi8D05* and *Mh8D05* and each had a secretion signal peptide (underlined) at the N terminus as predicted by SignalP (6). A conserved coil-coil motif predicted using SMART (49) was identified (underlined) at amino acids 53 to 90 and 159 to 194 of *Mi8D05* and at amino acids 58 to 99 and 178 to 206 of *Mh8D05*.

Fig. 2. Quantitative expression of *Mi8D05* within developmental life stages of the root-knot nematode *Meloidogyne incognita* as determined by quantitative real-time reverse-transcription polymerase chain reaction. The relative fold-change values were calculated from three biological replicates using the $2^{-\Delta\Delta CT}$ method (36) and represent changes in mRNA expression in nematode preparasitic J2 (Pre-J2), late parasitic J2 (Par-J2), J3, J4, and adult female life stages. The *M. incognita* actin gene (BE225475) was used as an internal control to normalize gene expression levels for all samples. Bars represent the standard error of the mean and significant ($P \leq 0.05$) differences between values (*) were derived by paired *t* test.

Purified Mi8D05 antiserum bound to secretory granules specifically within the subventral gland cells of preparasitic and parasitic J2 and their cytoplasmic extensions and expanded ampullae, which are located posterior to the pump chamber of the metacarpus (Fig. 3). No specific labeling with the rabbit preimmune serum was observed in any nematode specimens.

Overexpression and RNAi of *Mi8D05* in *Arabidopsis*. Constitutive expression of *Mi8D05* was confirmed in four homozygous transgenic lines of *Arabidopsis* using RT-PCR (data not shown). Expression of *Mi8D05* with and without its native signal peptide significantly accelerated shoot growth and early flowering (Fig. 4A and B). Siliques also matured earlier in the *Mi8D05* overexpression plants, but no visible differences in final plant heights or phenotypes were observed between *Mi8D05* overexpression plants and wild-type *Arabidopsis* once all plants had reached maturity. Interestingly, no differences in root growth rate or phenotype were observed between *Mi8D05* overexpression plants and wild-type *Arabidopsis* (Fig. 4C). An increase in susceptibility to infection by *M. incognita* was observed in all *Mi8D05* overexpression lines compared with wild-type *Arabidopsis* although the values were not significantly ($P \leq 0.05$) different (Fig. 4D).

The expression of the *Mi8D05* hairpin dsRNA was confirmed in seven homozygous transgenic RNAi lines of *Arabidopsis* by RT-PCR of the PDK intron (data not shown) that separates the expressed complementary dsRNA arms when using the pHANNIBAL vector (57). No visible differences in root or shoot development and phenotype were observed between *Mi8D05*-RNAi lines and wild-type *Arabidopsis*. Infection of all *Mi8D05*-RNAi lines by *M. incognita* was reduced compared with wild-type *Arabidopsis* with six *Mi8D05*-RNAi lines having significantly ($P \leq 0.05$) reduced infection rates (Fig. 5). Reduction in *M. incognita* infection among all *Mi8D05*-RNAi lines compared with wild-type plants ranged from 27 to 90%, with four lines that reduced *M. incognita* infection between 73 to 90%.

The interaction of *Mi8D05* with plant host proteins. In the initial yeast two-hybrid screen that used *Mi8D05* as bait and proteins expressed from a cDNA library constructed from unchallenged tomato roots (28) as prey, multiple interacting plant proteins in the major intrinsic protein (MIP) family (37) were isolated. The most highly represented *Mi8D05*-interacting MIP proteins included the plant aquaporins tonoplast intrinsic protein 2 (TIP2; AY731066) in truncated and full-length forms, tonoplast intrinsic protein 4 (TIP4; AB211518), and plasmalemma intrinsic protein 2 (PIP2; X73847, X73848). A subsequent yeast two-hybrid screen using *Mi8D05* as bait and prey proteins expressed from a cDNA library that was constructed from tomato roots at

7 days postinfection with *M. incognita* isolated multiple interactions with TIP2 (AY731066) almost exclusively (data not shown). Cotransformation assays in yeast between *Mi8D05* and TIP2 confirmed the specificity of the interaction.

The expression of *tip2* (AY731066) in tomato roots was monitored by qRT-PCR over several time points during infection by *M. incognita* and compared with tomato actin (U60481) expression to normalize the data. Expression levels of *tip2* were each about onefold higher than baseline (actin) expression levels in both uninfected (check) tomato roots and tomato roots at 7 days postinfection by *M. incognita*, but *tip2* expression was significantly reduced compared with uninfected roots of check plants by 14 and 24 days postinfection by *M. incognita* (Fig. 6).

DISCUSSION

The results of the functional analyses presented here suggest that the *Mi8D05* parasitism gene produces a novel secreted effector protein that plays an important role in the parasitic interaction of root-knot nematodes with host plants. *Mi8D05* peaks in expression in the parasitic J2 stage of *M. incognita*, the time when induction and early formation of giant-cells occurs (34). Relatively rapid induction in expression in parasitic J2 that have entered plant roots has been observed with several nematode parasitism genes to date (23–25), suggesting a developmental role in the early stages of the parasitic process. Immunodetection of the translated *Mi8D05* protein specifically within the subventral gland cells of early stages of *M. incognita* indicates the potential for the effector to be secreted into host plant tissues (15,31). Only one database match of *Mi8D05* was identified, a 1,602-bp genomic fragment (*Mh8D05*) on contig 2374 (ABLG01002375) of the *M. hapla* genome (39), indicating that *8D05* is a novel protein that may exist only within species of root-knot nematodes. The presence of a predicted secretion signal peptide, a relatively high similarity in protein size and sequence, and two conserved predicted coil-coil motifs suggest that *Mi8D05* and *Mh8D05* are homologs.

Constitutive expression of individual nematode parasitism genes in transformed *Arabidopsis* plants has served as a crude visual indicator of effector activity within host plant cells (24,25, 35,54–56). As in plants that express other nematode parasitism genes (23–25,41), plants that overexpressed *Mi8D05* were more susceptible to nematode infection (although not statistically significant with *Mi8D05*). Visible changes in shoot phenotype in *Arabidopsis* that overexpressed *Mi8D05* have been observed with other parasitism genes (25,54–56), although the physiological basis of the accelerated shoot growth during *Mi8D05* expression

Fig. 3. Binding of antisera raised to predicted peptides within the *Mi8D05* sequence within a parasitic J2 of *Meloidogyne incognita* that was excised from infected plant roots. **A**, Fluorescent (FITC) labeling indicates specific binding of the *Mi8D05* antisera to proteins in granules within the two subventral esophageal gland cells (SvG) posterior to the nematode metacarpus (M). **B**, Light micrograph of the specimen shown in panel A. Scale bar represents 10 μ m.

remains unknown at present. It is interesting that effectors of root parasitic nematodes can have specific effects in plant shoot growth, confirming hypotheses that root-knot nematodes recruit a number of diverse plant developmental processes to form giant-cells (1,8,20,21). The specific acceleration of shoot growth (but not root growth) in *Mi8D05* overexpression plants is just the opposite of the specific acceleration in only root growth observed in *Arabidopsis* plants that overexpress *Mi16D10* (28). The *Mi16D10* protein, however, was demonstrated to interact with members of the SCARECROW-like transcription factor family (28) that specifically regulate root cell growth and differentiation (43).

Very dramatic and significant reductions in infection rates of *M. incognita* were demonstrated in multiple lines of transgenic *Mi8D05*-RNAi *Arabidopsis* plants. Up to 90% reduction in gall formation was observed in *Mi8D05*-RNAi *Arabidopsis* plants, almost to the levels of reduction in *M. incognita* infection observed in *Mi16D10*-RNAi plants (27). Quantitative investigations of *8D05* siRNA production in the different transgenic RNAi plant lines using high-throughput RNA sequencing technologies (16,52) may shed light on the differential nematode infection rates observed among *Mi8D05*-RNAi lines. Quantitative assessment of PDK intron expression in RNAi constructs (not conducted here) could be used to provide a relative comparison of transgene ex-

Fig. 5. Infection of plant roots (galls/gram of root) by *Meloidogyne incognita* decreased in all lines (L5, L7, L4, L11, and L1) of transgenic *Arabidopsis thaliana* that expressed double-stranded RNA (RNAi) complementary to the *Mi8D05* parasitism gene coding sequence compared with wild-type (WT) *Arabidopsis*. Bars represent the standard error of the mean and significant ($P \leq 0.05$) differences between values (*) were derived by paired *t* test.

Fig. 4. Growth and nematode infection of transgenic *Arabidopsis thaliana* plants that constitutively expressed cDNA of the *Mi8D05* parasitism gene of *Meloidogyne incognita*. **A**, Accelerated plant shoot development, early flowering, and **B**, increased plant height were observed at 26 days after germination in transgenic *Arabidopsis* lines (L2, L10, L11, and L12) that constitutively expressed the *Mi8D05* parasitism gene compared with wild-type (WT) *Arabidopsis* (Col-0). **C**, No significant differences in root growth were observed between WT and any transgenic *Arabidopsis* lines that constitutively expressed the *Mi8D05* parasitism gene at any developmental stage. **D**, An increase (not statistically significant) in root infection rate (gall number) by *M. incognita* was observed in all transgenic *Arabidopsis* lines that constitutively expressed the *Mi8D05* parasitism gene compared with WT. Bars represent the standard error of the mean and significant ($P \leq 0.05$) differences between values (*) were derived by paired *t* test.

Fig. 6. Relative expression of the endogenous tomato *tip2* (AY731066) gene that encodes the tonoplast intrinsic protein 2 (TIP2) aquaporin found to interact with the Mi8D05 effector protein of *Meloidogyne incognita*. Expression of tomato *tip2* was monitored in roots of noninfected check (CK) roots and tomato roots at 5, 14, and 24 days postinfection with the root-knot nematode (RKN), *M. incognita*. The relative fold-change values were calculated using the $2^{-\Delta\Delta CT}$ method (36) and represent differences in mRNA expression levels between infected and noninfected roots at each time point. The tomato actin gene (U60481) was used as an internal control to normalize gene expression levels for all samples. Bars represent the standard error of the mean and significant ($P \leq 0.05$) differences between values (*) were derived by paired *t* test.

pression levels among lines, but assay of the siRNA levels would provide the most accurate measure of processed bioactive RNA molecules to relate to nematode infection levels. Essentially every *Mi8D05*-RNAi line tested had significant reductions in *M. incognita* infection rates, however, indicating that the *Mi8D05* parasitism gene (and its encoded effector) plays a critical role in the parasitic process of the root-knot nematode.

The specific interaction of the Mi8D05 protein with plant aquaporins (37), primarily tonoplast intrinsic protein 2 (TIP2) from tomato roots (AY731066) that had been infected with *M. incognita*, suggests a potential role for the 8D05 effector protein in regulating water and solute transport in giant-cells. Interestingly, one of the first root-knot nematode-inducible plant promoters identified (40) increased specific expression of the *TobRb7* aquaporin gene in giant-cells, and RB7 aquaporins are now considered members of the TIP family of plant proteins (47,53). Given the similarities in metabolic upregulation within both syncytia formed by cyst nematodes and giant-cells formed by root-knot nematodes, high solute concentrations (7,26) that lead to increased turgor pressure in feeding cells would require careful regulation to maintain feeding cell integrity. This potential regulation may occur at the protein interaction level in the early stages of parasitism when transcripts encoding both the Mi8D05 effector and plant TIP2 proteins are most highly expressed and giant-cell formation has commenced. By 14 days postinfection with root-knot nematodes, the giant-cells are fully formed (34) and *Mi8D05* and *tip2* expression have been minimized. Subsequent high-resolution localization experiments of Mi8D05 and TIP2 interactions within host plant cells combined with functional data using *tip2* mutant plants, *tip2* overexpression, and physiological indicators of cellular water and solute transport (37) will refine our understanding of the functional role of the Mi8D05 effector in parasitism.

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