Ingestion of Double-Stranded RNA by Preparasitic Juvenile Cyst Nematodes Leads to RNA Interference

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RNA interference is of value in determining gene function in many organisms. Plant parasitic nematodes are refractory to microinjection as a means of introducing RNA and do not show any oral uptake until they are within plants. We have used octopamine to stimulate uptake by preparasitic second stage juveniles of two cyst nematodes, *Heterodera glycines* and *Globodera pallida*. This new technique was used to facilitate uptake of double stranded RNA (dsRNA) together with fluorescein isothiocyanate as a visual marker. Targeting cysteine proteinases did not reduce the number of parasites but caused a shift from the normal female/male ratio of 3:1 to 1:1 by 14 days postinfection (dpi). Exposure of *H. glycines* to dsRNA corresponding to a newly characterized protein with homology to C-type lectins did not affect sexual fate, but 41% fewer parasites were recovered from the plants. As expected, treatment with dsRNA corresponding to the major sperm protein (MSP) had no effect on either parasite development or sexual fate over 14 days. Northern analysis showed lower transcript abundance for the two targeted mRNAs that occur in J2, plus a later inhibition for MSP transcripts when males developed sperm at 15 dpi. These findings establish a procedure for RNAi of plant parasitic nematodes.

Nematodes show highly complex parasitic interactions with plants and cause over \$100 billion of annual crop losses to world agriculture (Sasser and Freckman 1987). Cyst-nematodes such as *Globodera pallida* and *Heterodera glycines* are important examples and key pests of potato and soybean, respectively. Cyst-nematodes are obligate parasites and take several weeks to complete their life cycle. The second-stage juvenile (J2) of a cyst nematode hatches from a previously dormant egg, often following stimulation by a chemical factor diffusing from host roots. Its small size (approximately 450 µm long and 18 µm wide) enables the J2 to migrate through plant root cells from the zone of elongation to a site by the vascular tissue where feeding is initiated. The established J2 modifies the plant cell, causing progressive wall loss and a merging of cytoplasm. The resultant large syncytial cell, essential for female development, acts as a transfer cell providing nutrient flow to the now sedentary parasite. Both sexes feed as second and third stage juvenile (J2, J3) parasites and sexual dimorphism arises. The males do not feed in later stages, and they regain

mobility as adults before fertilizing the sedentary, saccate female. The J3 female remains sedentary and grows at a rapid rate to reach J4 stage. A mature female is approximately 100-fold larger than the male. It is the female whose feeding causes much of the crop loss associated with these major pests. After fertilization, the female body wall tans to form a protective cyst around many of the several hundred eggs, each containing a J2. Many of this next generation can enter dormancy until another host crop is grown.

As obligate parasites with a life cycle of several weeks, cyst nematodes prove refractory to studies aimed at defining genes essential for development and plant parasitism. The full range of genetic approaches that has been deployed so incisively with the microbivorous nematode *Caenorhabditis elegans* is not readily applied to them. Fire and associates (1998) reported that RNA interference (RNAi) is a means by which double stranded RNA (dsRNA) induces sequence-specific posttranscriptional gene silencing. In *C. elegans* postgenomic studies have utilized the power of RNAi to determine gene function. RNAi has been used to systematically analyze 90% of the predicted genes on chromosome I (Frasier et al. 2000), 96% of the predicted genes on chromosome III (Gonczy et al. 2000), approximately 2,500 nonredundant cDNAs (Maeda et al. 2001), and the function of 350 oocyte cDNAs (Piano et al. 2000). These projects have rapidly advanced the understanding of gene function in *C. elegans*. The mode of action by which RNAi operates is being elucidated. Long dsRNA sequences are more active than short dsRNAs in effecting gene silencing (Sharp 2001). The long dsRNA is processed into short interfering RNAs (siRNAs) of 21 to 23 nucleotides by an RNase III-like activity. These siRNAs mediate cleavage of mRNA through sequence complementarity (Elbashir et al. 2001; Hammond et al. 2000; Zamore et al. 2000). The mode of action of RNAi has been reviewed in detail (Sharp 2001).

Fire and associates (1998) demonstrated that RNAi abolished expression of targeted genes in *C. elegans* by injecting dsRNA. Subsequently, others have shown that the effect occurs when *C. elegans* is fed the bacterium *Escherichia coli*, which transcribes the recombinant dsRNA (Timmons and Fire 1998). It also results from simply soaking the animals in dsRNA preparations (Tabara et al. 1998). In animal parasites, electroporation has been used as a means of delivering dsRNA to *Trypanosoma brucei* (Ngo et al. 1998). These modes of dsRNA delivery are not readily applied to plant parasitic nematodes. The small size and well-formed cuticle of a cyst nematode J2 make microinjection both difficult and dependent on special equipment, and this technique has yet to be routinely employed. Delivery of dsRNA by ingestion is difficult, as the nematode only feeds following the establishment of a feeding site inside the root and does not ingest substances prior

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to this stage. Here, we report a procedure that results in the uptake of dsRNA molecules by J2s, thus enabling RNAi to be applied to cyst nematodes and the approach to be used to study the effects of RNAi on three genes. The approach should help define nematode gene expression that is essential for their successful interaction with plants.

RESULTS

Uptake of fluorescein isothiocyanate (FITC) by cyst nematodes stimulated by octopamine.

Analogues of the serotonergic neurochemical 5-hydroxy tryptamine (5HT, serotonin) induce plant parasitic nematodes to release pharyngeal gland secretions (Willats et al. 1995). In *C. elegans*, a biologically active amine, octopamine, has the opposite effect of 5HT (Horvitz et al. 1982). In the expectation that the opposing effect may be true in tylenchids, we examined the possibility that octopamine may affect pharyngeal pumping and ingestion. This was demonstrated by the observation of fluorescence in the pharyngeal lumen following soaking in 50 mM octopamine and FITC (Fig. 1). The lumen of the pharynx is not autofluorescent and, in the absence of octopamine, no fluorescence can be observed because second stage preparasitic J2s do not ingest. Occasionally, in the absence of octopamine, uptake of the dye by preparasitic J2 cyst nematodes was limited to the anterior sense organs (amphids) and some of their neurones (Atkinson et al. 2001). Uptake of FITC was observed in the pharyngeal lumen and in the excretory/secretory system; entry by either route may facilitate RNAi as dsRNA has the ability to cross cellular boundaries (Fire et al. 1998).

Stages of *C. elegans* readily swallow the aqueous medium around them, and this can be monitored using the fluorescent dye FITC (Fig. 1A). Uptake of FITC by *H. glycines* and *G. pallida* was induced by the presence of 50 mM octopamine, a biologically active amine. Following treatment, FITC was present in the lumen of the mouth stylet, which is normally used to pierce plant cell walls, and is clearly passed posteriorly to the pharyngeal lumen (Fig. 1B, C). The excretory/secretory system of the parasite also showed uptake (Fig. 1D). Uptake was typically limited to 15 to 20% of individuals through the stylet but was often >50% via the excretory/secretory system. We have concentrated on studying cyst nematodes but are exploring the possibility of developing the technique for application with *Meloidogyne incognita*.

RNAi of target genes.

We have explored the potential of the new RNAi technique to deliver dsRNA molecules targeted against three very different cyst nematode genes. First, we targeted cysteine proteinases because their inhibition by specific protein inhibitors as transgenes in plant hosts affects growth of established parasites (Urwin et al. 1997a; 1998; 2001). J2 of *H. glycines*, *G. pallida*, and mature *C. elegans* were treated for 4 h with combinations of dsRNA corresponding to a cysteine proteinase from each species (*hgcp-I*, *gpcp-I*, or *gcp-I*, respectively) with or without 50 mM octopamine. FITC was included as a reporter of uptake. The relative transcript abundance immediately after treatment was determined by virtual Northern analysis (BD-Clontech, Oxford). All the cDNA was electrophoresed concurrently on one agarose gel that was subsequently probed with a mixture of radiolabeled *hgcp-I*, *gpcp-I*, or *gcp-I* at the same time. Both *H. glycines* and *G. pallida* showed reduced cysteine proteinase transcript abundance, but only when octopamine was present with the dsRNA (Fig. 2A). As expected,

Fig. 2. Virtual Northern analysis of transcript abundance. Abundance of **A**, a cysteine proteinase; **B**, actin; **C**, *hgcp-I* transcript in preparasitic juvenile *Heterodera glycines* immediately after soaking; and **D**, MSP in male *Globodera pallida* first treated with dsRNA as juveniles prior to being used to infect plants. oct = the presence of octopamine; RNA = the presence of dsRNA in the soaking solution.

Fig. 1. Fluorescence microscopy showing ingestion of fluorescein isothiocyanate (FITC). **A**, After soaking in 1 mg of FITC per ml, FITC in the pharyngeal lumen (pl) of *Caenorhabditis elegans*. **B**, After soaking in 1 mg of FITC per ml and 10 mM octopamine, FITC in the lumen of the stylet of *Globodera pallida*. m = mouth opening, sl = stylet lumen, skl = lumen of the stylet knobs. **C**, FITC in the pharyngeal lumen of *Heterodera glycines*. dpg = duct of the dorsal pharyngeal gland. **D**, FITC in the length of the excretory/secretory duct (esd) of *H. glycines*. Inset: the opening of the excretory/secretory duct. No fluorescence was apparent in these structures when *G. pallida* and *H. glycines* were treated solely with FITC. Bar represents 20 μ m.

the neurohumor was not required for *C. elegans* to show the effect, because the animal readily swallows the medium in which it is bathed. Stripping the virtual Northern blot and reprobing it with an actin probe confirmed equal loading of cDNA for all samples (Fig. 2B).

The second gene that was selected was a novel *H. glycines* gene (*hgctl*). It was chosen to explore the potential of RNAi to help determine if a gene of unknown function must be expressed for normal parasite development. This possibility is explored later, using J2 that had received RNAi treatments to challenge host plants. *hgctl* was isolated following monoclonal antibody (MAb) screening of a cDNA expression library (C. J. Lilley, *unpublished data*). *hgctl* is expressed most abundantly by adult female cyst nematodes during their period of rapid growth. It encodes a protein of 77 kDa with two C-type lectin (ctl) domains that are functional when expressed in *E. coli*. Its two lectin domains provide sequence homology with a cobra venom coagulation factor, macrophage mannose receptor, and proteoglycan core proteins such as aggrecan. *C. elegans* has a number of genes that are

predicted to contain ctl domains, but none have high homology to *hgctl* (C. J. Lilley, *unpublished data*). RNAi suppressed the level of the *hgctl* transcript abundance in J2 (Fig. 2C).

The final transcripts we targeted for RNAi were those of the major sperm protein (MSP). MSP genes show a high degree of conservation, and sequence information on the five MSP genes of *G. pallida* (Novitski et al. 1993) was used to amplify the corresponding sequences from *H. glycines*. MSP genes were chosen for two reasons: any RNAi effect on these male-specific transcripts should not influence development of females, and secondly, MSP is only expressed in the sperm of males, which form more than 6 days after establishment of the J2 as a parasite (Wyss 1992). A pool of approximately 100 recovered male nematodes at 15 days postinfection (dpi) that had been treated with dsRNA as J2 did show a suppression of MSP transcript abundance (Fig. 2D). Therefore, an RNAi effect can be achieved for a cyst nematode gene that is first expressed several days after dsRNA treatment.

Fig. 3 Growth and sexual dimorphism of *Heterodera glycines* following RNAi of cysteine proteinases. **A**, The control represents animals treated with only octopamine and FITC; RNAi treatment represents animals treated with octopamine, fluorescein isothiocyanate (FITC), and dsRNA of a cysteine proteinase. Filter values for roundness (R) and length (L) provide a basis to discriminate between developmental stages (Atkinson et al. 1996). At 14 days two groups are apparent: enlarged saccate animals, typically large feeding females (J4 and adult) and fusiform animals that may include females that are developmentally compromised, J2 animals that have not reached a stage of sexual differentiation, and males. **B**, Visual identification of fusiform *H. glycines* ($R > 2$; $L \leq 469 \mu\text{m}$) showed that nearly all had a vermiform shape inside the J3 cuticle, indicative of males. The remaining animals were developmentally less advanced but had the correct cuticular shape to suggest maleness. Bar = 20 μm . **C**, An example of amplification of major sperm protein (MSP) from individual animals using rtPCR. Lane 1, λ DNA restricted with *Pst*I; lane 2, fusiform animal, visually a mature male; lane 3, no visual sign of vermiform shape, but malelike cuticular shape; lane 4, cuticular shape less distinctive of a male. A band of the predicted size of MSP was apparent in lanes 2 and 3 but not in lane 4. A fragment of the correct size corresponding to actin (approximately 1.2 kb) was amplified in all the samples, indicating that polymerase chain reaction (PCR) had been successful. It should be noted that less developed males might not provide a PCR fragment representative of MSP.

Determination of postparasitic phenotype.

The consequence to subsequent plant parasitism of RNAi was studied for each of the three genes targeted at the J2 stage. Preparasitic juveniles of *H. glycines* and *G. pallida* were again stimulated to ingest and were exposed to dsRNA. RNAi treatment of cysteine proteinases of both cyst nematodes and the C-type lectin domain protein of *H. glycines* had an effect on subsequent development as parasites. After RNAi of cysteine proteinases, there was no decline in the number of nematodes established at 14 dpi, and normal growth rates were observed. However, the sexual fate of some individuals was changed. The proportion of *H. glycines* developing as females declined significantly from the expected value of 75% on controls to 50% after RNAi of the cysteine proteinase ($P < 0.001$) (Fig. 3A, Table 1). The same effect occurred for *G. pallida* with a fall from 77% in the control population to 56% ($P < 0.01$) for RNAi-treated animals (Table 1). New males have a distinct appearance that allowed visual identification to confirm that filter values used in image analysis correctly defined the sex of an animal (Fig. 3B). In addition, reverse transcriptase-polymerase chain reaction (RT-PCR) was used to detect MSP in individual animals, some of which were not as advanced in development as the animal shown in Figure 3B (Fig. 3C). Improved confidence in determination of males helped establish that changes in sexual fate did occur. In a further study involving RNAi of cysteine proteinases, nematodes recovered from plants 21 dpi showed a 40% reduction in the number of females relative to that in the controls ($P < 0.05$) (Table 1). This is consistent with a larger proportion of males for the RNAi treated population. Most males leave the plant by 21 dpi, whereas females are still sedentary parasites. At both 14 and 21 dpi there was no appreciable difference in the size of the females treated as juveniles with dsRNA corresponding to cysteine proteinases and that of the control females. In contrast, dsRNA directed at the transcript of *hgct1* did not affect the sexual fate of the nematodes, but it did reduce the number of parasites at 14 dpi by 41% ($P < 0.01$) (Table 1). The reduction in parasite numbers represents a failure of the nematodes to establish on the host plant. The results detect effects on either sexual fate or parasitic establishment arising from RNAi-targeted inhibition of gene expression. Neither effect occurred following RNAi of MSP, but males showed reduced transcript abundance of the corresponding transcript several days later.

DISCUSSION

While the current procedure already provides a basis for applying dsRNA to cyst nematodes, the inclusion of 3 mM spermidine and 0.05% gelatin in the soaking solution (Maeda et al. 2001) may improve the penetrance of molecules such as

dsRNA and FITC. Phenotypes obtained by soaking and feeding have been considered weaker than those obtained by injection of *C. elegans* (Kuwabara and Coulson 2000; Tabara et al. 1998). However, ingestion of bacteria transcribing dsRNA has been shown to be at least as effective as microinjection. Embryonic lethal phenotypes are detected with similar efficiency with ingestion, leading to the detection of 50% more postembryonic phenotypes (Kamath et al. 2000). Soaking has been shown to have >97% penetrance for a number of genes whose loss of phenotype was well documented (Maeda et al. 2001). *C. elegans* postgenomic studies have also utilized high-throughput soaking RNAi protocols (Gonczy et al. 2000, Maeda et al. 2000).

The three initial genes to be targeted by RNAi were selected to show the power of RNAi in revealing the importance of gene function to parasitism. Biochemical inhibition of cysteine proteinases has revealed a dependence of cyst nematodes on this class of enzyme (Urwin et al. 1997a) and was therefore selected for the first demonstration of RNAi experiments. The recently identified c-type lectin gene was targeted by RNAi to determine if the importance for plant-nematode interactions could be addressed and whether RNAi could be used as an indication for prioritizing further analysis of genes. Finally, we analyzed MSP in an attempt to determine the period over which RNAi could have an effect. Transcriptional analysis of MSP following RNAi treatment establishes that the RNAi effect persists for several days, therefore the technique has the potential to treat J2s and then to study those genes first expressed only after the nematodes become established parasites. In *C. elegans*, RNAi effects can persist throughout an entire life cycle of the progeny of the injected animals (Kuwabara and Coulson 2000); however, the *C. elegans* life cycle is shorter than the duration of the RNAi effect described here for cyst nematodes.

RNAi is a sensitive technique, as dsRNA has a surprising ability to cross cellular boundaries, and the mode of action appears to involve an amplification of the original signal (Fire et al. 1998). All plant parasitic nematodes are obligate parasites. For cyst and other sedentary endoparasites, the invasive juveniles do not ingest and feed only as a postparasitic animal following formation of specialized feeding structures in the plant. It is possible that dsRNA can be delivered in planta to the nematode, providing the dsRNA is not excluded on a size basis, as occurs for dextrans (Bockenhoff et al. 1994) and proteins (Urwin et al. 1998). RNAi has been applied to plants, e.g., *Arabidopsis thaliana* (Chuang and Meyerowitz, 2000), and targeted at plant genes. RNAi may also be a basis for disrupting plant-nematode interactions. The application of RNAi described here will help improve understanding of genes involved in the interaction of cyst nematodes with their host.

Table 1. Number of females (J4 and adult) and all other stages collected from plants post-RNAi^a

RNAi Target	Species	dpi	Number of parasites				P value	
			Female ^b		Other stages ^c			
			RNAi	Control	RNAi	Control	Ratio female/others	Total nematode number
Cysteine proteinase	<i>Heterodera glycines</i>	14	39	65	40	20	<0.001	NS
Cysteine proteinase	<i>H. glycines</i>	21	32	53	8	*	NS	<0.05
Cysteine proteinase	<i>Globodera pallida</i>	14	48	61	37	18	<0.01	NS
C-type lectin	<i>H. glycines</i>	14	32	54	14	24	NS	<0.01
MSP	<i>G. pallida</i>	14	41	34	19	20	NS	NS

^a *P* values are for χ^2 test RNAi treatments relative to control; * No animals would be expected in this group at 21 dpi; sexual development is complete and males have left the plant.

^b J4 and adult female.

^c J2, J3, and adult male.

MATERIAL AND METHODS

RNA synthesis.

Full-length cDNA clones encoding a cysteine proteinase from both *H. glycines* (*hgcp-I*) (Urwin et al. 1997b) and its homologue from *G. pallida* (*gpcp-I*, isolated by screening the *G. pallida* cDNA library with *hgcp-I*) (C. J. Lilley, unpublished data) were available in the phagemid pBluescript. The coding region of the cysteine proteinase gene of *C. elegans*, *gcp-I* (Ray and McKerrow 1992), was also available in pBluescript (Urwin et al. 1995). *hgctl* (GenBank accession number AF498244) was isolated by screening a cDNA expression library (Lilley et al. 1997) with a monoclonal antibody (N46C10) as described by Sambrook and associates (1989). The MAb is one of many hundreds originally obtained using a shotgun approach with immunogens of homogenized females of *H. glycines* (Atkinson et al. 1988). Approximately 440 bp of the MSP open reading frame was amplified using the primers 5' AATTAACCCCTCACTAAAGGGATGGCGCAACTTCTC 3' and 5' TAATACGACTCACTATAGGGACGTTGTACTCCGATCGGCAAG3' that incorporate the RNA primer sites T3 and T7 (underlined), respectively. The vectors harboring *hgcp-I* and *gpcp-I* were linearized with *XhoI* or *EcoRI*, the vector harboring *gcp-I* was linearized with *Sall* or *PstI*, the vector harboring *hgctl* was linearized with *SacI* or *KpnI* when driving transcription from the T3 or T7 promoters, respectively. To produce both sense and antisense RNA strands, in vitro transcription using the T3 and T7 promoters was carried out as specified by the manufacturer (Megascript; Ambion, Oxfordshire, U.K.).

RNAi by soaking.

Parasitic J2 *G. pallida* and *H. glycines* were collected from sterilized cysts as described by Urwin and associates (1995). *G. pallida* were hatched at 20°C, and *H. glycines* were hatched at 25°C. Approximately 10,000 animals were soaked in 50 mM octopamine made up in M9 salts (43.6 M Na₂HPO₄, 22 mM KH₂PO₄, 2.1 mM NaCl, 4.7 mM NH₄Cl) together with 1 mg FITC Isomer I (Sigma, Poole Dorset, U.K.) per ml (stock made up at 20 mg of FITC per ml DMF) and between 2 and 5 mg dsRNA per ml. Nematodes used as a control were soaked in only FITC and octopamine. The animals were left at room temperature for 4 h before being collected by brief microcentrifugation and then washed copiously with water to remove any external exogenous dsRNA. Uptake of FITC was viewed by fluorescence using a Leica microscope (model DMRB) with suitable filters. We found it beneficial to sort (done manually on the slide) animals showing uptake of FITC in the lumen of the stylet, pharynx, or in the excretory/secretory duct. Images were captured with a software package (Quantimet 500c; Leica, Cambridge) through either a color (Kappa CF15 MCC) or a black and white (COHU) camera mounted on the microscope.

Analysis of transcript abundance.

Approximately 10,000 parasitic J2 animals were stimulated to ingest at any one time. Those showing FITC uptake were collected to analyze transcript abundance. PolyA⁺ RNA was isolated from J2 animals using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia, Bucks, U.K.). Transcript abundance was determined by virtual Northern analysis that utilizes SMART PCR cDNA synthesis technology (Clontech, Hampshire, U.K.). A modified oligonucleotide (dT) primer was used to prime the synthesis of first strand cDNA. At the 5' end of the mRNA, the terminal transferase activity of reverse transcriptase adds a few cytidine residues at the 3' end of the first strand cDNA. An oligonucleotide primer with polyG sequence at its 3' end hybridizes to the polyC sequence

and extends the template. The reverse transcriptase switches template and continues replicating to the end of the oligonucleotide. The oligonucleotide sequences at the 3' and 5' ends are then used to amplify cDNA by long-distance PCR; the number of cycles is determined empirically for each experiment but is often 18. The cDNA is then size-fractionated by agarose electrophoresis, transferred to an immobilized support, and probed with a suitably radiolabeled-DNA sequence using standard protocols (Sambrook et al. 1989).

Infection of plants.

FITC-positive J2 animals were used to infect plants grown in pouches, as described by Atkinson and Harris (1989). Commonly, between four and six infection points were established on the host plant, with approximately 60 nematodes being used at each infection point. The percentage of infection was about 10% for both the controls and for those nematodes treated with dsRNA. Two plants, either *Solanum tuberosum* spp. *tuberosum* cv. Desiree or *Glycines max* were placed in a pouch and infected with *G. pallida* Pa2/3 or *Heterodera glycines*, respectively. Pouches (8 to 10) were set up for both the treatment, nematodes exposed to octopamine, FITC, and dsRNA, and the control groups, nematodes exposed to only octopamine and FITC. Studies on all genes targeted by RNAi were replicated.

Observation of phenotypes.

Animals were collected from the roots after 14 or 21 dpi and studied by image analysis (Atkinson et al. 1996). Presumptive males were subject to RT-PCR using an ABgene kit (ABgene, Surry, U.K.). Individual animals were first dissected from root material and ground in a microcentrifuge tube. Hybaid Recovey amplification reagent was used as described by the manufacturer (Hybaid, Middlesex, U.K.). Primers were designed against the published sequence of MSP (Novitski et al. 1989) with the sequence 5' ATGGCGCAACTTCTTCC3' and 5' ACGTTGTACTCGATCGGCAAG3'. As a control in RT-PCR, primers were designed to amplify an actin from *G. pallida*, 5' AGTACCCGATTGAGCACGGC3' and 5' GGCGAATGGGTCGGCGGATGG3'. It was possible to use all the primers together in the same standard RT-PCR reaction.

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