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PII: S0014-4894(12)00349-9
DOI: http://dx.doi.org/10.1016/j.exppara.2012.11.011
Reference: YEXPR 6557

To appear in: Experimental Parasitology

Received Date: 15 August 2012
Revised Date: 21 September 2012
Accepted Date: 15 November 2012

Please cite this article as: T.J-A. Hwa, M.G.K. Jones, J. Fosu-Nyarko, Gene silencing in root lesion nematodes (Pratylenchus spp) significantly reduces reproduction in a plant host, Experimental Parasitology (2012), doi: http://dx.doi.org/10.1016/j.exppara.2012.11.011

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Gene silencing in root lesion nematodes (*Pratylenchus* spp) significantly reduces reproduction in a plant host

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Abbreviations:
1. PPN- Plant parasitic nematode
2. RLN- Root lesion nematode
3. RNAi- RNA interference
4. dsRNA- double stranded RNA
5. FITC- Fluorescein isothiocyanate
Abstract

Root lesion nematodes (RLNs, *Pratylenchus* species) are a group of economically important migratory endoparasitic plant pathogens that attack host roots of major crops such as wheat and sugarcane, and can reduce crop yields by 7-15%. *P. thornei* and *P. zeae* were treated with double stranded RNA (dsRNA) to study gene silencing, (RNA interference, RNAi), as a potential strategy for their control. Mixed stages of nematodes of both species ingested dsRNA when incubated in a basic soaking solution in the presence of the neurostimulant octopamine. Incubation for up to 16 hours in soaking solutions containing 10-50 mM octopamine, 0.1-1.0 mg/mL FITC, and 0.5-6 mM spermidine did not affect vitality. Spermidine phosphate salt hexahydrate rather than spermidine or spermidine trihydrochloride increased uptake of FITC by nematodes, and this resulted in more effective gene silencing. Silencing *pat-10* and *unc-87* genes of *P. thornei* and *P. zeae* resulted in paralysis and uncoordinated movements in both species, although to a higher degree in *P. thornei*. There was also a greater reduction in transcript of both genes in *P. thornei* indicating that it may be more susceptible to RNAi. For *P. thornei* treated with dsRNA of *pat-10* and *unc-87* there was a significant reduction (77-81%) in nematode reproduction on carrot mini discs over a 5 week period. The results show that RLNs are clearly amenable to gene silencing, and that *in planta* delivery of dsRNA to target genes in root lesion nematodes should confer host resistance. Moreover, for the two genes, dsRNA derived from either nematode species silenced the corresponding gene in both species. This implies cross-species control of nematodes via RNAi is possible.

Keywords: RNA interference (RNAi), double stranded RNA (dsRNA), *Pratylenchus* spp, Troponin C, Calponin, carrot mini disc culture
1. Introduction

Root lesion nematodes (*Pratylenchus* spp) are economically important migratory endoparasitic pests of agricultural, horticultural and industrial crops (Oliviera et al. 1999; Seinhorst, 1998; Smiley et al. 2005). Both juvenile and adult nematodes can enter and leave root tissues, move between cortical cells and feed from them: their life cycle takes 3 – 8 weeks to complete (Castillo and Vovlas, 2008). Feeding by *Pratylenchus* spp. deprives host plants of essential nutrients, whilst entry wounds make plant roots more susceptible to bacterial and fungal pathogens present in the soil. The focus of control strategies for these and other nematodes has depended on application of expensive and environmentally unfriendly chemicals, deployment of natural resistance genes and cultural practices. The current emphasis is to move to nematode-centred environmentally friendly approaches. One such strategy involves the application of RNA interference (RNAi), first to understand the function of genes essential to nematode development and parasitism and second to confer resistance to host plants engineered to express dsRNA and small interfering RNAs (siRNA) to target and silence specific nematode genes.

The discovery of RNAi in *Caenorhabditis elegans*, in which double stranded RNA (dsRNA) induces the degradation of cognate endogenous mRNA and so prevents synthesis of the encoded protein, has provided a significant new tool to study gene function (Mello and Fire, 1995). RNAi has been investigated in many organisms including mammals, insects, fungi and plants (Bucher et al. 2002; Elbashir et al. 2001; Kennerdell and Carthew, 1998; Ngô et al. 1998; Romano and Macino, 1992). In *C. elegans*, RNAi can be triggered by exogenous dsRNA (100-500 bp long) delivered via soaking in buffered-solutions, by microinjection of dsRNA into the adult worm or by feeding on *Escherichia coli* engineered to produce dsRNA (Fire et al. 1998; Fraser et al. 2000; Tabara et al. 1998; Timmons and Fire, 1998). This technology, and sequencing of the genome of *C. elegans*, has enabled functional analysis of almost all of its genes, making it the best annotated multicellular organism (*C. elegans* Sequencing Consortium, 1998, www.wormbase.org).
RNAi now provides new opportunities for research on plant parasitic nematodes (PPNs). Delivery of dsRNA to nematode juveniles via ‘soaking’ in solutions containing dsRNA has been used successfully to investigate the function of some genes in cyst nematodes (e.g. *Globodera pallida* and *Heterodera glycines*), root knot nematodes (e.g. *Meloidogyne incognita*, *Meloidogyne hapla* and *Meloidogyne javanica*) and migratory nematodes such as *Radopholus similis* and *Bursaphelenchus xylophilus* (Adam et al. 2008; Cheng et al. 2010; Haegeman et al. 2009; Huang et al. 2006; Park et al. 2008; Rosso et al. 2005; Urwin et al. 2002). For these obligate parasites, uptake of exogenous dsRNA is enhanced by neurostimulants such as octopamine, resorcinol or serotonin in the soaking solution (Bakhetia et al. 2005; Dubreuil et al. 2007; Rosso et al. 2005). Uptake of soaking solution containing dsRNA in the stylet, pharynx and intestinal tract can be monitored either with fluorescently labelled dsRNA or the fluorescent dye, fluorescein isothiocyanate (FITC) as a marker, which can be included with dsRNA in soaking solutions. Appropriate controls are needed because adverse effects on nematodes have occasionally been reported from FITC and some components of the soaking solution (Adam et al. 2008; Huang et al. 2006; Rosso et al. 2005; Schroeder and MacGuidwin, 2007; Sukno et al. 2007; Urwin et al. 2002). There is now good evidence that RNAi can be used as a control strategy for PPNs in that *in planta* delivery of siRNA/dsRNA reduces nematode establishment and development (Fairbairn et al. 2007; Huang et al. 2006; Sindhu et al. 2009; Yadav et al. 2006). However, there has been no published work to show whether RLNs are also amenable to RNAi.

Until recently, RLNs were relatively neglected pests of crop plants, but with the availability of transcriptome data for some of these species, research on host interactions leading to potential control can be pursued more readily (Haegeman et al. 2011; Nicol et al. 2012). Unlike other PPNs, for which several of their *in vitro*, axenic culture and experimental hosts can be genetically transformed relatively easily, suitable experimental systems are not readily available for root lesion nematodes. Most migratory endoparasitic nematodes have been cultured on alfalfa callus,
but monoxenic cultures of root lesion and burrowing nematodes on callusing carrot discs provide a convenient culture system (Kaplan and Davis, 1990; Krusberg, 1961; Lownsbery et al. 1967; Reise et al. 1987).

In this study, the efficacy of RNAi in *P. thornei* and *P. zeae* has been studied using soaking to introduce dsRNA. Possible effects of different components of the soaking solution on the activity of nematodes have been examined. To investigate long-term effects of RNAi on *P. thornei*, the carrot disc technique was adapted to culture and extract RLNs for higher throughput experimentation, using mini discs in 24 well plates, for which only 50 nematodes were used as an initial inoculum on each disc. This system was used to assess how silencing calponin and troponin C genes delivered by soaking nematodes in dsRNA affected establishment and reproduction of *P. thornei*. In *C. elegans*, these two genes are required to maintain structure and contraction of muscles, and hence their orthologues in RLNs would be important genes in these migratory nematodes. Here we confirm that root lesion nematodes are amenable to gene silencing by RNAi, and that a significant reduction in nematode reproduction after soaking in dsRNA of two target genes was obtained.

2. Materials and Methods

2.1. Identification of target genes

*P. thornei* and *P. zeae* orthologues of the *C. elegans* troponin C (*pat-10*) and calponin (*unc-87*) genes were used to test the amenability of these RLNs to RNAi. The genes were obtained using amino acid sequences of *C. elegans pat-10* (wormbase ID F54C1.7) and *unc-87* (wormbase ID F08B6.4c) to query several databases including the National Centre for Biotechnology Information (NCBI) GenBank (http://www.ncbi.nlm.nih.gov/), Nembase (www.nematode.org) and Nematode.net using TBLASTX 2.2.26+ (Altschul et al. 1997). Translated amino acid sequences of two ESTs from mixed stages of *P. penetrans* with high identities and sequence coverage were
selected: BQ627209 (557 nucleotides, nt) was 84 % identical to *C. elegans pat-10* whereas BQ626831 (524 nt) was 80 % identical to *C. elegans unc-87*. Primers Pppat-10F and Pppat-10R, and Ppunc-87F and Ppunc-87R, were designed based on sequences BQ627209 (NCBI) and BQ626831 (NCBI) to amplify the equivalent sequences in *P. thornei* and *P. zeae* using cDNAs generated from mixed stages of *P. thornei* and *P. zeae*. PCR products for both genes differed in sizes and are designated with the prefixes Pt or Pz for *P. thornei* and *P. zeae* respectively (Table 1). Sequences of the ESTs have been deposited in GenBank: Ptpat-10 (NCBI: JX122489), Ptunc-87 (NCBI: JX122490), Pzpat-10 (NCBI: JX122491) and Pzunc-87 (NCBI:JX122492).

### 2.2. RNA extraction, reverse transcription and PCR amplification of target genes

RNA used for both reverse transcription-polymerase chain reaction (RT-PCR) of target genes and quantitative RT-PCR was extracted from mixed stages populations of *P. thornei* and *P. zeae* using TRIzol® Reagent (Life Technologies Corporation) and ethanol precipitation. The number of nematodes used depended on the purpose of the experiment. Nematodes were first macerated with liquid nitrogen in a 1.5 mL RNAse-free centrifuge tube after which 800 µL of TRIzol® Reagent was added and vortexed for 5 min. RNA was extracted with 200 µL of chloroform. The aqueous phase was ethanol-precipitated for 30 min at 12, 000 g, resuspended in DEPC-treated water and treated with DNase I (Qiagen, Australia). cDNA preparations were made using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Australia) according to the manufacturer’s protocol. PCRs of target genes, amplified from 300 ng of cDNA, were done with MyTaq DNA Polymerase (Bioline, Australia) following the manufacturer’s recommendation in a 20 µL reaction volume using the following temperature profile: 95°C for 3 min, followed by 35 cycles of 95°C for 15 sec, 55°C for 15 sec and 72 °C for 30 sec followed by 72°C for 10 min in a Veriti® 96-Well Thermal Cycler (Applied Biosystems).
2.3. Generation of double stranded RNA

Double stranded RNA corresponding to *Pzpat-10* and *Ptunc-87* was used in soaking experiments to test the efficacy of RNAi in *P. thornei* and *P. zeae* (Table 1). A 745 nucleotide long dsRNA corresponding to the *gfp* gene of *Aequorea victoria* was used as control. These were synthesised using the MEGAscript® RNAi Kit according to the manufacturer’s protocol (Ambion, Austin, USA) from PCR products as templates. The DNA templates for the nematode genes were generated with primer pairs, T7Pzpat-10-F and T7Pzpat-10-R and T7Ptunc-87-F and T7Ptunc-87-R, each with the T7 promoter sequence upstream of the gene specific portion for *in vitro* transcription with the T7 RNA polymerase promoter (Table 1). Primers used to amplify the *gfp* gene were T7GFP-F and T7GFP (Table 1). Briefly, 2 µg of DNA was incubated with the T7 enzyme mix and 75 mM each of ribonucleotides for 16 h at 37°C, followed by 1 h of DNase-1 treatment at 37°C. An extra annealing step at 75°C for 5 min was done to ensure the longer dsRNA of *gfp* was completely annealed before nuclease treatment. DsRNAs were purified and checked for integrity on a 1.5 % nondenaturing agarose gel prepared with 1X TBE as described by the manufacturer.

2.4. Nematode soaking and dsRNA treatments

For all soaking experiments, nematodes were incubated in a basic soaking solution consisting of M9 buffer (22 mM KH₂PO₄, 42.3 mM Na₂HPO₄, 85.6 mM NaCl, 1 mM MgSO₄), 0.05 % gelatine and 3 mM spermidine. Different concentrations of the following compounds were added to optimise uptake or determine their effects on nematode activity: i) neurostimulants - octopamine or resorcinol; ii) spermidine derivatives- spermidine (Sigma Aldrich, 85561), spermidine trihydrochloride (STH, Sigma Aldrich, S2501) and spermidine phosphate salt hexahydrate (SPSH, Sigma Aldrich, S0381); and iii) Fluorescein isothiocyanate isomer I (FITC) (Sigma Aldrich, 46952). When dsRNA was added to the soaking solution, a final concentration of
1.0 or 2.0 mg/mL was used. Different numbers of nematodes were treated in 200 µL of soaking solution in replicates, covered and incubated in the dark at 21-23°C for the appropriate length of time (see specific experiments). After incubation, nematodes were transferred to a 15 mL Falcon tube and washed three times with sterile water by centrifugation at 1, 200 g for 2 min to remove the soaking solution.

2.5. Quantitative RT-PCR of target genes

Analyses of gene expression in nematodes were done with the GoTaq® qPCR Master Mix (Promega Corporation, Australia) in a Corbett RotorGene Quantitative Thermal Cycler (Qiagen Pty Ltd, Australia). Briefly, 1 µl of cDNA, converted from 200 ng of RNA of experimental and control samples, was added to 1X GoTaq qPCR master mix, 10 µM each of the gene specific primer pair and sterile deionised water to make the reaction mixture to 20 µL. All PCRs were done in triplicate and the mean of Ct values determined. Expression of pat-10 and unc-87 was quantified with primer pairs qPzpat-10-F and qPzpat-10-R and qPtunc-87-F and qPtunc-87-R, which amplified 121 bp and 134 bp respectively in both P. thornei and P. zeae (Table 1). PCRs were done at 95°C for 10 min, followed by 45 cycles of 95°C for 15 sec and 55°C for 60 sec. Gene expression was normalised with actin and/or 18S ribosomal RNA (rRNA) genes using primer pairs qActin-F and qActin-R and q18SrRNA-F and q18SrRNA-R that amplified 126 bp and 147 bp respectively in both nematode species (Table 1). Relative gene expression was determined using the ΔΔCt method as described in the ABI PRISM 7700 Sequence Detection System Bulletin 2 (Applied Biosystems).

2.6. Stock carrot cultures

Stock nematode cultures were maintained on large discs made from carrot tap roots (Daucus carota cv Stefano) obtained fresh from a local wholesale market. Preparation of sterile carrot pieces involved washing them with lukewarm soapy water and then submersion in a 5 % (v/v) bleach solution for 30 min. The outer tissues were then removed with a sterile kitchen knife.
and the remaining tissues were soaked in 95% ethanol for 10 min, followed by washing five times with sterile water. The tissues were then flamed lightly using a Bunsen burner, and peeled again before being cut into 5 cm wide and 3-4 cm thick pieces under sterile conditions in a laminar flow bench. These large carrot pieces were placed in sterile 250 mL culture vessels (7.0 x 7.5 cm) without nutrient solution or agar, and closed with sterile plastic caps. The vessels were then incubated at 25°C in the dark for up to four weeks to check for contamination and/or to allow some callus formation before inoculation with 200-300 nematodes of mixed stages. Inoculated carrot discs were then incubated at 25°C for up to 8 weeks before sub-culturing onto freshly prepared callusing carrot pieces. Sub-culturing either involved transferring a piece of infected tissue onto a new piece or infecting the carrot piece with clean nematodes.

2.7. Mini disc carrot cultures

To establish a higher throughput experimental system to culture and extract nematodes, carrots were surface-sterilised as described in section 2.6, and using a 10 mm diameter cork borer, mini discs of 10 mm thickness were cut from the core and each disc placed in one well of a 24-well plate (Greiner Bio-One, Germany) containing half strength (2.2g/L) Murashige and Skoog basal solution with Gamborg’s vitamins (Sigma Aldrich, Australia) solidified with 0.7% agar (Fig. 1a). The plates were covered with a sterile lid, sealed with parafilm and incubated at 25°C for 2 weeks to ensure sterility and to initiate callusing. When callus started to form, discs were inoculated with 50 sterile nematodes of either *P. thornei* or *P. zeae* and incubated in the dark at 25°C. For each nematode species, one plate with 24 mini discs was infected and three replicates were analysed for numbers of nematodes present at weekly intervals over a period of 8 weeks.
2.8. Nematode inoculum

Cultures of *P. thornei* were started from individual nematodes isolated from commercial wheat plants growing in Western Australia and then maintained on partially sterilised carrot cultures *in vitro*. *P. zeae* was originally isolated from sugarcane plants, and subsequently maintained on roots of sorghum plants and carrot pieces *in vitro*. Nematode inocula used to establish stock nematode cultures on large carrot pieces consisted of mixed juvenile and adult stages. They were surface-sterilised before use: surface sterilisation involved suspending nematodes in 1 % chlorhexidine gluconate (hibitan) for 20 min, followed by 1 % streptomycin sulphate for 5 min: they were then washed five times with sterile water. Washing involved suspending nematodes in water in 1.5 mL or 15 mL tubes, inverting the tube 3-5 times and centrifuging at 1,000 g for 3 min. This was repeated 4-5 times. The nematodes were then observed using a light microscope to check for viability before use. Nematode inocula for mini disc cultures and for RNAi feeding experiments were obtained from axenic stock cultures without any further cleaning, using the extraction method described in section 2.9.

2.9. Nematode extraction

Nematodes were extracted from carrot cultures by cutting the tissue into smaller pieces with a sharp sterile scalpel, followed by sieving and centrifugation or using a mist apparatus. Large infected carrot pieces were cut into 0.1 - 0.2 cm pieces in a glass Petri dish with enough water to cover them; the dishes were then placed on a rotator at 80 rpm for 24 h. The suspended nematodes were poured through a 250 µm sieve and collected in a glass beaker. Nematodes from mini discs used for RNAi infection assays were extracted using a mist chamber: cut discs were placed on coffee filters on a 5 cm sieve with 0.1 cm aperture, which were sprayed with a fine mist of water for 10 seconds every 10 minutes for 24 h (Fig. 1b). The sieve was placed on a funnel attached to a 2.5 mm wide rubber tubing. Nematodes moved down the tube and accumulated in
a U-shaped section of tubing sealed with a clip until collected. Collected nematodes were cleaned by repeated washes with sterile water followed by centrifugation as described above. The nematode numbers were then quantified using a dissected microscope. Descriptive statistics of all data including average numbers of nematodes and standard errors were estimated using Microsoft Excel.

2.10. Analysis of RNAi phenotypes and FITC fluorescence intensity in nematodes

Nematodes were suspended in 100 μL of sterile water in wells of a 96 well plate; their viability and RNAi phenotypes were assessed using an inverted light microscope (Olympus LH50A). Fluorescence from nematodes resulting from FITC uptake was observed using an Olympus BX-51 microscope with FITC filter at 10X magnification. Fluorescence intensities were quantified from captured images using the NIH software ImageJ (http://rsb.info.nih.gov/ij/) (Abramoff et al. 2004). Pixel counts were used to determine the differences in intensity of fluorescence. The fluorescent images were first converted to 8-bit greyscales and a threshold set to obtain the number of fluorescent pixels for each image. The threshold used was the level required to remove all background and auto-fluorescence in control non-fluorescing images, which were kept constant throughout the analysis. Fluorescent intensity, expressed as percent fluorescence, for representative images (nematodes) was determined by dividing the number of fluorescent pixels for a given nematode by the total pixel area.

3. Results

3.1. Ingestion of soaking solution by P. thornei and P. zeae using FITC as a marker

To find an optimum concentration of FITC that reflected uptake of solutes through the stylet and gut with minimal detrimental effects on nematodes, six concentrations of FITC (0.1
mg/ml; 0.2 mg/ml; 0.4 mg/ml; 0.8 mg/ml; 1.0 mg/ml; 2.0 mg/ml) were added to the basic soaking solution and nematode behaviour and uptake was observed. The effect of FITC on nematodes was estimated by counting active and inactive or dead nematodes after incubation at 21 °C for 16 h. For each concentration of FITC, 500 mixed stages of *P. thornei* were soaked in basic soaking solution with 50 mM octopamine. For controls, a similar number of nematodes was soaked under the same conditions but without FITC. Two replicates were prepared for each soaking experiment. Nematodes soaked with FITC all fluoresced after 16 h incubation (Fig. 2). Fluorescence was present in the stylet and oesophageal gland cells, indicating that the nematodes ingested soaking solution via the stylet (Fig. 2i-iii). After uptake, the pattern of fluorescence in the nematodes suggested that FITC might also partition in lipid layers, and it is possible that the FITC might also enter nematodes via other apertures (e.g. anus or amphids) (Fig. 2i-iii). The proportion of *P. thornei* that fluoresced increased with increasing concentration of FITC after 16 h of incubation, except that there was no obvious difference in fluorescence of nematodes incubated in solutions with 0.4, 0.8 and 1.0 mg/mL FITC (Fig. 2). However, at 2 mg/mL, fluorescence was very high and similar in all nematodes (Fig. 2 K, L). Also at this concentration, 98 % of the treated nematodes were motionless (Fig. 2 K, L and Fig. 3A): these nematodes were considered dead if they did not move after exposure to light and swirling. This observation indicates the fluorescent dye could be toxic to *P. thornei* at 2 mg/ml. However, there was no statistical difference between the average viability of *P. thornei* exposed to any of the other five FITC concentrations (0.1 - 1.0 mg/ml) (p<0.05), and there was only a 16 % reduction in viability at 1 mg/mL compared to nematodes soaked without FITC (Fig. 3A).

To examine possible effects of longer incubation of nematodes with FITC, a time course experiment was done in which mixed stages of both *P. zeae* and *P. thornei* were separately incubated in basic soaking solution with 1.0 mg/mL FITC and the viability of nematodes assessed
at 4, 8 and 24 h. The number of nematodes that showed FITC fluorescence increased with time, with almost half of the nematodes exhibiting obvious fluorescence 8 h after incubation. After 24 h of incubation, almost all nematodes in soaking solutions with FITC fluoresced. On average, there was a 23% reduction in viability of *P. thornei* soaked with FITC at the three time points when compared with the controls (without FITC) (Fig. 3B). However, for *P. zeae*, a significant loss of viability (average of 37%) was observed when soaked in solutions with FITC after 4, 8 and 24 h indicating that they were adversely affected by the fluorescent dye (*p* < 0.05, Fig. 3B). More than half of *P. zeae* incubated in the basic soaking solution with 1.0 mg/ml FITC were inactive after 24 h. All FITC used in experiments was dissolved in 70% of dimethylsulfoxide (DMSO) before addition to soaking solutions. When 2% dimethylformamide (DMF) was used instead of DMSO almost 100% loss in nematode activity was observed for *P. thornei* after 16 h incubation (data not shown).

### 3.2. Octopamine but not resorcinol is effective in stimulating uptake of solution by *P. thornei* and *P. zeae*

In addition to the 50 mM octopamine used in soaking solutions to investigate optimum FITC levels, four other concentrations of octopamine (5 mM, 10 mM, 30 mM and 100 mM) and 1% resorcinol were added to soaking solutions with 1 mg/mL FITC to test the possibility of enhancing ingestion by nematodes and to determine whether increased pharyngeal pumping caused by neurostimulants had adverse effects on nematode viability. For each concentration, the number and activity of fluorescing nematodes and the intensities from two replicates of 500 nematodes were observed and compared to nematodes incubated in basic soaking solution without neurostimulants. After 16 h soaking, fluorescence was observed in almost all nematodes and eggs soaked at all the different concentrations of octopamine (Fig. 4). In contrast almost no fluorescence was observed in the stylet of nematodes when no octopamine was added to the soaking solution, although very weak fluorescence (autofluorescence, Goto et al, 2010) could be
seen in the pharynx and oesophageal glands of some *P. thornei* (data not shown). Fluorescence intensity in *P. thornei* increased with increasing concentration of octopamine. Strong FITC signals were observed in the stylet, pharynx and the excretory/secretory pore of *P. thornei* when soaked in the presence of octopamine. Concentrations of up to 50 mM octopamine did not seem to affect viability of *P. thornei* since there was no significant difference in the activity of nematodes incubated with or without octopamine (p<0.05). Activity of *P. thornei* was only slightly reduced (by 12 %) when soaked with 100 mM octopamine compared to controls (p<0.05) (Fig. 5A).

As the number of active *P. thornei* soaked with 50 mM octopamine did not differ significantly from exposure to 5, 10 or 30 mM octopamine, the effect of longer incubations on viability of both *P. thornei* and *P. zeae* soaked with and without 50 mM octopamine was investigated 4, 8 and 24 h after incubation. For each time point, two replicates of 500 mixed stages of nematodes were set up separately and their activity monitored. Both *P. thornei* and *P. zeae* appeared normal after 8 h incubation with or without 50 mM octopamine (p<0.05) (Fig. 5A). A pronounced adverse effect of 50 mM octopamine on nematodes soaked for 24 h is indicated in that there was a 37 % and 41 % reduction in viability respectively for *P. thornei* and *P. zeae* (Fig. 5B). When 1 % resorcinol, another neurostimulant, was used in place of octopamine and incubated for 4, 8 and 24 h, a drastic reduction in viability of nematodes was observed. More than 90 % of both *P. thornei* and *P. zeae* were inactive, and scored as dead, after only 4 h incubation (Fig. 6). Analysis of independent soaking experiments for the different time points showed that 1 % resorcinol is highly detrimental to both nematode species. In addition, fluorescence microscopy of nematodes which appeared dead after treatment with resorcinol showed intense fluorescence throughout their bodies with a pattern that was clearly different from that observed for live nematodes. Dead nematodes stained diffusely, whereas live nematodes exhibited a granular localisation of fluorescence. This suggests that the FITC may diffuse into the body of dead
nematodes via relaxed sphincters. Lower concentrations of resorcinol in soaking solutions were not studied because addition of octopamine provided the desired levels of ingestion via the stylet.

3.3. Spermidine enhances FITC fluorescence intensity in P. thornei

The effect of different concentrations and derivatives of spermidine on uptake of external solution by RLNs was also investigated. The intensity of fluorescence of P. thornei incubated for 16 h in basic soaking solution plus 1.0 mg/mL FITC and 50 mM octopamine with 5 different concentrations of spermidine (0.5 mM, 1 mM, 2 mM, 3 mM and 6 mM), was compared to soaking without spermidine. Nematodes from all the treatments showed increased fluorescence compared to those soaked without spermidine, indicating enhanced uptake of the soaking solution. However, there was greater fluorescence intensity at 3 mM and 6 mM concentrations of spermidine. Similar results were obtained when two derivatives; spermidine trihydrochloride (STH) and spermidine phosphate salt hexahydrate (SPSH) were substituted for spermidine at 3 mM and 6 mM concentrations. Moreover, nematodes soaked with SPSH gave a characteristically sharper, slightly diffuse and intense fluorescence that outlined the shape of their internal structures more clearly (Fig. 7). Fluorescence intensities from representative P. thornei nematodes soaked in the three spermidine derivatives were quantified using the Image J software. Nematodes soaked in buffers with 3 mM spermidine displayed 33 % fluorescence intensity compared to 45 % in 3 mM STH and 86 % in 3 mM SPSH. When soaked in buffers with 6 mM of the compounds, P. thornei showed 38 % fluorescence for spermidine, 53 % for STH and 96 % for SPSH.

3.4. RNAi of calponin [unc-87] of P. thornei

To test the amenability of RLNs to RNAi and whether the differences in fluorescence intensities demonstrated by P. thornei soaked in buffers containing SPSH correlated with
increased dsRNA uptake and reduction in mRNA, RNAi of the calponin gene (*Ptunc-87*) was undertaken for *P. thornei*. Three RNAi feeding experiments were set up, where for each, 2000 nematodes were fed with 2 mg/mL of dsRNA corresponding to *Ptunc-87*, *gfp* or with no dsRNA in a basic soaking solution that contained 10 mM octopamine. For each feeding experiment, three replicates were set up each with 3 mM spermidine, STH or SPSH and incubated at 21 °C for 16 h, after which nematode viability and behaviour was observed. Nematodes soaked in dsRNA of *gfp* showed no behavioural differences from those soaked without dsRNA. About 90 % of nematodes soaked with each of the three spermidine derivatives showed effects of dsRNA ingestion, and exhibited abnormal behaviour consistent with silencing of the *unc-87* gene, including twitching, slow movement, repeated banging of the head against the body and loss of orientation, in which treated nematodes moved in circles and sometimes in waves (Fig. 8).

The reduction in expression of mRNA of *Ptunc-87* in nematodes treated with dsRNA was quantified using qRT-PCR using expression of actin as an endogenous reference for normalisation. Reduction in transcript accumulation was calculated using the Comparative Ct method (*ΔΔ*Ct) with reference to expression of the gene in nematodes fed with dsRNA of *gfp* and those soaked with no dsRNA. Nematodes soaked in dsRNA of *Ptunc-87* with all three spermidine derivatives showed drastic silencing of the gene. When compared to expression of *Ptunc-87* in *P. thornei* soaked with no dsRNA, there was 3,373-fold, 3,983-fold and 32,541-fold reduction in transcript accumulation respectively for soaking solutions containing spermidine, STH and SPSH. The trend was similar when reduction in transcript accumulation in *P. thornei* was calculated relative to nematodes treated with dsRNA of *gfp*: with gene knockdown of 10,085-fold, 11,910-fold and 97,289-fold in nematodes soaked in solutions containing spermidine, STH and SPSH respectively (Table 2). When expression was normalised using 18s rRNA, the extremely high fold reduction in *unc-87* transcripts in dsRNA-fed nematodes did not differ from results obtained when actin was used. From both analyses, there is a strong indication that increased fluorescence intensity demonstrated when
dsRNA was fed in the soaking solution with SPSH correlated with greater gene silencing, with about a 10-fold greater effect than soaking with STH and spermidine.

### 3.5. RNAi of troponin C [pat-10] of *P. thornei*

When dsRNA corresponding to *pat-10* gene of *P. zeae*, *Pzpat-10*, was fed to 2000 vigorous mixed stages of *P. thornei*, the effect on nematode behaviour was monitored over time by observing the percentage of nematodes exhibiting abnormal behaviour and quantifying the extent of gene silencing. Nematodes were fed with 2 mg/ml *Pzpat-10* dsRNA in a basic soaking solution with 10 mM octopamine, 0.1 mg/ml FITC and the effects compared with those incubated under the same conditions with 2 mg/ml *gfp* dsRNA and with no dsRNA. The effect of ingesting *Pzpat-10* dsRNA was estimated by observing three samples of nematodes, approximately 650 nematodes at a time, using an inverted light microscope, and counting the number of active nematodes after 2, 4, 8, 10, 12, 16, 24, 30, 36, 48, 60 and 72 h. *P. thornei* nematodes exhibited clear and observable effects on ingesting dsRNA of *Pzpat-10* eight hours after incubation. This included decreased activity manifested in very slow or wavy movement compared to their vigorous activity before feeding (Fig. 8). In extreme cases nematodes were paralysed and did not move even after exposure to intense light. No such observations were made for nematodes fed with dsRNA of *gfp* and with no dsRNA. The number of active *P. thornei* fed with *Pzpat-10* dsRNA decreased significantly with time: from 100/650 (about 15 %) at 8 h to almost 85 % (550/650) 72 h after incubation (Fig. 9). There was no significant effect of soaking nematodes in dsRNA of *gfp* and without dsRNA (p<0.05) (Fig. 9).

Evidence of *Pzpat-10* transcript knockdown was obtained from assessing transcript levels 16 h after feeding, normalised against expression of 18S rRNA. In addition, to determine the effect of longer incubations in dsRNA and possible recovery of gene expression when the dsRNA trigger was removed, transcript levels were quantified for nematodes kept in water for 5 days in
the dark at 25°C following 16 h incubation in dsRNA, and for nematodes kept in water for 2 days in the dark at 25°C following 72 h of incubation. After 16 h, expression of Pzpat-10 in dsRNA-treated nematodes showed 155-fold and 261-fold reduction respectively compared to expression in nematodes fed with dsRNA of gfp and with no dsRNA (Table 2). Gene expression in similar nematodes left in water for 5 days (after initial soaking for 16 h in dsRNA) was 365-fold and 194-fold lower compared to expression in nematodes treated similarly with gfp dsRNA and with no dsRNA (Table 2). In general, after 72 hours of feeding on dsRNA, nematodes were alive but very weak compared to controls and ingestion of dsRNA for the duration resulted in higher levels of gene silencing than shorter incubation times (16 h): 826-fold and 765-fold decrease compared to expression of nematodes fed with gfp-dsRNA and no dsRNA treatments respectively (Table 1).

3.6. Relative sensitivity of P. thornei and P. zeae to RNAi

To assess the relative sensitivity of P. thornei and P. zeae to RNAi, dsRNA of Pzpat-10 and Ptunc-87 were fed to both nematode species under the same conditions, their behaviour observed and the extent of transcript reduction assessed. About 500 active nematodes of each species (in triplicate) were incubated for 16 h at 22 °C in a basic soaking solution with 50 mM octopamine, 1 mg/mL FITC and 1 mg/mL dsRNA of either Pzpat-10 or Ptunc-87. The fold decrease in transcripts in dsRNA-treated nematodes was compared to the level of expression in nematodes soaked with dsRNA of gfp. Nucleotides corresponding to dsRNA of Pzpat-10 were 94 % similar to Ptpat-10 whereas the Ptunc-87 fragment used to construct dsRNA was 87 % similar to Pzunc-87.

The effects of RNAi on the two genes, both of which were associated with locomotion, were consistent with previous observations where nematodes became sluggish and paralysed (mainly for pat-10) and uncoordinated (Fig. 8). In both cases the effects on RNAi were more pronounced for P. thornei than for P. zeae, with about 70 % of P. thornei becoming inactive
compared to 50 % for \textit{P. zeae} when fed with dsRNA of \textit{Pzpat-10}. Similarly, about three times more \textit{P. thornei} were observed to have been affected by feeding on dsRNA of \textit{Ptunc-87}.

The RNAi phenotypic observations were confirmed when reduction of transcript accumulation of both genes was assessed using expression of 18s RNA for each species for normalisation and the fold decrease quantified against expression of both genes from each nematode species fed dsRNA of \textit{gfp}. Ingestion of \textit{Pzpat-10} dsRNA resulted in 50.6-fold reduction in gene expression in \textit{P. thornei} compared to only 3.6-fold reduction in \textit{P. zeae} (Table 2) Similarly, there was a greater down-regulation of \textit{unc-87} for \textit{P. thornei} (29.9-fold) than for \textit{P. zeae} (7.8-fold). These results, combined with the observed phenotypes indicate that \textit{P. thornei} could be more susceptible to RNAi than \textit{P. zeae} (Table 2).

3.7. Establishment and reproduction of nematodes in mini disc cultures

We aimed to develop a high throughput culture system that could be used to assess the fitness of RLNs after gene silencing via RNAi. To do this, the establishment and reproduction of \textit{P. thornei} and \textit{P. zeae} was assessed over 8 weeks after inoculating carrot mini discs in 24 multi-well plates with 50 nematodes per disc. The reproduction of the nematodes on the discs was quantified by extracting eggs, juveniles and adults weekly from 3 replicates of mini discs for each species. One week after inoculating mini discs, browning of the carrot tissue, characteristic of root lesion nematode infection and feeding, appeared on all discs infected with either nematode species. Mini disc cultures continued to produce calli for 5 weeks after nematode infection, and remained firm until about the 6th week, when they started to appear degraded and to shrink in size.

One week after infection, an average of 34 \textit{P. thornei} nematodes and 37 \textit{P. zeae} nematodes (68 % and 74 % of the initial inoculum respectively), was extracted. These numbers indicated that the nematodes survived and were active, but that the extraction method used was less than 100 % efficient. For both species of nematodes, the numbers extracted from mini discs
increased weekly and peaked at week 5 representing 8.4 and 19.2 fold increases over the original inoculum respectively for *P. thornei* and *P. zeae* (Fig. 10). The numbers then declined from the 6th week after infection, when the discs began to dry, until the 8th week when last readings of the experiments were taken. All larval and adult stages of nematodes were extracted from mini discs each week. Included in the latter were eggs (20 – 63 % of total nematode weekly counts for *P. zeae* and 5 - 52 % for *P. thornei*) (Fig. 10). The retrieval of eggs from mini discs, especially in week 1 and 2 after infection, indicates that the initial inoculum included adults or gravid females.

More *P. zeae* (both vermiform and eggs) were extracted each week from the mini discs than *P. thornei* (p<0.05), suggesting that the former was better adapted to the growing conditions on the carrot mini discs, or that its life cycle was shorter (Fig. 10). Over the experimental period of 8 weeks, an average of 1,627 nematodes including 596 eggs were extracted for *P. thornei* cultures, whereas 3,650 nematodes (of which about half were eggs), were retrieved for *P. zeae*. These represent a reproduction factor of 32.54 and 73 respectively for *P. thornei* and *P. zeae*.

3.8. Silencing of *pat-10* and *unc-87* reduces establishment and reproduction of *P. thornei* and *P. zeae*

The mini disc culture system was then used to assess the fitness of nematodes treated with dsRNA of *pat-10* and *unc-87*, to determine their ability to establish and reproduce on the carrot host. To assess the effects of silencing *pat-10*, four replicates of self-callusing mini carrot discs were each infected with 125 mixed stages *P. thornei* previously fed with 2 mg/mL dsRNA of *Pzpat-10* in a basic soaking solution with 50 mM octopamine for 16 h. The infected discs were kept at 21°C for six weeks and the number of nematodes, extracted using a mist apparatus were compared to that of similar nematodes fed with dsRNA of *gfp* or no dsRNA. Whilst there was no significant difference in the average number of nematodes extracted from discs infected with
nematodes fed with \textit{gfp} dsRNA and no dsRNA, on average, there was an 81 \% reduction in the number of nematodes retrieved from discs infected with \textit{Pzpat-10} dsRNA-treated nematodes \((p<0.05)\) (Fig. 11A).

In the case of \textit{unc-87}, the infection assays were also used to assess whether the greater reduction of transcript expression in nematodes fed in the presence of SPSH would affect their establishment and reproduction to a greater extent than those fed in the presence of spermidine and STH. Four replicates of self-callusing carrot discs were each infected with 50 mixed stages \textit{P. thornei} previously fed with 2 mg/mL dsRNA for 16 h. Like \textit{pat-10}, the effect of reduction in transcript accumulation of \textit{unc-87} in dsRNA-fed nematodes persisted, and strongly reduced establishment and reproduction on the carrot host. This is evidenced in the significantly lower average number of nematodes (29 \%) retrieved from discs infected with dsRNA-treated nematodes (from all spermidine derivative feeding solutions) compared to those obtained from discs infected with \textit{gfp} dsRNA-treated and no dsRNA-treated nematode infection assays \((p<0.05)\) (Fig. 11B). However, there was no significant difference in the number of nematodes retrieved from discs infected with nematodes previously soaked with the three spermidine types \((p<0.05)\). This result indicates that the increased fluorescence intensities and the corresponding greater down-regulation in nematodes soaked with SPSH did not translate into lower establishment and reproduction of nematodes compared to those soaked in STH and spermidine.

4. Discussion

This study provides the first detailed report of dsRNA-mediated gene silencing in RLNs by artificial feeding. Significant silencing of two genes, calponin and troponin C, was observed in \textit{P. thornei} and \textit{P. zeae} following optimisation of soaking conditions with dsRNA sequences from these two genes. Both genes are essential for structural integrity and proper muscle contraction in
nematodes (Goetinck and Waterston, 1994; Matthews et al. 2004; Ono and Ono, 2004; Terami et al. 1999). Vermiform/infective stages of RLNs migrate and feed from cell to cell largely in the root cortex of their hosts and any paralysis or immobility will affect their feeding ability and subsequent survival or reproduction in a host.

We observed that RLNs, like J2s of root knot and cyst nematodes, could ingest external solutions with the help of neurostimulants. Basic soaking solutions containing M9 buffer, 0.05% gelatine, 0.5-6 mM spermidine, 10-50 mM octopamine and 0.1 – 1.0 mg/mL FITC, were optimal for uptake by mixed stages of *P. thornei* and *P. zeae*, and allowed fluorescence to be observed in the stylet and intestine without significant detrimental effects on nematode activity after incubation for up to 16 h. However, concentrations of some components of the soaking medium and incubation times did affect their viability. Resorcinol was not a good neurostimulant for dsRNA uptake by these nematodes, because soaking mixed stages of both *P. thornei* and *P. zeae* in 1 % resorcinol for only 4 h reduced their viability by ~100 % and ~80 % respectively. A less detrimental effect was reported for *M. incognita* where, although incubation of J2s for up to 4 h in 1 % resorcinol induced uptake of dsRNA without reducing viability, incubation over 6 h results in about 10 % lethality (Rosso et al. 2005). However, for *M. javanica* overnight incubation of J2s in lower concentrations (0.5 %) killed nematodes without uptake of dsRNA (Adam et al. 2008). When FITC is included in dsRNA soaking solutions for PPNs, concentrations at 1.0 mg/mL or lower are used to trace uptake of external solutions or dsRNA for between 4 and 24 h. It is known that even at these low concentrations some nematodes are affected. For example, the cumulative hatching rate of *H. glycines* was reduced when soaked with 0.1 mg/mL FITC (Schroeder and MacGuidwin, 2007). For the RLNs used in our study, we did not observe any significant phenotypic effects of FITC at lower concentrations, but soaking in 1.0 mg/mL for longer than 16 h appeared to affect viability of *P. zeae* more than *P. thornei*. 
Addition of spermidine to soaking solutions containing dsRNA for RNAi significantly reduces expression of target genes. One explanation is that it neutralises the negative charge of nucleic acids thereby facilitating uptake of dsRNA through the intestine (Maeda et al. 2001). In our experiments, all three spermidine derivatives used increased the proportion that fluoresced and the fluorescence intensity of *P. thornei*, which was highest at 3 and 6 mM concentrations. A significant observation was that soaking solutions containing spermidine phosphate salt hexahydrate resulted in a more intense fluorescence than was the case for spermidine and spermidine trihydrochloride, and this suggested increase in dsRNA uptake by nematodes. The latter was confirmed with a 10-fold decrease in transcript accumulation of *Ptunc-87*. However, the greater reduction in transcript was not associated with a corresponding reduction in reproduction of *P. thornei* on carrot discs compared to nematodes soaked with spermidine and spermidine trihydrochloride. This is partly because RNAi is not a stochastic process, and it is possible that a threshold level of dsRNA/siRNA is needed to trigger silencing (of this particular gene) and any additional dsRNA molecules taken up may not necessarily increase the effect. Such a threshold level may have been achieved in each of the three experiments with the spermidine derivatives. Despite this observation, substituting spermidine phosphate salt hexahydrate could provide better phenotypic characterisation of nematodes in RNAi soaking experiments and enhance uptake of dsRNA, which could enhance RNAi effects depending on the target gene.

The effectiveness of RNAi in nematodes depends on several factors, including the turnover of the target gene, susceptibility of the organism to RNAi, the amount of dsRNA delivered, the base composition and position of dsRNA construct in a target gene, the length of dsRNA sequence, mode of delivery and type of target tissue (Fire, 1999; Haegeman et al. 2009; Huang et al. 2006; Kimber et al. 2007; Orii et al. 2003). We showed that gene silencing induced by soaking nematodes with dsRNA effectively reduced the expression levels of both troponin C and calponin of *P. thornei* and *P. zeae*, but the extent depended on the nematode species, the target gene and
the concentration of dsRNA used. Transcript knockdown of *Ptpat-10* in *P. thornei* induced by 2 mg/mL after 16 h was three times higher than when 1 mg/mL of dsRNA was used. Similarly, for *unc-87*, an increase in dsRNA concentration from 1 mg/mL to 2 mg/mL resulted in a more effective gene knockdown than for the *pat-10* gene. Increasing the level of dsRNA of *Ptunc-87* resulted in an almost complete absence of target gene expression: results were similar whether expression was normalised with 18s rRNA or actin. However, feeding with a higher concentration of dsRNA does not always ensure an increase in RNAi effect. In a similar experiment when expression of a parasitism gene in *H. glycines* was downregulated using 2.5 mg/mL and 5 mg/mL of dsRNA, the two-fold increase in concentrations of the dsRNA trigger did not enhance the level of gene silencing, an observation attributed to the nature of the gene and possibly the position of the dsRNA construct in relation to the target gene sequence (Sukno et al. 2007).

In our experiments, there was a clear difference in susceptibility between *P. thornei* and *P. zeae* to RNAi. When both nematodes were fed with the same amounts and dsRNA constructs of *pat-10* and *unc-87* under the same conditions, the behaviour of more *P. thornei* were affected and there was also a greater reduction in transcript for both genes in this species. The sequence of the dsRNA of *Pzpat-10* used for the soaking experiments was 94 % similar to *Ptpat-10* whereas that of *Ptunc-87* was 87 % similar to *Pzunc-87*. To find a possible explanation for this result, we used si-Fi [http://labtools.ipk-gatersleben.de/] to determine the number of possible siRNAs of 18-25 nucleotides that could be generated from each dsRNA, and the number of efficient siRNAs that could target the ESTs of *P. thornei* and *P. zeae* corresponding to the genes. The parameters used were: (1) A siRNA must have a G/C content of between 35-60 (2) the antisense strand should start with an A/U and (3) at least 3 of the first 7 nucleotides at the 5’ end of the antisense strand are A/U (Lück and Douchkov, 2009). Because the *Ptunc-87* dsRNA sequence differed by 13 % from that of *P. zeae*, it was expected that more siRNAs with perfect matches would be generated for *P. thornei* (Table 3). This is consistent with the greater knockdown of *unc-87* in *P. thornei* (29.9-fold
reduction) compared to the 7.8 fold reduction in mRNA transcript in *P. zeae*. The sequence of *Pzpat-10* dsRNA used in the soaking experiments was 100 % and 94% similar to the ESTs of *P. zeae* and *P. thornei*. Consequently, there were consistently more significant total and efficient siRNAs of all sizes generated as possible triggers for RNAi of the *P. zeae* target gene (Table 3)(p<0.5). However, this result was not consistent with the response of *P. zeae* to soaking in *Pzpat-10* dsRNA. A possible explanation is that there is a higher siRNA threshold for gene knockdown in *P. zeae*, at least for the *pat-10* gene. Such results are not surprising since differences in susceptibility to RNAi have been reported for species of *Caenorhabditis*, and it cannot be assumed that RNAi will be equally effective in even closely related nematode species (Descotte and Montgomery, 2003; Nuez and Felix, 2012). Such differences could reflect the diversity in RNAi effectors/genes in different nematode groups and that RNAi pathways may differ between nematode species (Dalzell et al. 2011; Rosso et al. 2009). One conclusion is that it provides experimental support that sequences used for dsRNA constructs to silence genes in nematodes do not need to be 100 % identical to a target gene. It also follows that, depending on the gene targeted, a sequence from one nematode species (of a similar genus) may be used to silence orthologous genes in another nematode species irrespective of whether the gene sequence(s) of the latter is known.

Soaking *P. thornei* in dsRNA of *pat-10* for 24 h resulted in 3 fold reduction in transcript level compared to expression after soaking for 16 h, indicating that longer soaking times can increase gene knockdown. This must be balanced by possible deleterious effects of longer soaking times on the viability of nematodes. Although there appears to be some recovery in *pat-10* transcript levels when nematodes soaked for 16 h were removed from the dsRNA trigger, the effect of the target knockdown persisted and affected the reproduction of the treated nematodes cultured on carrot discs such that there was an 81 % reduction in numbers 5 weeks after infecting the carrot discs. Similarly, *P. thornei* soaked with dsRNA of *Ptunc-87* failed to replicate normally after soaking, and only 29 % of control values were obtained 5 weeks after infection. The mini carrot
disc method for culturing and extracting all stages of both *P. thornei* and *P. zeae* was convenient and suitable for assessing the longer term effects of gene silencing after soaking in dsRNA. The culturing on mini discs will be a useful tool for functional analysis of RLN genes, for which two transcriptomes have recently been published (Haegeman et al. 2011; Nicol et al. 2012).

We have demonstrated here that the RLNs *P. thornei* and *P. zeae* are amenable to dsRNA-mediated RNAi via soaking in solutions containing dsRNA. Therefore, RLNs, which are migratory endoparasites, do not differ in this respect from other plant parasitic nematodes, studied. This result agrees with the recently published transcriptomes of *P. thornei* and *P. coffeae* in which evidence is provided for the presence an efficient exogenous RNAi pathway and mechanism (Haegeman et al. 2011; Nicol et al. 2012). The results presented here show that RNAi can be used to study gene function in root lesion nematodes, and has potential to be used as a control strategy for this important group of crop pests.

**Acknowledgments**

We thank Dr Vivien Vanstone, Department of Agriculture and Food Western Australia, for providing the initial inoculum of *P. thornei*, and the Australia India Strategic Research Fund for financial support. We appreciate the efforts of Ms Jyoti Rana in establishing the nematode mini disc cultures and Mr. Gordon Thomson, Biological Sciences, Murdoch University for his help in all the microscopy work. The Australia-India Strategic Research Fund (BF030027) supported this work.
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Vitae

Jo-Anne Tan
I completed a Bachelor of Science with Honours in Biotechnology in 2008 at Murdoch University, Perth, Western Australia, on a research project involving RNA interference (RNAi) of genes required for moulting of root knot nematodes. I am currently a PhD candidate at Murdoch University investigating the application of RNAi to root lesion nematodes and the use of the technology as a control strategy for the plant pest.

Michael Jones

My research interests are on the molecular basis of plant host-pathogen interactions, focussing on nematode pests and virus diseases. The aim is to gain a better understanding of both host plant responses and genes that pathogens need to be successful in invading plant hosts. This information can then be used to develop new methods for their control.

John Fosu-Nyarko

My research focus is plant-pathogen interactions. I have previously investigated relationships between fungi, phytoplasma, viruses and now nematodes and their hosts. After completing a PhD studies into an RNA virus as a gene silencing vector, I moved on to nematode research starting with the application of laser microdissection to capture and study gene expression in nematode feeding cells. Currently, my focus is on the application of bioinformatics to nematode research (recently published the first transcriptome of Pratylenchus thornei) and RNA interference to study gene function and control of root knot, cyst and root lesion nematodes, and insects.

Figures Captions

Fig. 1: Carrot mini disc culture and extraction system for root lesion nematodes. (A). Carrot mini discs in a 24-well plate. (B). Mist chamber set-up in a glasshouse for extraction of mixed stages of root lesion nematodes.
Fig. 2: FITC fluorescence of *P. thornei* incubated in basic soaking solution. (i), (ii) and (iii) Fluorescence resulting from FITC uptake in nematodes, soaked with 1 mg/mL FITC at 2h, 4h and 16 h respectively. Images in A-L show FITC fluorescence of mixed stages of *P. thornei* incubated for 16 h with different concentrations of FITC: A and B = 0.1 mg/ml; C and D = 0.2 mg/ml; E and F = 0.4 mg/ml; G and H = 0.8 mg/ml; I and J = 1.0 mg/ml; K and L = 2.0 mg/ml. Scale bar represents 200 µm.

Fig. 3: Effects of soaking mixed stages of *P. thornei* and *P. zeae* with FITC. (A) Average percentage of active *P. thornei* after soaking with different concentrations of FITC for 16 h. (B) Average percentage of active *P. thornei* and *P. zeae* after soaking with 1 mg/mL of FITC for 4, 8 and 24 h.

Fig. 4: FITC fluorescence of mixed stages of *P. thornei* incubated for 16 h in soaking solution with different concentrations of octopamine: A = no octopamine; B = 5 mM; C = 10 mM; D = 30 mM; E = 50 mM; F = 100 mM. Scale bar represents 200 µm.

Fig. 5: Effects of soaking mixed stages of *P. thornei* with the neurostimulant octopamine. (A) Average percentage of active *P. thornei* after soaking with different concentrations of octopamine for 16 h. (B) Average percentage of active *P. thornei* and *P. zeae* after soaking with 50 mM octopamine for 4, 8 and 24 h.

Fig. 6: Viability of *P. thornei* and *P. zeae* after soaking in a solution containing 1% resorcinol for 4, 8 and 24 h.

Fig. 7: FITC fluorescence from mixed stages of *P. thornei* soaked with 3 and 6 mM of three derivatives of spermidine for 16 h. (A and B) soaking with spermidine. (C and D) soaking with spermidine trihydrochloride. (E and F) soaking with spermidine phosphate salt hexahydrate. (G) soaking with no form of spermidine. (H) Water only control. Scale bar represents 200 µm.

Fig. 8: Phenotypes displayed by *P. thornei* and *P. zeae* following soaking in dsRNA of *Ptunc-87* and *Pzpat-10*. (A) Loss of sense of direction in *P. zeae* caused by dsRNA of *Ptunc-87* (B) *P. thornei* banging its head onto body caused by dsRNA of *Ptunc-87* (C) Wavy movement in *P. thornei* exposed to dsRNA of *Pzpat-10* (D) Paralysis in *P. thornei* after exposure to dsRNA of *Pzpat-10*. Scale bar represents 200 µm.

Fig. 9: Reduction in activity of *P. thornei* after soaking with dsRNA of *Pzpat-10*.

Fig. 10: Growth analysis of *P. thornei* and *P. zeae* in carrot mini discs over an 8 week period. (A) Number of eggs and vermiform *P. thornei* extracted at weekly intervals. (B) Number of eggs and vermiform *P. zeae* extracted at weekly intervals (C) Growth rates of *P. thornei* and *P. zeae* in carrot mini disc culture.

Fig. 11: Reduction in reproduction of *P. thornei*, treated with dsRNA of *pat-10* and *unc-87*, on carrot mini discs over a 5 week period (A) Average number of *P. thornei* extracted from carrot mini discs, nematodes were previously soaked with 2 mg/ml dsRNA of *Pzpat-10* (B) Average number of *P. thornei* extracted from carrot mini discs, nematodes were previously soaked in 1 mg/ml dsRNA of *Ptunc-87* with 3 mM spermidine, spermidine trihydrochloride and spermidine phosphate salt hexahydrate for 16 h.
Fig. 1
Fig. 2

A B C D

E F G H

I J K L
Fig. 3

(A) Average % of active nematodes with respect to FITC concentration (mg/ml).

(B) Average % of active nematodes with respect to incubation time (h).

- P. thornei without FITC
- P. thornei with FITC
- P. zeae without FITC
- P. zeae with FITC
Fig. 5

A

B

Average % of active nematodes

Octopamine concentration (mM)

Average % of active nematodes

Incubation time (h)

P. thornei without octopamine
P. thornei with octopamine
P. zeae without octopamine
P. zeae with octopamine
Fig. 6

- P. thornei without resorcinol
- P. thornei with resorcinol
- P. zeae without resorcinol
- P. zeae with resorcinol

Average % of active nematodes vs. Incubation time (h)
Fig. 9
Fig. 10

A

Number of nematodes or eggs extracted

Period of culture (number of weeks)

Total nematodes

Number of eggs

B

Number of nematodes or eggs extracted

Period of culture (number of weeks)

Total nematodes

Number of eggs

C

Total number of nematodes extracted

Number of weeks

P. zeae

P. thornei
Average number of nematodes extracted

### A

- **Pzpat10 dsRNA**
- **GFP dsRNA**
- **No dsRNA**

### B

- **Ptunc-87 dsRNA**
- **Ptunc-87 dsRNA + spermidine trihydrochloride (STH)**
- **Ptunc-87 dsRNA + spermidine phosphate salt hexahydrate (SPSH)**
- **GFP dsRNA + spermidine**
- **No dsRNA + spermidine**

---

**Fig. 11**
Table 1: Primers for reverse transcription, quantitative-PCR, and for generating templates for dsRNA synthesis.

<table>
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<tr>
<th>Gene (accession number)</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product length (bp)</th>
<th>Primer use</th>
<th>Position of primers in gene sequence</th>
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<td>P. penetrans pat-10</td>
<td>PpPat10-F</td>
<td>5’ GAATGGCTACATCATGCG 3’</td>
<td>393 (P. thornei)</td>
<td>RT-PCR</td>
<td>75-93</td>
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<td></td>
<td>PpPat10-R</td>
<td>5’ AACAATCTTGGTACAGTC 3’</td>
<td>418 (P. zeae)</td>
<td></td>
<td>501-521</td>
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<tr>
<td></td>
<td></td>
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<tr>
<td>P. penetrans unc-87</td>
<td>Ppunc87-F</td>
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<td>Ppunc87-R</td>
<td>5’ TTTGTTTGACCCGACTGGAG 3’</td>
<td>310 (P. zeae)</td>
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<tr>
<td>P. thornei (EU130826)</td>
<td>q18S rRNA-F</td>
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<td>5’ ACGTGATGGTGCGAGAGAGG 3’</td>
<td>134</td>
<td>qPCR</td>
<td>151-171</td>
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*The T7 polymerase promoter sequence is underlined.
Table 2: Quantification of reduction in gene expression after dsRNA-mediated gene silencing.

<table>
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<tr>
<th>Target Gene</th>
<th>Concentration of dsRNA</th>
<th>Target nematode</th>
<th>Treatment</th>
<th>Fold decrease in transcript levels after dsRNA soaking compared to expression in nematodes fed with dsRNA of GFP compared to expression in nematodes fed with no dsRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pzpat-10</td>
<td>2 mg/mL</td>
<td>P. thornei</td>
<td>Soaking for 16 h</td>
<td>155.4</td>
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<tr>
<td>Pzpat-10</td>
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<td>Soaking for 16 h + 5 days in water</td>
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<td>Pzpat-10</td>
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<td>Pzpat-10</td>
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<td>Soaking for 16 h</td>
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<tr>
<td>Pzpat-10</td>
<td>1 mg/mL</td>
<td>P. zeae</td>
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<td>Ptunc-87</td>
<td>1 mg/mL</td>
<td>P. zeae</td>
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Table 3: Predicted siRNAs generated from dsRNA of *Ptunc-87* and *Pzpat-10* for silencing the corresponding genes in *P. thornei* and *P. zeae*.

<table>
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<tr>
<th>Gene</th>
<th>siRNA size (bp)</th>
<th>All siRNA hits</th>
<th>Efficient siRNA hits</th>
<th>All siRNA hits</th>
<th>Efficient siRNA hits</th>
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<tbody>
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<td><em>P. thornei</em></td>
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<td><em>P. zeae</em></td>
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Graphical Abstract

Solution uptake optimised with FITC marker

Abnormal movement of *P. thornei* resulting from RNAi of *pat-10* gene

![Graphical Abstract Image]
Highlights

- *P. thornei* and *P. zeae* are amenable to double stranded RNA-induced gene silencing via soaking

- Using spermidine phosphate salt hexahydrate in soaking media results in more effective gene silencing

- Silencing *pat-10* and *unc-87* of *P. thornei* reduces reproduction by 77-81% in carrot mini discs

- Double stranded RNA from either nematode species silenced the corresponding gene in both species

- RNA interference is demonstrated to be a potential control strategy for root lesion nematodes