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Effects of ced-9 dsRNA on *Caenorhabditis elegans* and *Meloidogyne incognita*

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Abstract: Problem Statement: In metazoans Programmed Cell Death (PCD) is essential for proper development. Suppression of PCD is needed to guarantee cell survival and in the nematode *Caenorhabditis elegans* the regulation of PCD is accomplished by the function of the ced-9 gene. **Approach:** In this work the use of double stranded RNA (dsRNA) to knock-down ced-9 gene function was tested as means to induce PCD. **Results:** Our results indicate that dsRNA targeting the cell death protection gene ced-9 is effective at decreasing the fecundity of *C. elegans* by up to 21%. The decreased fecundity correlated with an increased presence of cell corpses in developing embryos. Endogenous ced-9 transcript levels were reduced in progeny of ced-3 mutant nematodes fed bacteria expressing ced-9 dsRNA. These data suggest that nematode fecundity can be reduced by ingestion and exposure to dsRNAs targeting regulation of the cell death pathway. In an attempt to determine if plant parasitic nematodes are susceptible to the targeting of the PCD regulatory pathway we exposed *Meloidogyne incognita*, a plant parasitic nematode, to ced-9 dsRNA; here we show that this exposure results in decreased gall formation in the tobacco plants. **Conclusion/Recommendations:** Our results provide the first steps toward using RNAi technologies to attempt nematode control by targeting cell death pathways. Ongoing research with transgenic plants designed to express dsRNA for ced-9-like sequences will further test the feasibility of generating plants with RNAi-based resistance to parasitic nematodes.

Key words: Gene silencing, Programmed Cell Death (PCD), *Caenorhabditis elegans*, *Meloidogyne incognita*, ectopic cell death, RNA interference (RNAi), *E. coli*, Green Fluorescent Protein (GFP), plant parasitic nematodes.

INTRODUCTION

Nematode parasitism of animals and plants remains a significant global problem (Chitwood, 2003; De Silva *et al*., 2003). Research conducted using the free-living nematode *Caenorhabditis elegans* has contributed tremendously to our understanding of other nematode species and as such, *C. elegans* provides highly suitable model for testing potential methods of parasitic nematode control, particularly as older ineffective, or environmentally detrimental methods are phased out or become ineffective (Britton and Murray, 2006). In particular, *C. elegans* has contributed to our understanding of Programmed Cell Death (PCD). PCD in *C. elegans* eliminates a total of 131 cells (Kinchen and Hengartner, 2005). The genes egl-1, ced-4 and ced-3 are required for PCD in somatic cells of *C. elegans* and a loss-of-function (lf) mutation in any of them results in increased survival rates in cells that would otherwise be eliminated (Metzstein *et al*., 1998). The gene ced-9, responsible for inhibiting cell death, is epistatic to ced-3 and ced-4 and shares significant similarity in structure and function with the mammalian proto-oncogene bcl-2 (Chinnaiyan *et al*., 1997; Hengartner and Horvitz, 1994). A gain-of-function (*gf*) mutation in ced-9 prevents normally occurring cell deaths, while lf mutants lead to increased cell deaths, maternal-effect lethality and sterility (Hengartner *et al*., 1992).

RNA interference (RNAi) has been used extensively in recent years for the experimental targeting and silencing of specific genes in many species (Bakhetia *et al*., 2005; Geldhof *et al*., 2007; Wang and Barr, 2005). In this study we tested the effectiveness of Double Stranded (DS) RNAs for targeting and silencing the cell death protection gene ced-9 in *C. elegans*. We hypothesized that feeding or soaking *C. elegans* with double stranded (ds) ced-9 RNA would silence endogenous ced-9 expression and phenocopy a loss-of-function ced-9 mutant. Our data

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suggest that fecundity is significantly reduced in *C. elegans* treated with double stranded ced-9 RNA. We also tested the possibility that ds ced-9 RNA might be used to control reproduction in parasitic nematodes. Our work indicated that the plant root-knot nematode *Meloidogyne incognita* was also sensitive to ced-9 dsRNAs, demonstrated by reduced gall formation in tobacco plants after treating infective J2 *M. incognita* nematodes with ced-9 dsRNAs. These data suggest that targeting the cell death pathway of nematodes by RNAi may be an effective method for their control.

MATERIALS AND METHODS

Nematode and bacterial strains: The strains of *C. elegans* (obtained from the *Caenorhabditis* Genetic Center, University of Minnesota) used in this work were: wild-type N2; ced-3 mutant strain (n717) IV; ced-4 mutant strain (n1162)III and gfp transgenic line (PD4251). Nematodes were cultured and synchronized according to standard protocols (Sulston and Hodgkin, 1998). *E. coli* strain OP50 was used as a food source for maintenance of the different free-living nematodes. The plant-parasitic nematode species *Meloidogyne incognita* (root-knot nematode) was kindly provided by Dr. Valerie Williamson, UC-Davis, CA.

Molecular biology techniques: All reagents unless otherwise indicated were purchased from Fisher Scientific Company. All molecular biology techniques were conducted using standard protocols (Ausubel, 2003; Sambrook and Russell David, 2001) with the following modifications: cloning was initially carried out in *E. coli* strains DH5α or Top10 (Invitrogen) and plasmids were later transformed into *E coli* strain HT115(DE3) for *C. elegans* dsRNA feeding. The following primers were designed using ced-9 cDNA sequence (L26545) and were synthesized by Operon (Qiagen): P1 5'-ATAAGAATGCGGCCGCAGATGACACGCTGC ACGGCG-3'; P2 5'-GAAGGCCTTTACTTCAAGCTGAACATCATC-3'; P3 5'-CGAGATGAAGGAGTTTCTGG-3'; P4 5'-GCCAATCATCGACCACCGTC-3'. A cDNA of the ced-9 gene was cloned (908 bp) and its identity confirmed by DNA sequencing. The ced-9 gene was subcloned into the pLitmus28i vector (New England Biolabs). Constructs pLitmus28i::ced-9 (Fig. 1A) and pLitmus::Mal (positive control provided in New England Biolabs RNAi Synthesis Kit) were tested for their ability to produce dsRNAs under *in vitro* and *in vivo* conditions; transcripts of the correct size were obtained using both *in vitro* (Fig. 1B) and *in vivo* (Fig. 1C) conditions.

RNAi of the *C. elegans* ced-9 gene*:* *In vitro* dsRNA synthesis was conducted as follows: pLitmus28i::ced-9, pLitmus28i::gfp and pLitmus::Mal plasmid constructs were used as templates in PCR reactions with a T7 primer (5'-TAATACGACTCACTATAGG-3'), which flanks the insert in the pLitmus28i vector (New England BioLabs). pLitmus::Mal shares no homology with the *C. elegans* genome and was used to generate non-specific dsRNAs. 100 ng of purified plasmid DNA was added to PCR reactions with *Pfu-turbo* polymerase (Stratagene). PCR products were purified and 1 μg of DNA template was used in dsRNA synthesis reactions according to recommended protocols (New England BioLabs). dsRNA reactions were treated with DNase1 (Qiagen) and S1 nuclease (Promega) and purified dsRNA was resuspended in nematode soaking buffer (10.9 mM Na_2HPO_4, 5.5 mM KH_2PO_4, 2.1 mM NaCl, 4.7 mM NH_4Cl, 3 mM spermidine and 0.05% gelatin in diH2O).

For *in vivo* dsRNA synthesis the plasmids pLitmus28i, pLitmus28i::ced-9, pLitmus28i::gfp and pLitmus::Mal, were transformed into *E. coli* strain HT115(DE3) and cultured as described (Timmons *et al.* 2001). Single colony-derived cultures were grown to an OD_{600} of ~0.4 and dsRNA expression was induced with 1 mM IPTG for 4 h at 37°C. RNA was extracted from an aliquot of each sample, treated with DNase I and S1 Nuclease and resolved in 1% agarose to identify bacterial clones producing dsRNA bands of the correct molecular weight.

Feeding and soaking of *C. elegans*: The feeding of *C. elegans* to monitor fecundity was conducted as follows: bacterially seeded NGM plates (24-well Falcon plates with NGM containing IPTG and 100 μg mL^{-1} ampicillin when appropriate) were incubated at 37°C overnight. Replica wells were seeded with 5 μL of HT115 (DE3) bacteria containing pLitmus28i empty vector, pLitmus::Mal, or pLitmus28i::ced-9. Two or 3 synchronized L3 or L4 stage hermaphrodites were placed in each well. After ~72 h, 1 treated nematode was selected at random from each well and placed into a well of a 96-well round-bottom plate containing 30 μL of liquid nematode media (3% yeast extract, 3% peptone and 1% dextrose supplemented with 0.5 mg mL^{-1} of hemoglobin and 40 μg mL^{-1} of cholesterol). Nematodes were transferred to fresh media every 48 hours and total progeny counted at 120 h. Each randomly selected nematode was considered a biological replicate, a minimum of 12 biological replicates were analyzed per trial and at least 2 independent trials were carried out on each genotype.

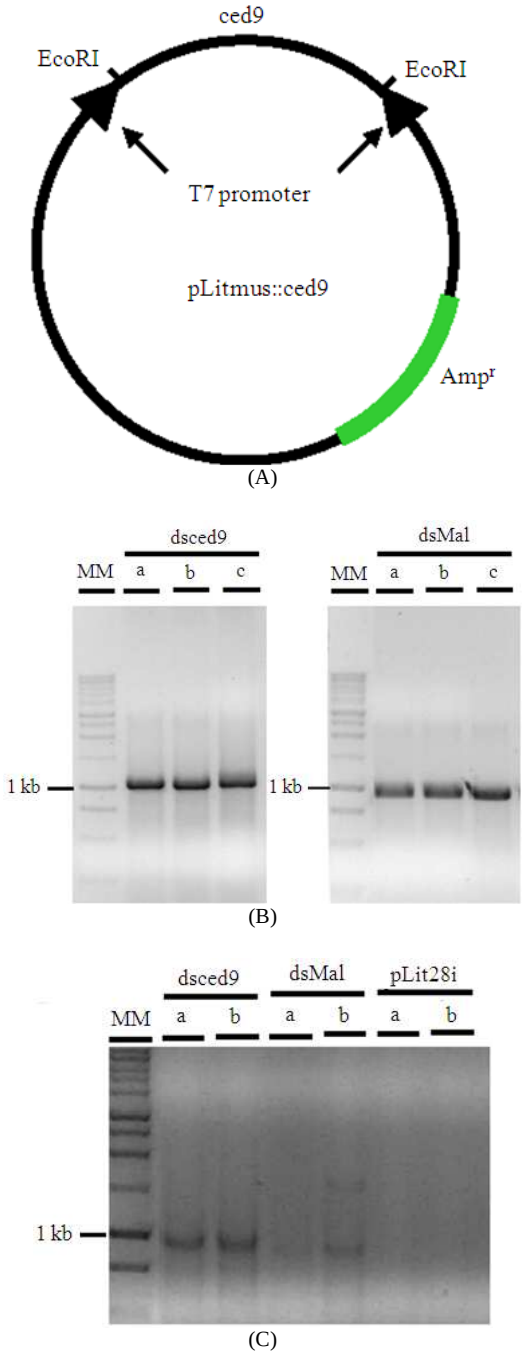


Fig. 1: Construct and transcripts used in the present work. (A) Is a schematic representation of the pLitmus::ced-9 plasmid; similar empty vector, or vector containing the nonspecific Mal gene were also used. For *in vitro* generated transcripts (B) PCR amplified genes or entire plasmids were used as template in transcription reactions and dsRNA corresponding to the expected sizes were obtained: ~1 kb dsRNAs for ced-9 and ~0.9 kb for Mal). Lanes a, b and c represent transcripts from independent clones. Transcripts were also generated *in vivo* (C) in *E. coli* strain HT115(DE3). Independent clones (lanes a and b) expressing ced-9 and Mal revealed dsRNAs of the expected sizes (~0.9 kb and ~0.8 kb respectively), while bacterial samples containing empty pLitmus28i plasmids did not.

For each genotype the two negative controls were each used in at least one trial (pLitmus28i empty vector and pLitmus::Mal) and had similar effects on treated nematodes. Trials showed similar trends and replicates were pooled for final analysis (for wt, n=136 and n=132 for control and test treatment, respectively; for ced4, n=122 and n=120 for control and test treatments; for *ced3* worms n=59 and n=60 for control and test treatments). PD4251 nematodes were fed HT115(DE3)::pLitmus28i::gfp or HT115(DE3)::pLitmus28i for a period of 72 hours and were mounted for observation by epi-fluorescence microscopy. The total number of GFP positive muscle and vulval cell nuclei were counted (n=10 and n=38 for control and test treatments, respectively).

For soaking experiments, 2-3 wild-type, ced-4(lf), or ced-3(lf) *C. elegans* L3 stage nematodes were added to 1 μL droplets (in closed 200 μL PCR tubes) of each treatment. Treatments were composed of soak buffer containing ced-9 or Mal dsRNA at a concentration of ~1 μg/μL, or soak buffer with no dsRNA. Nematodes were soaked for 24 h and then transferred to NGM plates seeded with OP50 for a two-day recovery. As hermaphrodites matured and began to produce eggs (~72 h), they were placed in 50 μL of liquid nematode media (in wells of 96-well flat-bottom plate), transferred to fresh media after 48 hours and total progeny counted at 96 hours. Each treated hermaphrodite was a biological replicate, a minimum of 10 biological replicates were analyzed per trial and at least two independent trials were conducted on each genotype. Replicates from all trials were pooled for final statistics as described above (for wt, n = 40 and n = 34 for control and test treatment, respectively; for ced4, n = 48 and n = 43 for control and test treatments; for ced3 worms n = 45 and n = 46 for control and test treatments). Similarly, PD4251 nematodes were treated with double stranded gfp dsRNA or soak buffer (n >30 for both treatments). Two sample T-tests were conducted using the Microsoft Excel data analysis function.

Feeding of mutant ced-3 *C. elegans* with ced-9 or Mal dsRNA and competitive PCR (cPCR) assay: Mutant ced-3 nematodes were plated in NGM plates containing OP50 and incubated for 72 h. Matured hermaphrodites were washed with M9 media (48 mM Na_2HPO_4, 22 mM KH2PO4, 9 mM NaCl, 19 mM NH4CL), incubated in a hypochlorite solution (final concentration of 5.25% Na-O-Cl and 250 mM KOH) for 10 min and the recovered embryos washed three times with M9. Embryos were transferred to NGM plates and incubated overnight with ced-9 or Mal dsRNA producing bacteria (NGM plates were supplemented with 1mM IPTG, 50 μg mL^{-1} ampicillin and 25 μg mL^{-1} tetracycline) and allowed to develop and feed for 72 hours. Embryos were extracted from these fed nematodes and divided in two fractions; the first fraction was used for RNA extraction; the second fraction was plated on NGM plates seeded with OP50 bacteria and incubated for an additional 24 hours for RNA extraction from developed nematodes. Embryo fractions were treated with hypochlorite solution and washed with M9 prior to RNA extraction to eliminate bacteria and avoid dsRNA cross-contamination. Total RNA was isolated from ced-3 mutant *C. elegans* nematodes or embryos using Trizol reagent protocol as recommended by the manufacturer (Invitrogen). Total RNA was treated with DNase I (1 unit μg^{-1}) and purified by phenol chloroform extraction. Two micrograms of RNA was combined with 1 μg of ced-9 P4 primer for first-strand cDNA synthesis with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV) according to manufacturer instructions (Promega). The cDNA was used as template for PCR with ced-9 primers (P3 and P4) in the presence of known quantities of the ced-9Δ competitor. The competitor was derived by fill-in and blunt-end ligation of pLitmus28i::ced-9 plasmid after digestion with Bpu10I and SapI restriction enzymes. The resulting plasmid (pLitmus28i::ced-9Δ) contains a deletion of 88 base pairs in the ced-9 gene sequence.

To quantify cDNA production during ced-9 cDNA synthesis we used the *C. elegans* gamma-tubulin gene, tbg-1 (F58A4.8), since gamma-tubulin is a single copy gene and is constitutively expressed in *C. elegans* (Consortium, 1998; Strome *et al*., 2001). The gamma-tubulin primers used were: upstream primer=5'ATGTCCGGTACGGGTGCCTTGA3' and downstream primer=5'TGGAGGCCTTCTGATTGCGAAATC3'. The PCR amplification was performed for 30 cycles as follows: hot start at 95°C for 5 min; denaturation at 95°C for 30 sec.; annealing at 49°C for 30 sec and extension at 72°C for 1 min. At the end of the 30 cycles products were extended at 72°C for 7 min. PCR products were visualized by ethidium bromide staining on 1% agarose gels and digitized using the 1D Image Analysis Software v3.5 (Eastman Kodak Company). The results shown are representative of three independent experiments.

Nomarski microscopy of *C. elegans*: Nomarski (Differential Interference Contrast) microscopy was used to study the morphology of *C. elegans* embryos

derived from hermaphrodites fed *E. coli* expressing ced-9 or Mal dsRNA. Cell corpses were identified by their increased refractivity as described (Hengartner *et al*., 1992; Sulston *et al*., 1983). Embryos were mounted on agar-pad slides and visualized using an Olympus IX-70 research microscope. Images were recorded using MagnaFire camera and software (Optronics). Observations were conducted in multiple focal planes on the lateral sides of embryos ranging in age from ~350-420 min (n = 51 and n = 56 for test and control treatments, respectively). A two sample T-test was conducted as described above for testing the null hypothesis of equal means.

Maintenance and soaking of *M. incognita*: Susceptible tomato plants (Rutger's Select, Tomato, Augusta, GA) were used to maintain *M. incognita* as follows: five to ten days old sand-germinated tomato plants (10 cm shoot and approximately 4-5 cm long roots) were infected with pre-parasitic J2s. Five to eight week old heavily galled plant roots were cut into small pieces using a razor blade. The chopped plant roots were vigorously shaken for 5 min with 10% bleach and subsequently poured onto a 250 micron mesh screen. Eggs were collected from the flow-through on a 25 micron mesh screen and further purified by centrifugation in 35% sucrose at 500 g for 10 min. Purified eggs were subjected to two additional treatments in 10% bleach (10 min each) followed by centrifugation at 500 g for 5 min and several rinses in sterile water. Sterilized eggs were hatched at 28°C. During this incubation period, juveniles were allowed to crawl through a sterile mesh strainer into a water container. Freshly hatched juveniles *M. incognita* J2 were re-suspended in soaking buffer for treatment with dsced-9 or dsMAL RNA.

***M. incognita* susceptibility assay:** Tobacco plants were germinated on half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) and transferred to square (10x10mm) plates containing MS medium and 1% Agargel (Invitrogen). The tobacco seedlings were placed on the surface of media in square plates and allowed to grow in the incubator for 2 weeks at 28°C and at a photoperiod of 16/8h (light/dark). 30 tobacco plants were used per treatment; 5 plants/plate and 2 roots per plant were inoculated at the root apex with approximately with 20 *M. incognita* J2 nematodes. Prior to infection, *M. incognita* J2s were soaked 24 hours in soaking buffer containing dsced-9 or dsMAL RNA at a concentration of 1 μg μL^{-1}. Positive infections (root thickenings caused by growth of adult females) were scored every 48 hours for 10 days. Each root thickening developed into a gall after about 20 days (not shown). Sodium-hypochlorite-acid- fuchsin staining methods (Byrd *et al*. 1983) were used to confirm nematode infections.

RESULTS

***C. elegans* fed or soaked with ced-9 dsRNA exhibited decreased fecundity:** in feeding experiments with wild-type nematodes, ced-9 dsRNA fed nematodes produced 21% fewer offspring than negative controls (Fig. 2A; $p<0.001$). No reduction in fecundity was observed in ced-4(lf) individuals fed *E. coli* expressing ced-9 (Fig. 2A). Similarly, ced-3(lf) nematodes showed no decrease in fecundity following treatment (Fig. 2A). Wild-type *C. elegans* soaked in ced-9 dsRNA produced significantly fewer offspring than control treatments (Fig. 2B), although the effect was not as strong as that observed in feeding assays. An average decrease of ~16% in fecundity was observed ($p<0.05$). Conversely, ced-4(lf) and ced-3(lf) *C. elegans* mutants showed no difference among treatment groups (Fig. 2B).

Gfp silencing in *C. elegans* by RNAi confirmed the effectiveness of dsRNA delivery methods: the effectiveness of our RNAi delivery methods was confirmed by silencing the expression of Green Fluorescent Protein (GFP) in the gfp transgenic *C. elegans* strain PD4251 (Fig. 2C). In wild-type *C. elegans*, ced-9 was more efficiently silenced by dsRNA feeding; however in *C. elegans* strain PD4251, the gfp transgene was more effectively silenced by dsRNA soaking (Fig. 2C).

Endogenous levels of ced-9 mRNA are reduced following treatment with ced-9 dsRNA: we quantified the expression level of the endogenous ced-9 mRNA in progeny of treated nematodes using a cPCR assay (Brutnell and Dellaporta, 1994; Death *et al*., 1999). Since lf alleles of ced-9 lead to sterility and maternal-effect lethality and ced-9; ced-3 double mutant nematodes are viable (Hengartner *et al*., 1992), we tested the specificity of RNAi directed at ced-9 expression in a ced-3 background.

The endogenous concentration of ced-9 mRNA in wild-type nematodes was approximately 0.5 picograms per 5 μL of cDNA template used for competitive PCR (not shown). We then quantified the concentration of ced-9 and gamma-tubulin, as an internal control, in progeny of mutant ced-3 *C. elegans* that were fed bacteria expressing ced-9 or Mal dsRNA. The expression levels of gamma-tubulin were similar in both ced-9 and Mal dsRNA fed individual nematodes (Fig. 3A lanes 3 and 4) and embryos (Fig. 3A lanes 7 and 8).

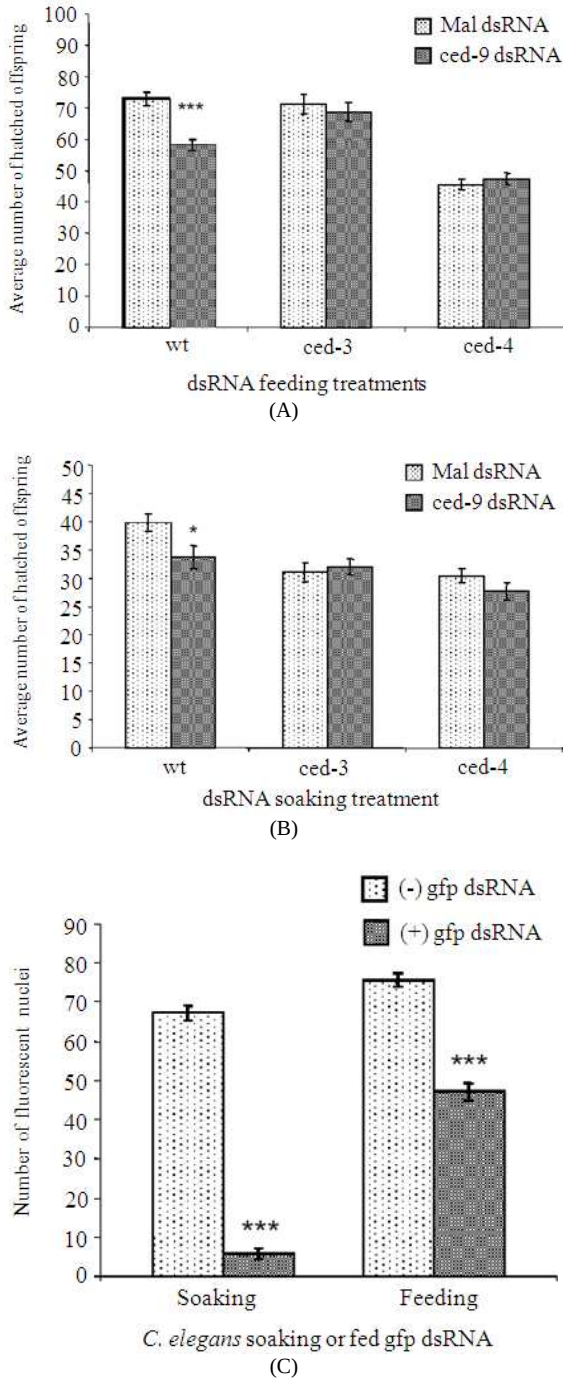


Fig. 2: Fecundity of wild-type and ced-3 and ced-4 mutant *C. elegans* fed (A) or soaked (B) with ced-9 dsRNA. (C) Efficiency of gfp silencing by soaking or feeding in strain PD4251. Values are means and error bars the Standard Error of the Mean (SEM) (t-test, p≤0.001*** and p≤0.05*)

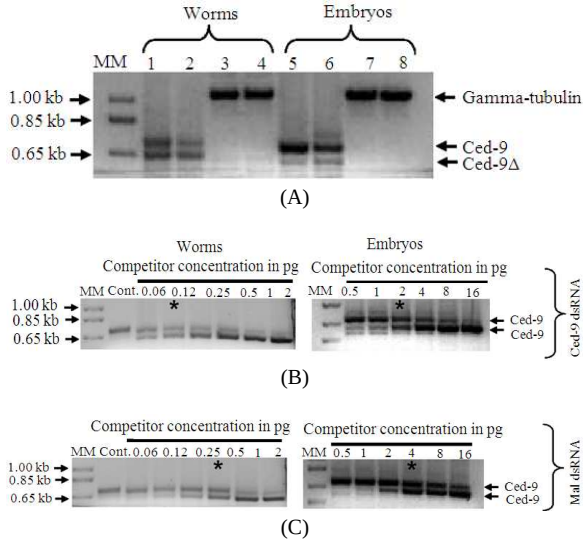
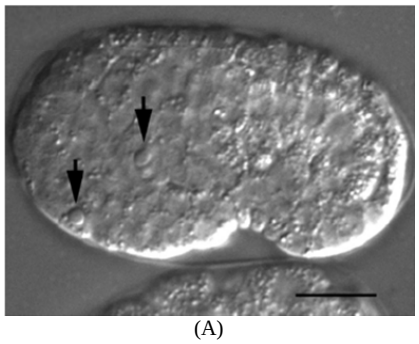


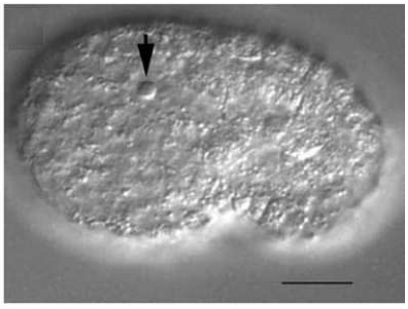
Fig. 3: Competitive PCR analysis of ced-9 transcript levels on treated *C. elegans* ced-3 mutant nematodes. (A) Results of cPCR on RNA extracted from nematodes fed bacteria expressing Mal dsRNA (lanes 1, 3, 5 and 7) or ced-9 dsRNA (lanes 2, 4, 6 and 8). Quantification of ced-9 and gamma-tubulin expression in nematodes (lanes 1-4) or embryos (lanes 5-8). The competitor concentration used in this experiment was 0.5pg. (B) and (C) show results from cPCR on RNA extracted from ced-3 mutant *C. elegans* feed *E coli*. Strain HT115 (DE3) expressing ced-9 or Mal dsRNA, respectively in both nematodes (left panels) and embryos (right panels). Pg: picograms; MM: molecular marker (1 kb ladder); Cont.: PCR amplification of plasmid control. The asterisk marks the competitor concentration at which the target to competitor bands ratio is closest to one.

However, endogenous ced-9 mRNA was lower in both adult nematodes (compare lanes 1 and 2 of Fig. 3A) and embryos (compare lanes 5 and 6 of Fig. 3A) progeny of the ced-9 dsRNA fed nematodes.

We then conducted cPCR assay and found that ced-3 mutant *C. elegans* fed ced-9 dsRNA consistently demonstrated reduced (0.12 picograms; Fig. 3B) ced-9 levels compared to nematodes fed Mal dsRNA (0.5 picograms; Fig. 3C). Results were similar when the endogenous ced-9 mRNA was measured in progeny embryos (2 picograms in ced-9 dsRNA fed nematodes compared to 4 picograms in Mal dsRNA fed nematodes; Fig. 3B and C). There were no differences in endogenous ced-9 concentrations between wild-type or ced-3 mutant *C. elegans* fed Mal dsRNA (not shown).



(A)



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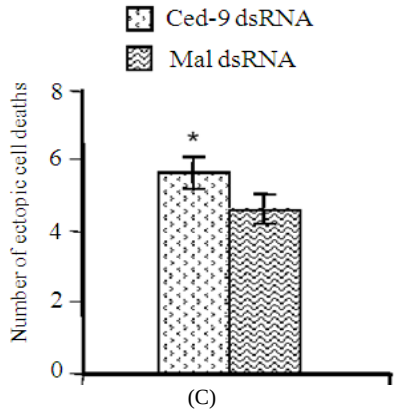
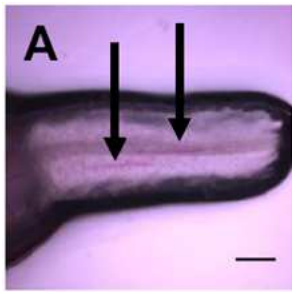
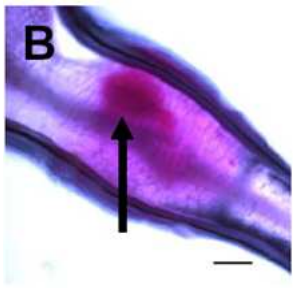


(C)

Fig. 4: Mean number of ectopic cell deaths counted in *C. elegans* embryos exposed to ced-9 dsRNA were observed by Nomarski microscopy. One focal plane of a treated embryo (A) and control embryo (B) at the comma stage. Arrows in (A) and (B) point to cell corpses; identified by their increased refractivity under Nomarski microscopy as described previously (Hengartner *et al*., 1992; Sulston *et al*., 1983). (C) The graph shows a comparison between ced-9 and Mal dsRNA treatments. Values are means +/- SEM. Both comma stage (350-380 min) embryo data and 1½ fold stage (~380-420 min) embryo data were combined and ced-9 dsRNA treatments were compared to Mal dsRNA. Scale bar is 10 µm. (t-test, p≤0.05*)



(A)



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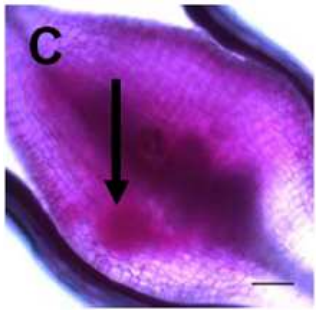
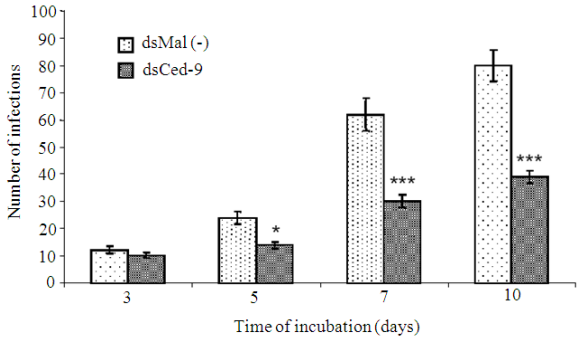


(C)



(D)

Fig. 5: Response of tobacco plants infected by *M. incognita* J2 worms soaked in Mal or dsCed-9 dsRNA. (A), (B) and (C) show the appearance of nematodes infected roots. (A) Shows nematodes within 24 hours of root penetration (see arrows). (B) and (C) show different levels of root thickening due to nematode infection; arrows point to developing females. Scale bar is 200 µm. (D) Number of galls developed at 3, 5, 7 and 10 days. Values are means of two independent experiments and in each experiment 30 plants were used. ced-9 dsRNA treatment showed a statistically significant reduction in the number of galls when compared to the control Mal dsRNA treatment t-test, p≤0.05* at 5days post-infestation and t-test, p≤0.001*** at 7 and 10 days post-infestation.

Decreased fecundity was associated with increased cell (extra space between increased and cell) death corpses in *C. elegans* embryos: we detected a significant increase (19%) in ectopic cell deaths occurring in comma stage (350-380 min) embryos treated with ced-9 dsRNA and a 16% increase in the number of cell deaths occurring in the 1½-fold stage (400-420 min) embryos relative to controls. Overall, a 17% increase in cell deaths was detected in 350-420 min embryos derived from ced-9 dsRNA fed wild-type *C. elegans* relative to, (delete comma, space corrected between elegans and relative) Mal dsRNA fed nematodes (Fig. 4; $p<0.05$).

M. incognita* juveniles soaked in ced-9 dsRNA prior to plant inoculation demonstrate decreased efficiency of gall formation in infected tobacco plants:** Juveniles of the plant parasitic nematode *M. incognita* were soaked with ced-9 or Mal dsRNA prior to inoculation of tobacco roots. In two independent experiments, we observed that *M. incognita* J2s soaked with ced9 dsRNA prior to inoculation of tobacco plants, showed a reduction of 40% in the number of galls generated when compared to worms soaked with dsMal RNA (Fig. 5). This (edited) reduction in the number of galls was statistically significant between two treatments; t-test, p0.05* at 5 days post-infestation and t-test, $p\leq0.001$ at 7 and 10 days post-infestation, (SPSS 13.0 statistical analysis software for windows was used) values were means of two independent experiments and in each experiment 30 plants were used per treatment.

DISCUSSION

Plant-parasitic nematodes pose a serious worldwide threat to agriculture and as older or environmentally unfriendly methods for their control are phased out or become ineffective, new methods must be actively sought. Our research suggests that nematode numbers can be affected by exposure to dsRNAs encountered in their environment and food, and indicates that cell death regulatory genes may be useful targets for engineering nematode resistance in host crop species. Wild-type *C. elegans* was susceptible to dsced-9 RNA in both feeding and soaking experiments, with up to a 21% reduction in fecundity following treatment. The reduced fecundity correlated with an (delete) increased (d added) in (delete) ectopic cell deaths in treated embryos. A similar analysis in a (the instead of a?) plant parasitic nematode *M. incognita* also demonstrated susceptibility to ced-9 dsRNA.

The effectiveness of our system was confirmed by silencing the expression of green fluorescent protein (gfp) with greater than 90% efficiency in the gfp transgenic *C. elegans* strain PD4251 (data not shown). These results were comparable to those achieved in other laboratories (Timmons *et al*., 2001). When comparing the silencing of gfp and ced-9 in *C. elegans*, it appeared that silencing efficiency was dependent on the method of RNAi treatment employed and the gene targeted. Gene-specific differences in RNAi susceptibility have been reported between feeding and microinjection procedures (Kamath *et al*., 2001).

The fact that ced-9 dsRNA treated *C. elegans* continued to produce offspring throughout their life indicates that the penetrance of the phenotype was incomplete. It is not uncommon that weaker phenotypes are achieved using RNAi, or that silencing occurs in only a subset of cells in comparison to genetic mutants (Tabara *et al*., 1998). We observed high expression of the gfp transgene in embryos within dsRNA treated hermaphrodites, supporting the idea that some embryonically expressed transcripts may be difficult to silence (Kamath *et al*., 2001). It has been shown that maternally-derived ced-9 is passed into the developing egg (Hengartner *et al*., 1992) and it is possible that this subset of RNAs was more efficiently degraded by RNAi of ced-9. Consequently, the techniques employed in this study may have been insufficient for silencing embryonically expressed transcripts, leading to increased survival. In *C. elegans*, stronger ced-9(lf) mutants have been characterized as having massive ectopic cell deaths in developing embryos, maternal effect lethality and partial sterility (Hengartner *et al*., 1992). On the other hand, weak loss-of-function alleles have also been described in which nematodes are viable (Riddle, 1997). Early high throughput RNAi assays did not report phenotypes associated with the ced-9 gene (Gonczy *et al*., 2000; Maeda *et al*., 2001), while other studies have reported increased apoptosis, embryo lethality, and other cell death phenotypes (Lettre *et al.*, 2004; Sonnichsen *et al.*, 2005). The results presented here support previous studies and indicate that a quantifiable reduction in fecundity followed ced-9 RNAi treatments.

It is possible that non-specific silencing of other genes occurred in *C. elegans* treated with ced-9 dsRNA. The ced-9 locus transcribes four mRNA species two of which include both ced-9 and cyt-1. Cyt-1 encodes a protein similar to cytochrome b560 and is involved in electron transport in mitochondria (Senoo-Matsuda *et al*., 2003). Mutants in cyt-1, such as mev-1, lead to increased sensitivity to oxidative stress and aging (Senoo-Matsuda *et al*., 2003). In *C. elegans*, cyt-1

mutants display structural abnormalities of the mitochondria, anion generation, loss of membrane potential, changes in ced-9 protein levels and ced-3 dependent increases in cell death occurring in 400-min embryos. Thus it is plausible that non-specific degradation of cyt-1 contributed to the phenotypes we observed. However, three lines of evidence suggest that the decrease in fecundity we observed in *C. elegans* was due to specific silencing of ced-9 and consequent up-regulation of the cell death pathway. First, decreased fecundity was only observed in wild-type *C. elegans* and not in ced-3 or ced-4 mutants, suggesting the effect was due to an up-regulation of the proteins CED-4 and CED-3 through reduced ced-9 levels. Second, endogenous levels of ced-9 RNA were reduced in ced-3 mutants treated with ced-9 dsRNA. Third, a significant increase in ectopically occurring cell deaths was observed in treated nematodes.

C. elegans shares common ancestry with plant-parasitic nematodes (Riddle, 1997) and it has been demonstrated that much like *C. elegans*, *Meloidogyne artiellia*, *Heterodera glycines* and *Globodera pallida* are sensitive to RNAi (Fanelli *et al*., 2005; Urwin *et al*., 2002). This suggests that RNAi mechanisms are conserved among nematode species. Homology search across Nematoda phylum using the cyt-1/ced-9 full-length transcripts (L26545) identified homology to cyt-1 and ced-9 sequences in some species of plant parasitic nematodes (not shown). PCR-Southern blot hybridizations to *C. elegans* ced-9 probe detected similar sized sequences in other nematode genera, including *Pristionchus*, *Zeldia* and *Meloidigyne* (our unpublished data). Our susceptibility assay on *M. incognita* suggests that this species may contain an orthologous ced-9 gene susceptible to RNAi; however, our inability to clone a ced-9-like gene using the ced-9 primers suggests sequence divergence among the two species. Consequently, it remains to be seen whether or not genes with similar sequences from unrelated biochemical pathways were silenced by double stranded ced-9 RNAs, contributing to reduced gall formation.

CONCLUSION

In conclusion, our results provide the first steps toward using RNAi technologies to attempt nematode control by targeting cell death pathways. Ongoing research with transgenic plants designed to express dsRNA for ced-9-like sequences will further test the feasibility of generating plants with an RNAi-based resistance to parasitic nematodes.

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