

Article

Silencing of *Mg-pat-10* and *Mg-unc-87* in the Plant Parasitic Nematode *Meloidogyne graminicola* Using siRNAs

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Abstract: Until recently, the standard method for RNA interference (RNAi)-based reverse genetics in plant parasitic nematodes (PPNs) was based on the use of long double-stranded RNA (dsRNA). This increased the chance of off-target gene silencing through interactions between different short interfering RNAs (siRNAs) and non-cognate mRNA targets. In this work, we applied gene-specific knockdown of *Mg-pat-10* and *Mg-unc-87* of the root knot nematode *Meloidogyne graminicola*, using discrete 21 bp siRNAs. The homologue of *Mg-pat-10* in *C. elegans* encodes body wall troponin C, which is essential for muscle contraction, whereas the homologue of *Mg-unc-87* encodes two proteins involved in maintenance of the structure of myofilaments in the body wall muscle of *C. elegans*. The knockdown at the transcript level, as seen by semi-quantitative RT-PCR analysis, indicates that the *Mg-pat-10* gene was silenced after soaking the nematodes in a specific siRNA for 48 h. At 72 h post-soaking, the *Mg-pat-10* mRNA level was similar to the control, indicating the recovery of expression between 48 h and 72 h post-soaking. For *Mg-unc-87* the nematodes started to recover from siRNA silencing 24 h after thorough washing. A migration assay showed that for the nematodes that were soaked in the control (siRNA of β -1,4-endoglucanase), 77% of the nematodes completed migration through the column in a 12 h period. By comparison with the control, nematodes incubated in the siRNA of *pat-10* or *unc-87* were significantly inhibited in their motility. After 12 h, only 6.3% of the juveniles incubated in the *Mg-pat-10* siRNA and 9.3% of those incubated in *Mg-unc-87*

siRNA had migrated through the column, representing 91.8% and 87.9% inhibition respectively compared to the control. In the present work, we demonstrated that *M. graminicola* is readily susceptible to siRNAs of two genes involved in nematode motility. This is an important contribution to the progressive use of siRNA for functional analysis. Moreover, the application of RNAi in PPNs opens the way for environmentally friendly control of *M. graminicola*.

Keywords: short interfering RNA (siRNA); *Meloidogyne graminicola* (Mg); *Mg-pat-10*; *Mg-unc-87*; migration assay; expression analysis

1. Introduction

Plant-parasitic nematodes (PPNs), which include the genus *Meloidogyne*, are responsible for some of the most damaging biotic stresses on crops [1]. Rice plants infected by the root-knot nematode *M. graminicola* are characterized by a number of distinctive signs; the development of abnormal swellings of the root tip known as galls, as well as yellowing, stunting and wilting of the plants [2]. The current practice operating to control PPNs is through the integrated application of several procedures; nematicides, resistant crop varieties, and biological control strategies such as the use of trap crops, natural enemies and cultural practice [3]. However, these approaches are becoming increasingly unsatisfactory due to the proliferation of PPNs through the continuous and intensive cultivation of susceptible varieties. Significant progress has been made with the use of RNAi in PPNs and this may be advantageous for parasite control through plants engineered to express PPN-specific transcripts. Recently it was demonstrated that RNAi application was a successful approach to functional genomics and nematode control [4]. The RNAi pathway elicits sequence-specific silencing of target mRNA by means of introduction of homologous double stranded RNA [5,6]. For the free living nematode *C. elegans*, dsRNA can be delivered by feeding the nematodes with bacteria producing dsRNA from a plasmid. In contrast to *C. elegans*, PPNs rarely feed outside of their host. Rosso *et al.* [7] stated that for PPNs, eggs and hatched J2 can be used for soaking. Soaking of J2 of the cyst nematode (CN) *Globodera pallida* in dsRNA was used to target five *flp* genes and contrary to *C. elegans*, in which neuronal tissues are refractory to RNAi, soaking J2 in dsRNA induced a strong depletion in *flp* transcripts and motility alteration phenotypes [8]. It was noted surprisingly that *flp* genes from *G. pallida* seem particularly sensitive to RNAi because dsRNA concentrations as low as 10^{-4} – 10^{-9} µg/µL were sufficient to trigger silencing. *Mi-CRT*, a calreticulin gene expressed in the esophageal glands of *Meloidogyne incognita* can be silenced using siRNAs as shown by Arguel *et al.* [4], who further demonstrated that this knocking of *Mi-CRT* was not persistent.

In the present work, we evaluated the performance of discrete 21 bp siRNAs, targeting *unc-87* and *pat-10* in *M. graminicola*. In *C. elegans*, *pat-10* encodes body wall muscle troponin C, the calcium-binding component of actin filaments. RNAi targeting of this gene in *C. elegans* showed that the knocking down of *pat-10* leads to paralysis, embryonic lethality and maternal sterility [9]. In *C. elegans*, through alternative splicing *unc-87* encodes two proteins which are required to maintain the structure of myofilaments in body wall muscle cells. *Unc-87* resembles the C-terminal repeat

region of calponin and associates with thin F-actin filaments [10,11]. An RNAi study using dsRNA to *unc-87* in *C. elegans* by Simmer *et al.* showed that the knockdown of this gene results in uncoordinated locomotion of the nematode [12].

2. Materials and Methods

2.1. Oligonucleotides Design and siRNA Synthesis

Homologues of *C. elegans pat-10* (F54C1.7) and *unc-87* (F08B6.4) as well as a homologue of *M. incognita* β -1,4-endoglucanase (AAK21881.1) were identified from an EST dataset of *M. graminicola* by a tblastn search from Haegeman *et al.* [13]. Using DNA translation tools [14], eligible ORFs were selected to mark the region suited for siRNA target design. The siRNA target finder [15] and Silencer siRNA construction kit (Ambion) were used to design and synthesize siRNAs. The oligonucleotides used for the synthesis were supplied by Biolegio [16] and are shown in Table 1. Four pairs of oligonucleotides, unca, uncb, uncc and uncbM for the *unc-87* gene; pata, patb, patc and patbM for the *pat-10* gene were constructed. In the same way, the oligonucleotides for the β -1,4-endoglucanase (*Mg-eng*) gene were designed. The synthesis of siRNAs was performed according to the protocol provided by Ambion and all the reagents used were provided by the Silencer[®] siRNA Construction Kit.

Table 1. Oligonucleotides (senses and antisenses) used to synthesize short interfering RNAs (siRNAs).

SiRNA	Oligonucleotides
unca	Antisense: 5'-AAGAAAAAATCCGTGCTAGTGCCTGTCTC-3'
	Sense: 5'-AACACTAGCACGGATTTTTTCCCTGTCTC-3'
uncb	Antisense: 5'-AAAAAGGAATGGTCAGCTTCGCCTGTCTC-3'
	Sense: 5'-AACGAAGCTGACCATTCTTTCTGTCTC-3'
uncbM	Antisense: 5'-AAAAAGGAATGGTCAGCTTCGCCTGTCTC-3'
	Sense: 5'-AAGGAAGCTGACCATTCTTTCTGTCTC-3'
uncc	Antisense: 5'-AATATCCAGAGGAGGCTGAAACCTGTCTC-3'
	Sense: 5'-AATTTTCAGCCTCCTCTGGATACCTGTCTC-3'
pata	Antisense: 5'-AAATGGCCGAAAATATTGAAGCCTGTCTC-3'
	Sense: 5'-AACTTCAATATTTTCGGCCATCCTGTCTC-3'
patb	Antisense: 5'-AACCCTTCGAAAATTAATCCGCCTGTCTC-3'
	Sense: 5'-AACGATTAATTTTCGAAGGGCCTGTCTC-3'
patbM	Antisense: 5'-AAAAAGGAATGGTCAGCTTCGCCTGTCTC-3'
	Sense: 5'-AACCATTAATTTTCGAAGGGCCTGTCTC-3'
patc	Antisense: 5'-AATTAATGGCTGGAGAGACTGCCTGTCTC-3'
	Sense: 5'-AACAGTCTCTCCAGCCATTAACCTGTCTC-3'
enga	Antisense: 5'-AACCCGCCTTATGGAAAATTACCTGTCTC-3'
	Sense: 5'-AATAATTTTCCATAAGGCGGGCCTGTCTC-3'

Table 1. Cont.

SiRNA	Oligonucleotides
engb	Antisense: 5'-AATGGGAATGTTGTTTCGTGCTCCTGTCTC-3' Sense: 5'-AAAGCACGAACAACATTCCCACCTGTCTC-3'
engbM	Antisense: 5'-AATGGGAATGTTGTTTCGTGCTCCTGTCTC-3' Sense: 5'-AATGCACGAACAACATTCCCACCTGTCTC-3'
engc	Antisense: 5'-AACGCTGTTCTTACTCAAGTTCCTGTCTC-3' Sense: 5'-AAAACCTTGAGTAAGAACAGCGCCTGTCTC-3'

Table 1, both sense and antisense sequences of oligonucleotides were designed. The T7 promoter sequence (5'-CCTGTCTC-3') was added for the siRNA synthesis.

The fragments for siRNA synthesis were chosen inside the ORF. The fragment “a” was chosen in the beginning of the ORF, “b” in the middle and “c” at the end of the ORF. A mismatch (M) was designed in the fragment chosen in the middle (b) in an attempt to increase the chance for siRNA success [17].

2.2. Nematode Culture, Collection and Soaking in siRNA

The *M. graminicola* culture was provided by Dirk De Waele (Catholic University of Leuven, Leuven, Belgium) and was originally isolated in the Philippines. It has since been maintained on rice cv Nipponbare and on the grass *Echinochloa crus-galli*. The culture was kept in a plant room at 28 °C under a light regime of 12 h light/12 h darkness, and 70%–75% relative humidity. Stage 2 juveniles (J2) were extracted from a three month old culture using the modified Baermann method [18]. Approximately 1000 nematodes were mixed with siRNA (50 ng/μL) resuspended in 50 μL of distilled water and this was incubated for 24 h on a rotator at room temperature.

The nematodes were then washed copiously with tap water to remove external siRNA. Then the characteristic phenotypes were checked using a stereomicroscope at the following time points: immediately after washing and removal of external siRNA (0 h), at 6 h, at 24 h, at 48 h and at 72 h. The posture and activity of the nematodes incubated in siRNAs targeting β-1,4-endoglucanase was used as a control. About 300 nematodes from each treatment were used for expression analysis and another 300 for the migration assay. Three independent replicates for each treatment were performed.

2.3. Migration Assay and Expression Analysis

Migration assays were used in order to have a more quantitative measurement of the RNAi-effect on nematode mobility. Approximately 300 treated or control nematodes were added to the top of pre-moistened sand columns made by filling a 5 cm long tube (5 mm internal diameter) with washed, coarse river sand and covering the base of the tube with miracloth. The columns were placed vertically in collection vials containing sufficient water with rice root exudates and this covered the base of the column. To obtain rice root exudates, germinating rice seedlings were incubated for 24 h in a collection vial full of water.

The nematodes incubated in patb-, uncb- and in the control eng-siRNA were counted by microscope after migration. Four columns were used for each treatment and the number of nematodes migrating through the columns into the collection vials was counted after 12 h. A second nematode collection

and counting was done after 24 h. One-way ANOVA and Tukey's HSD test using the SPSS 16.0 package were used to analyze the results. For gene expression analysis, approximately 300 nematodes were thoroughly washed with sterile water and mRNAs were extracted using a sonication and trizol based protocol (Invitrogen Corporation 2003, Merlbeke, Belgium). The extracted RNA was treated with DNase I (Invitrogen, Merlbeke, Belgium) and was used as a template for cDNA synthesis using SuperScript™ II RNase H-Reverse Transcriptase, with an oligo (dT) primer and 10 mM dNTP mix (Invitrogen, Merlbeke, Belgium). The cDNA obtained was used for semi-quantitative PCR.

The following program was used: 2 min at 95 °C, followed by 30 cycles of (30 s at 95 °C, 45 s at 58 °C and 30 s at 72 °C), 2 min at 72 °C. At 30 cycles, 10 µL of the PCR reaction mix was withdrawn from the PCR tubes and the reaction proceeded for an additional three cycles. Due to the small amount of material (300 nematodes), 33 cycles were needed to get a good signal. The PCR products were loaded on a 1.5% agarose gel in 0.5 × TAE buffer and electrophoresis was applied for 20 min at 100 V. The different bands were visualized with ethidium bromide under UV light.

For expression analysis, RT-PCR was carried out. Tubulin (Mg-Contig 16812-71), a house-keeping gene whose amplification is easy to perform was used to normalize the amounts of different cDNA templates, while nematodes soaked in β-1,4-endoglucanase siRNA as well as in water (data not shown for water) were used as a negative control for general siRNA toxicity and for checking the specificity of gene silencing. Gene-specific forward and reverse primers to amplify a 291-bp fragment of *pat-10*, a 380-bp fragment of *unc-87*, a 158-bp fragment of tubulin were used (Table 2).

Table 2. Primers used for RT-PCR of *Mg-pat-10*, *Mg-unc-87*, *Mg-eng* and *Tubulin*.

Gene	Fragment Length	Primer Name and Sequence
<i>Mg-pat-10</i>	291-bp	<i>Mg-pat-F</i> 5'-CAACGTTTTCTCTCTTAATTTTTC-3'
		<i>Mg-pat-R</i> 5'-TTCGAAGGGTTTTCTCATCAA-3'
<i>Mg-unc-87</i>	380-bp	<i>Mg-unc-F</i> 5'-GATTTGGAGCCTCTTCCAGA-3'
		<i>Mg-unc-R</i> 5'-TATTCCGGATGGGCAGTATC-3'
<i>Mg-tubulin</i>	158-bp	<i>Mg-Tub-F</i> 5'-TCTGGCATAAATAAAATAAGCGAGT-3'
		<i>Mg-Tub-R</i> 5'-TCAAGATGCAACTGTTGAGGA-3'
<i>Mg-eng</i>	160-bp	<i>Mg-eng F</i> 5'-TAGCAGCTAACCCGCCTTATG-3'
		<i>Mg-eng R</i> 5'-TAGTGCCTCAGGGAAATTGC-3'

3. Results

3.1. *Mg-pat-10* and *Mg-unc-87* Genes Can Be Silenced Using siRNA

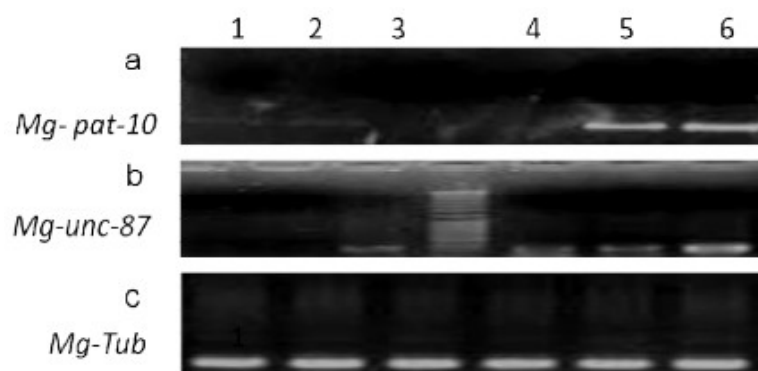
The two target genes for *M. graminicola* were selected because they have similarity with their homologues in *C. elegans*: *Mg-pat-10* is 91% similar, ($E_{\text{value}} = 2^{-23}$) while *Mg-unc-87* is 88% similar ($E_{\text{value}} = 2^{-8}$) [19].

Moreover, the silencing of *pat-10* and *unc-87* in *C. elegans* elicits an immediate visible characteristic paralysis of the model nematode. Previous reports on *in vitro* RNAi in the cyst nematode *G. pallida* and the root-knot nematode *M. incognita* have shown that high concentrations of non-specific dsRNA induced profound phenotypic changes in the infective juveniles of the nematodes [8,20]. With this in mind, we wanted to confirm that a disrupted motility was not due to any general toxic or inhibitory action of the siRNA rather than to the specific silencing of the genes.

Therefore, siRNAs designed against β -1,4-endoglucanase sequence of *M. graminicola* were used as a control during the investigation on the effect of siRNA of *Mg-pat-10* and *Mg-unc-87* in *M. graminicola*. It was demonstrated that β -1,4-endoglucanases, secreted through the stylet of PPN, play a role in the softening of cell walls during penetration of the root epidermis and migration of the juveniles [21], and silencing of this gene is not expected to affect motility. An initial screening was done to identify the most efficient siRNAs for each gene (Table 1) by incubating the nematodes in each siRNA. Based on the RT-PCR results and observations of the phenotypes of the incubated nematodes using stereomicroscopy (data not shown), we found that the nematodes soaked in siRNAs designed in the middle (patb and uncb) of the genes were good candidates for thorough investigation. For other siRNAs (pata, patc, unca, uncc), no difference in expression level was observed. While nematodes soaked in Mg-eng-siRNA (or water) showed normal sinusoidal movement, silencing of *Mg-pat-10* resulted in profound inhibition of motility. Some of the nematodes incubated in siRNA of *Mg-pat-10* were paralyzed and rigid with a hooked head; others barely moved. The nematodes incubated in siRNA of *Mg-unc-87* were coiled with less movement. Similar results were obtained in all three replicates.

The results of the expression analysis (RT-PCR) after incubation of the nematodes in siRNAs mentioned above are presented in Figure 1. Figure 2 shows the results of the nematode migration assay.

Figure 1. Expression analysis of *Mg-pat-10* and *unc-87* after siRNA-treatment. The first panel (a) shows the expression analysis of *Mg-pat-10*, the second panel (b) *Mg-unc-87* (the middle lane that is not labeled contains a ladder) and the third panel (c), Mg-tubulin mRNA levels following the treatment by siRNA of *Mg-pat-10* or *Mg-unc-87*. Lanes 1–5, panel (a) and (b): Expression status after treatment by siRNAs for target genes at the following time points: Lane 1, 0 h after treatment; lane 2, 6 h; lane 3, 24 h; lane 4, 48 h; lane 5, 72 h. For each panel lane 6 illustrates the control expression of the gene after Mg-eng siRNA treatment.



As can be seen in Figure 1 on panel (a), *Mg-pat-10* was silenced from 0–48 h after incubation with pat-siRNA. At 72 h (lane 5) the *Mg-pat-10* mRNA level was similar to the control (lane 6). This means that the recovery of *Mg-pat-10* expression occurred between 48 h and 72 h after washing. For *Mg-unc-87* in panel (b) partial recovery of mRNA was already visible after 24 h (lane 3), but this was still not complete at 72 h (lane 5). Lane 6 shows gene expression immediately after soaking in siRNA of *Mg-eng* demonstrating that expression of *Mg-pat-10* (a) or *Mg-unc-87* (b) was not affected by

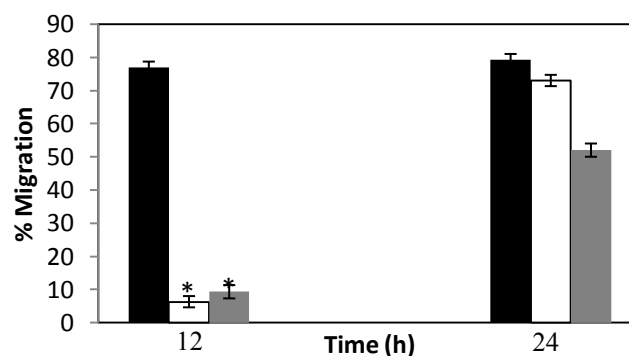
non-specific siRNA effects. With the panel (c) it was observed that expression of the house-keeping gene *tubulin* was not affected by siRNA treatment against *Mg-pat-10*, *Mg-unc-87*.

The siRNA synthesized using oligonucleotides with a mismatch did not produce any observable aberrant phenotypes. In addition, no difference was noticed in the expression of the target gene after incubation in siRNAs with a mismatch or siRNAs to the control gene (data not shown).

3.2. Silencing of *Mg-pat-10* and *Mg-unc-87* Impairs Migration Capacity of *M. graminicola*

To quantify the reduction in motility after silencing of *Mg-pat-10* and *Mg-unc-87*, *M. graminicola* juveniles were assessed for impaired motility in a sand column migration assay. Under normal conditions, nematodes migrate downward through the sand column. In contrast, nematodes with disrupted mobility have an impaired ability to complete this migration. For nematodes that were incubated for 24 h in siRNA of β -1,4-endoglucanase (negative control), a mean migration of $77\% \pm 3.15\%$ ($n = 4$) was recorded in a 12 h period. In contrast, nematodes incubated in siRNA of *Mg-pat-10* and *Mg-unc-87* showed significant inhibition of motility ($n = 4$, $p = 0.01$). After 12 h, only $6.3\% \pm 1.19\%$ of the juveniles incubated in *Mg-pat-10* siRNA and 9.3 ± 1.21 of those incubated in *Mg-unc-87* siRNA had migrated through the column representing a 91.8% and 87.9% inhibition of the control-soaked nematodes, respectively (Figure 2). However, after 24 h the number of nematodes that completed the migration through the sand column was high in all conditions and the difference between the siRNA treated nematodes and the control was not significant anymore in terms of their migration through the sand column.

Figure 2. Nematode migration assay. Migration assay results at two time points; 12 and 24 h. Three bars show the % of nematodes that migrated through the sand column at the given time points and read as follows from left to right: Black bar, nematodes incubated in siRNA of control *Mg-eng*; White, nematodes incubated in siRNA of *Mg-pat-10*; Gray, nematodes incubated in siRNA of *Mg-unc-87*. * indicates significant difference between the treatment and the control.



4. Discussion

In this work, the effect of small interfering RNA (siRNA) on functional gene knock-down was analyzed in *M. graminicola*. Two genes, *Mg-pat-10* and *Mg-unc-87* encoding body wall muscle proteins in nematodes were chosen as the siRNA targets in *M. graminicola*. The RT-PCR analysis of mRNA from nematodes incubated in siRNA of *Mg-pat-10* and *Mg-unc-87* for 24 h showed that the transcript levels of the genes were reduced. This reduction was reflected in the nematodes activity. Part of the nematodes incubated in siRNA of *Mg-pat-10* was paralyzed while part of those incubated in *Mg-unc-87* siRNA was coiled. *Pat-10* is essential for the initial assembly of the sarcomere and is involved in the attachment of muscle cells to the basement membrane [22]. Although nematodes were paralyzed after incubation in siRNA of *Mg-pat-10*, the pharyngeal pumping remained active. This agrees with the observation made by Terami *et al.* [23] who reported that pharyngeal pumping was unaffected in *pat-10* mutants of the nematode *C. elegans*. *Unc-87* serves as a structural component to maintain lattice integrity during contraction [24] and hence silencing of *unc-87* leads to disorganized body wall muscles affecting the contraction/relaxation cycle of the muscles (uncoordinated movement). The migration assay that was performed on siRNA treated nematodes showed that silencing of *Mg-pat-10* and *Mg-unc-87* significantly impedes nematode movement. These results demonstrate that the function of *unc-87* and *pat-10* has been evolutionarily conserved between the plant parasitic nematode *M. graminicola* and the free living nematode *C. elegans*. Recently, it was also reported that silencing of *unc-87* and *pat-10* give a similar phenotype in *Pratylenchus* species [25,26].

Incubation of the nematodes with siRNA at the concentration of 50 ng/μL for 24 h appeared to induce gene knock down in *M. graminicola*. However, recovery in transcript level of *Mg-pat-10* and *Mg-unc-87* was observed later. The recovery of transcript levels of *Mg-unc-87* was faster, but not as complete as that of *Mg-pat-10*. The transcript level of *Mg-pat-10* was the same as the control at 72 h after washing, while the transcript level of *Mg-unc-87* started to be less silenced earlier, but did not yet approach the level of the control after 72 h. This recovery from the siRNA treatment is similar to the observation of Soumi *et al.* [25] in *Pratylenchus coffeae* with the only difference being that they used long double stranded RNA. They found that most of the nematodes that were incubated in dsRNA of *Pc-pat-10* recovered normal sinusoidal movement after 24 h. On the contrary, the worms incubated in *Pc-unc-87* showed the aberrant phenotype after 24 h and very few worms could regain regular movement. This time-limited silencing effect was also reported by Rosso *et al.* [27] after *in vitro* soaking of *M. incognita* in dsRNA of *Mi-crt* (calreticulin) and *Mi-pg-1* (polygalacturonase) genes. While the silencing effect on *Mi-crt* lasted for 44 h, the effect on *Mi-pg-1* remained only for 20 h after soaking.

Dalzell *et al.* [20] revealed that on strategically incorporating base mismatches in the sense strand of a *G. pallida* specific siRNA, they could specifically increase or decrease the knockdown of its target (specific to the antisense strand). In our investigation, the siRNA with a mismatch did not produce any observable aberrant phenotypes and no difference was noticed in gene silencing between siRNAs with mismatch and the control treated nematodes.

Dalzell *et al.* [28] found that non nematode derived dsRNAs induced aberrant phenotypes and had an unexpected inhibitory effect on the motility of root-knot nematode *M. incognita* J2s following 24 h soaking in 0.1 mg/mL dsRNA, and established that this inhibitory phenomenon was both time- and

concentration-dependent. In our experiments, we included as a control the silencing of a β -1,4-endoglucanase under the same conditions and it could be demonstrated that the siRNA of this gene *Mg-eng* did not cause any inhibition of motility.

5. Conclusions

In conclusion, we have demonstrated that *M. graminicola* is susceptible to effective specific gene silencing by siRNAs. However, the persistence of the RNAi effect in *M. graminicola* is highly time limited and variable among the targeted genes. Therefore, it is essential to have a greater understanding of the RNAi regulatory pathway in plant parasitic nematodes to enhance the potency and persistence of RNAi.

This successful application of RNAi in *M. graminicola* could open the door for the identification of novel target genes that are essential to nematode biology and parasitism in order to control these root-knot nematodes in agriculture.

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Conflicts of Interest

The authors declare no conflict of interest.

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Appendix

ESTs used for target genes and control.

A1. >Mg_ *pat-10*

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GAATCAACGTTTTCTCTCTTAATTTTTCTTTAAATCCCTAAAATTATTTAATTTTTTAAAAT
ATTTATTTTCAGTACCTCTTTTCTATTTTATACTCATAAATATTTGAAATGGCCGAAAATAT
TGAAGAAATCCTTGCTGAAATTGACGGCTCTCAAATAGAGGAATATCAACGATTTTTTTGA
TATGTTTGACCGTGGAAAGAATGGTTACATTATGGCTACTCAAATTGGAACAATTATGAA
TGCTATGGAACAAGATTTTGATGAGAAAACCCCTTCGAAAATTAATCCGCAAATTCGACGC
AGACGGCAGTGGTAAAATCGAATTTGATGAGTTTTGTGCATTAGTATACACTGTAGCAAA
TACTGTAGATAAGGATACATTACGGAAAGAATTGAGAGAAGCTTTTCGTCTTTTCGACAA
GGAGGGTAATGGTTACATCTCCCGCCCTACTCTTAAAGGACTTCTTCACGAAATTGCACC
AGACCTAAGTGATAAAGATTTGGATGCTGCAGTAGATGAAATTGACGAAGACGGAAGCG
GTAAAATTGAATTTGAAGAATTTTGGGAATTAATGGCTGGAGAGACTGATTAAAATTTAA
AATAATTAATAATTAATATTTTGCCATAAAATAAAAAAGGTGCCCCCTAATTGCCTTTAAC
AATTTTATTATTTT
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A2. >*Mg_unc-87*

AGACCCTGCCGATTTGGAGCCTCTTCCAGAAGAAAAAATCCGTGCTAGTGATGGAATTGT
 TCGTTTACAATCCGGTACGAACAAGTTTGACTCACAAAAAGGAATGGTCAGCTTCGGTAC
 AAACCGACGCGAAACTACCAGAATGAAAGATACAAAACATCCGGAATATGATCACGAAC
 GCAATATTGATCAAAGCGAGATTCCTTTGCAATCCGGTACGAACAAATTTGCTTCACAAA
 AAGGAATGGTCAGCTTCGGTACAAACCGACGCGAAACTACAAAAATGTTGGATACTGCC
 CATCCGGAATACAGTCACGAAAGCAGTATTGACCAAACAAGTATTCCTTACCAAATGGGT
 TCAAATCGTTATGCTTCACAAAAGGGAATGACTTGTTTTGGACAACCACGTTGGGAGGTG
 CTTGACCCAAGTATTAGCTATCAGAATCGTAAATCACAAGGAATGGTTCGTCTTCAATCC
 GGTACAAACCGGTTTGCTTCACAAGCAGGAATGACAGGTTTTTGAACTCCAAGGAACACA
 ACATATGAAGCAGAAGCTGGAGAACTTCCTTATGAAGATATGAAAAAATCAGAAACAAT
 AATTCATCCCAGGCTGGTTGGAATAAGGGAGACTCTCAAAAATTAATGACTGGATTGG
 TACTCCACGTGACGTTAAAGGAAAACATTTGAAGCGTATATGGGAATTGGAATATCCAGA
 GGAGGCTGAAATTTCTTTAGATCGACTTTAAAGGAATTGAAAAGA

A3. >*Mg-eng*

ATGTTTAAAAATTGCTTAATATTAAATATTTTATTTATTTCTTATTATTTATCTATTATATTA
 GCAGCTAACCCGCCTTATGGAAAATTATCAGTACGTAAAGGGCAGTTAAAGGGGTCGAAT
 GGCCAAGTAGCTACATTGAGAGGAATTTCTCTCTGGTTTAGTCAATGGATAACTGAATTTT
 ATTCGCCTGGTGTTGTAAGGGCAATAAAATGTTTTTATAATGGGAATGTTGTTTCGTGCTTC
 AATTGGAACCTGTTGTTTCAGGTTATTTGGAAAACCCATCTGCTGCAATAAATGCTGCCAC
 AACTATTGGAGATGCAGCAATTGCTAATGGAATGTATTTTATAATTGACTGGCATGATGT
 AGGAAATCAGAAATGTAATAATGACGCAGATTTTAACAACCTTCATAAATAGTGCAATTAA
 ATTTTTTACAACAATTATTAACAAATATAAGGGCTCACCAAATATACTTTTAGAGCTTTGG
 AATGAACCTGCTTATACTTGTAGTTGGGCTAAATTGAAGCAATATTATAACGCTGTTCTTA
 CTCAAGTTAGAAAAATTGACCCAAATGTCGTCGTCATTCTCGGAACACCGAAACAGTCTA
 CTGGTCCAAGCGACGAAGTCATAAATAGTCCTGTTGGTGGTGCCAATATAATGTATGCCT
 TACATTTTTTATTGCGTACCGTACCAACAACATATTGAAAATCAAAAACAAATGATATTAA
 AAGCAAAAAGTAAAGGATTTGCAACATTCGTTAGTGAATATGGCGATGCAGATGCAACC
 CCTCCAGCACCTCTTCAACCAACAGCAATGAAGGCATTTTGGCAATTTATGGACCAAAAC
 CAACTTTCCTATGCAAAATGGTCACTTACTAACAAGATGAGGTTTATTCATTAATGGTGC
 CATGGTGTAGTGCATCGCAAGCATTTCAGAATCATGTCTTAGTGCCTCAGGGAAATTGC
 TTAGAGAACATATGTGGAGCTTAAATAATGGTATTAATGGTTGTAA