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ORIGINAL ARTICLE

Genomes of parasitic nematodes (*Meloidogyne hapla*, *Meloidogyne incognita*, *Ascaris suum* and *Brugia malayi*) have a reduced complement of small RNA interference pathway genes: knockdown can reduce host infectivity of *M. incognita*

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Abstract The discovery of RNA interference (RNAi) as an endogenous mechanism of gene regulation in a range of eukaryotes has resulted in its extensive use as a tool for functional genomic studies. It is important to study the mechanisms which underlie this phenomenon in different organisms, and in particular to understand details of the effectors that modulate its effectiveness. The aim of this study was to identify and compare genomic sequences encoding genes involved in the RNAi pathway of four parasitic nematodes: the plant parasites *Meloidogyne hapla* and *Meloidogyne incognita* and the animal parasites *Ascaris suum* and *Brugia malayi* because full genomic sequences were available—in relation to those of the model nematode *Caenorhabditis elegans*. The data generated was then used to identify some potential targets for control of the root knot nematode, *M. incognita*. Of the 84 RNAi pathway genes of *C. elegans* used as model in this study, there was a 42–53 % reduction in the number of effectors in the parasitic nematodes indicating substantial differences in the pathway between species. A gene each from six functional groups of the RNAi pathway of *M. incognita* was downregulated using in vitro RNAi, and depending on the gene (*drh-3*, *tsn-1*, *rrf-1*, *xrn-2*, *mut-2* and *alg-1*), subsequent plant infection was reduced by up to 44 % and knockdown of some genes (i.e. *drh-3*, *mut-2*) also resulted in abnormal nematode development. The information generated here will contribute to defining targets for more robust nematode control using the RNAi technology.

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Introduction

RNA interference (RNAi) or post transcriptional gene silencing (PTGS) involves several small RNA pathways: these include the exo and endo small interfering RNA (siRNA), and micro RNA (miRNA) pathways. Apart from their importance in developmental processes and in the regulation of gene expression, the small RNA pathway is also responsible for silencing viral messages and transposable elements (Ketting et al. 1999). Genome-encoded small RNAs and miRNAs enter the cell cytoplasm from the nucleus, whereas externally introduced double-stranded RNA (dsRNA) can enter the cell through trans-membrane protein channels. The process of RNAi occurs in distinct steps: in a eukaryotic cell, dsRNA is processed into siRNAs by a dicer complex consisting of helicases, dsRNA-binding enzymes and argonaute proteins which act together to dice the long dsRNAs into ~21–25 bp siRNAs. Unwinding of the siRNA duplex by the RNA-induced silencing complex (RISC) in an ATP-dependent step then leads to the activation of RISC (Nykänen et al. 2001). In *C. elegans*, an argonaute (*rde-1*), which is a part of the dicer complex, may also contribute to separating the two strands of siRNAs (Steiner et al. 2009). The processed antisense or guide strand then targets and hybridises to a perfect complementary mRNA, cleaving it at the centre of the ~21 bp siRNA leading to degradation of the mRNA (Elbashir et al. 2001). In nematodes, fungi and plants, there is an RNAi amplification system which recruits RNA-dependent RNA polymerases to synthesise more dsRNAs, using the sense strand as a primer (Vaistij et al. 2002; Alder et al. 2003; Calo et al. 2012). The process,

also referred to as transitive RNAi, leads to the production of secondary siRNAs, which have been associated with secondary argonautes which facilitate repetition of the RNAi process (Yigit et al. 2006).

Because most plant parasitic nematodes (PPNs) are obligate biotrophic soil pests, they are difficult to study and culture, and delivery of dsRNA to parasitic nematodes is far from routine. In animal parasitic nematodes (APNs), RNAi has been used successfully to downregulate gene expression in various species, including genes such as acetylcholinesterase (AChe B), tropomyosin and β-tubulin, but the extent of gene knockdown appears to depend on the cellular location of the expressed gene (Issa et al. 2005; Kotze and Bagnall 2006; Britton et al. 2012).

For PPNs, once the methods had been developed to stimulate J2 stage nematodes to take up exogenous dsRNA by *soaking* (Urwin et al. 2002; Bakhetia et al. 2008), an RNAi pathway was shown to be functional. With suitable choice of target gene, a reduction in viability and reproduction can be achieved when in vitro-treated nematodes are used to infect plants (e.g. Bakhetia et al. 2005; Tan et al. 2013). Targeted knockdown of nematode genes has been extended to host-delivered dsRNA as a potential method to confer plant resistance to PPNs (Rosso et al. 2009, Fosu-Nyarko and Jones 2015), and the same principle has also been applied to sucking insect pests (Pitino et al. 2011).

There has been a dramatic increase in genomic data available for many species, generated using different deep sequencing technologies, and these data can be combined with tools of bioinformatics to enable detailed study of the genetics of non-model organisms such as plant pests. When this study was undertaken, in addition to genome resources for the model nematode *C. elegans*, full genome sequence data were available only for the PPNs *M. hapla* and *M. incognita* and the APNs *A. suum* and *B. malayi*. However, the parasitic behaviour of PPNs and APNs means that during evolution to parasitism a subset of genes has evolved or been obtained by horizontal gene transfer which are specific to the parasitic life style. Genes involved in parasitism of different parasitic nematodes need further study, both to understand the molecular basis of their host-pathogen interactions, and to identify possible targets for their control. Current reports suggest that 35–70 % of the genes present in *C. elegans* have homologues in 28 different species of parasitic nematodes, and the data generated for *C. elegans* therefore provide a major resource that can be used to study most aspects of cellular functions and pathways of PPNs and APNs (Parkinson et al. 2004; Rosso et al. 2009; Dalzell et al. 2011).

The overall aim of this study was to identify genes involved in the RNAi pathway of parasitic nematodes, and to compare and contrast the gene structures with those of *C. elegans* and also between the different parasitic nematodes. Available genome sequences of *M. hapla*, *M. incognita*, *A. suum*, and *B. malayi*, were therefore compared with well-characterised genes of *C. elegans* involved in the RNAi pathway. Differences in protein domains encoded by specific genes in the PPNs and APNs were also explored. This analysis led to the identification of differences in the genes present in the RNAi pathway of PPNs and APNs compared to those present in *C. elegans*. Genes belonging to different functional groups of the RNAi pathway of the PPN *M. incognita* were then targeted by in vitro RNAi to assess their suitability as targets for nematode control: the effects of RNAi treatments on nematode behaviour and subsequent plant infection were studied.

Materials and methods

Identification of *effectors* of RNAi of *C. elegans*

Sequences of the effectors of the RNAi pathway of *C. elegans* were used as a primary source to identify orthologues in the parasitic nematodes, because these have been more highly characterised than those of any other species. Genes encoding 88 effectors directly involved in siRNA and miRNA processes of *C. elegans* were identified from the literature (Rosso et al. 2009; Dalzell et al. 2011; Kikuchi et al. 2011). Their nucleotide sequences were retrieved from Wormbase version WS241 (http://www.wormbase.org) and the National Centre of Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov). The effectors were grouped together, based on their functional roles in the RNAi pathway, into: those involved in transport of silencing triggers (e.g. dsRNA, siRNA and miRNA), the Dicer and RISC, amplifiers of silencing signals, RNAi inhibitors, nuclear RNAi effectors and argonaute proteins.

Identification of genomic contigs of parasitic nematodes mapped to RNAi effectors of *C. elegans*

Coding sequences (mRNA) of *C. elegans* effectors were used to query the NCBI dataset containing whole genomic sequence/contigs of *M. incognita*, *M. hapla*, *A. suum* and *B. malayi* using the BLAST suite of programs with an expected value (e-value) cut-off of 1E−05. In cases where there were no matching genomic contigs or the e-values were not higher than the threshold, sequences of effectors of parasitic nematodes were used for further confirmation for tblastx searches: blastn and blastx were also used where required for further analysis and these are indicated in the results. The resulting matching contigs were retrieved and sorted using bit scores: XML files were converted to csv formats for further analysis. Best matching contigs to specific effectors were identified after manual curation of alignments of all matching contigs to subject sequences.

In silico functional analysis of putative effectors of the parasitic nematodes

Genomic contigs of any of the parasitic nematodes identified to contain RNAi effector sequences were further analysed using a suite of bioinformatics tools. The aim was to determine the presence of functional domains and conserved motifs that characterise specific effectors and to determine their homology, structural and functional conservation amongst parasitic nematode groups and that of *C. elegans*. Functional protein domains of these contigs as well as full-length cDNA sequences of *C. elegans* effectors were analysed using the NCBI Conserved Domain Database (CDD v3.03, Marchler-Bauer et al. 2009; Marchler-Bauer et al. 2011) with default settings (expect value threshold 0.01), and the Pfam 27.0 program (http://www.pfam.sanger.ac.uk) using protein sequence queries with an e-value cut-off of 1.0 (default). In all cases, and to confirm that genomic contigs significantly matched effectors, and in specific cases where e-values were higher than the threshold, the tblastx search of the genomic contigs was conducted using sequences of specific functional domains of *C. elegans* RNAi effectors. Multiple sequence alignments and manual curation were undertaken using MultAlin (Corpet 1988) to compare matching contigs or orthologues of effectors. To obtain open reading frames from genomic contigs for any protein analysis, the Open Reading Frame Finder (http://www.ncbi.nlm.nih.gov/projects/gorf) and the Translate program at Expasy (http://web.expasy.org/translate) were used. The exon prediction in genomic sequences putatively encoding Dicer genes was done using FGENESH (Solovyev et al. 2006) followed by tblastx against corresponding *C. elegans* proteins, whilst the graphics were created on the Exon-Intron Graphic Maker version 4 at www.wormweb.org. Protein domain graphics were generated using protein sequences in Pfam 27.0.

Phylogenetic analyses

Multiple alignments for protein sequences using clustalW were created in MEGA version 6 (Tamura et al. 2013). Neighbour-joining trees with bootstrapping 1000 replicates were created based on those alignments. The RdRp domain was used for analysis of RNA-dependent RNA polymerase genes while PIWI domain was used for argonautes in all nematodes.

DsRNA synthesis

DNA templates corresponding to defined lengths of exons of the genomic sequences CABB01002056, CABB01000055, CABB01000474, CABB01001503, CABB01003815, CABB01000336 and putatively encoding protein domains of *drh-3*, *tsn-1*, *rrf-1*, *xrn-2*, *mut-2* and *alg-1* were generated by PCR using primers (Table 1) appended with restriction enzymes XhoI/AfeI and KpnI from *M. incognita* cDNA generated from RNA of mixed life stages. The PCR conditions were 95 °C for 5 min followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s followed by 72 °C for 7 min in a Veriti 96-Well Thermal Cycler (Applied Biosystems). The amplicons were ligated in a 3:1 insert:vector molar ratios to the vector pDoubler, which has T7 promoter regions on either side of the insert and allows a double-stranded DNA template to be digested with the T7 recognition sequence on either end which allows in vitro transcription with the T7 enzyme (Iqbal 2015). DNA templates were digested out of the plasmid with pDoubler backbone using EcoRI and used to synthesise dsRNA. DsRNAs were synthesised using 1 μg each of DNA template with the HiScribe T7 In vitro transcription kit (New England BioLabs Inc.) according to the manufacturer's instructions.

In vitro soaking of juveniles of *M. incognita* with dsRNA and analysis

M. incognita were maintained on the susceptible tomato cultivar Grosse Lisse. Freshly hatched J2s (juvenile stage 2) were obtained from egg masses and used for dsRNA soaking experiments because this is the stage at which nematodes enter the host plant and establish infection sites from which they feed during their subsequent life stages. Nematode-soaking treatments were set up as described by Tan et al. (2013), except that 1 mg/mL of dsRNA and 7,000 J2s per treatment were used. One gene from each of the functional groups was selected for in vitro soaking: these were *drh-3* (Dicer complex), *tsn-1* (RISC), *rrf-1* (RNAi amplification), *xrn-2* (RNAi inhibitor), *mut-2* (Nuclear RNAi) and *alg-1* (Argonaute). For controls, J2s were either soaked in a solution with dsRNA corresponding to 524 bp of the *gfp* gene of *Aequorea victoria* or in solution without dsRNA. To confirm that the J2s would take up the soaking solution, fluorescein isothiocyanate (FITC, 1 mg/mL) was added to a similar soaking medium without dsRNA for 16 h after which FITC uptake by J2s was monitored using an Olympus BX-51 microscope with FITC filter. Behaviour and activity of J2s 16 h after soaking was observed and recorded using bright field microscopy.

To assess the infectivity of J2s after soaking for 16 h in dsRNA, 2-week-old tomato seedlings (cv. Grosse Lisse) were infected with 400 nematodes from each treatment. Four weeks after inoculation, the number of galls on roots was counted for seven plants from each treatment and dry root weights recorded. Adult female nematodes were hand-dissected from the three other replicate plants 7 weeks after infection and their morphology was recorded.

Table 1 Primers used to amplify genes of *M. incognita*. Restriction enzyme sites were added to the primers (XhoI to the forward primer except *xrn-2* for which AfeI was added. KpnI was added to the reverse primers)

Gene	ID	Primer 5′–3′	Amplicon size (bp)
drh-3	Midrh3-F Midrh3-R	GTGGTGTCATAACCAAAATGTCTG C TTG TGC ACC AAC TGG AAG TG	284
tsn-1	Mitsn1-F Mitsn1-R	TTCCCTATCTACAGCGTTCGC CCT CAA CAT GAT GCT TCCATATACC	444
rrf-1	Mirrf1-F Mirrf1-R	TATGCTGACACGCTCCTTAATTG TCTGCCATGTAATCAAATGCATCC	682
xrn-2	Mixrn2-F Mixrn2-R	CTGTATCTCGATATGAACGG CAAGCATAATCAAGTCTGC	553
mut-2	Mimut2-F Mimut2-R	AACTTACAGGCACTATAATAAC TGCTGTTGTTGTTCTTTCTTC	204
alg-1	Mialg1-F Mialg1-R	CGGATCGAATGACATGTC ATTCCTGGTTGGTATCCTC	476
gfp	Hpgfp-F Hpgfp-R	TAACTCGAGTCTAGATTCACTGGAGTT TACGGTACCGGATCCTAATGATCAGC	524

Statistical analysis

The SPSSv20 software package (IBM Corporation, USA) was used for analysis of variance (ANOVA): for calculation of means, standard deviations, and standard errors. Significant differences between treatments were assessed at $p < 0.05$ and pair-wise comparisons were done post-hoc using the Tukey's test. The Kruskal-Wallis test was done to test the significance of data for female morphology and treatments were compared using the Mann-Whitney U test. Microsoft Excel Analysis ToolPak was used to generate bar charts with error bars representing standard error of the means for each treatment.

Results

Genomic contigs and ESTs of *M. incognita*, *M. hapla*, *A. suum* and *B. malayi* with homologies to effectors of the RNAi pathway of *C. elegans*

From the literature and database searches, 88 genes were identified as involved in the RNAi pathway of *C. elegans*, of which two argonautes *SAGO-2* (Wormbase ID: WBGene00018921) and *PPW-1* (Wormbase ID: WBGene00018921), were found to have 100 % identical nucleotide sequence with 78 bp longer sequence for PPW-1. The longer sequence was used as (PPW-1/SAGO-2) for this analysis. *Discontinued genes* (*rde-3*, *M03D4.6 and C06A1.4*) were also omitted from the list and the remaining 84 genes were divided into different groups based on their functions in the RNAi pathway of *C. elegans*.

For the 84 selected effectors of *C. elegans*, the percentage of full-length genes mapped to genome contigs/scaffolds and ESTs of *A. suum* were 1.2 and 0.23 % respectively. These percentages were 0.66 and 0.053 % for *B. malayi*, 3 and 0.31 % for *M. hapla* and 1.69 and 0.34 % for *M incognita*. Genes having homologues or encoding similar proteins in the genome of *C. elegans* mapped significantly (at 1E−05) to a large number of contigs for each of the four parasitic nematodes. These were the argonautes, spreading proteins *haf-6* and *sid-3*, and the genes encoding zinc finger domains i.e. *zfp-1* and *zfp-2*. For *M. incognita*, two genes (*tsn-1* and *mut-7*) belonging to different functional groups were present on the same contig (CABB01000055). Some genes of PPNs such as *pash-1*, *vig-1* and *smg*-6 were only identified after alignment with APN orthologues, indicating that these genes were more closely related to each other than to those of *C. elegans*.

Small RNA transport proteins

Four groups of small transport proteins have been characterised in *C. elegans.* The genes which encode them include those with systemic RNAi defective phenotypes (*sid-1*, *sid-2*, *sid-3*, *sid-5*), those with RNAi spreading defective phenotypes (*rsd-2*, *rsd-3*, *rsd-6*), exportins (*xpo-1*, *xpo-2*, *xpo-3*) and the *haf-6* gene. Blastn, blastx and tblastx analyses of the whole genome scaffold and contig sequences revealed that three sequences of *A. suum* (AMPH01007595; ANBK01003199; AEUI01011716) and one of *B. malayi* (AAQA01000131) had significant similarity to *sid-1* of *C. elegans.* A further assessment of these sequences indicated the presence of a type of sid-1 RNA channel similar to that encoded by *sid-1* of *C. elegans.* The amino acid homology is presented in Fig. 1a. No ESTs or genomic contigs from either *M. incognita* or *M. hapla* matched significantly to the *sid-1* full-length gene sequence or the signature sequence of the SID-1 RNA protein. Also, there were no sequences of ESTs or genomic contigs) from any other plant parasitic nematode

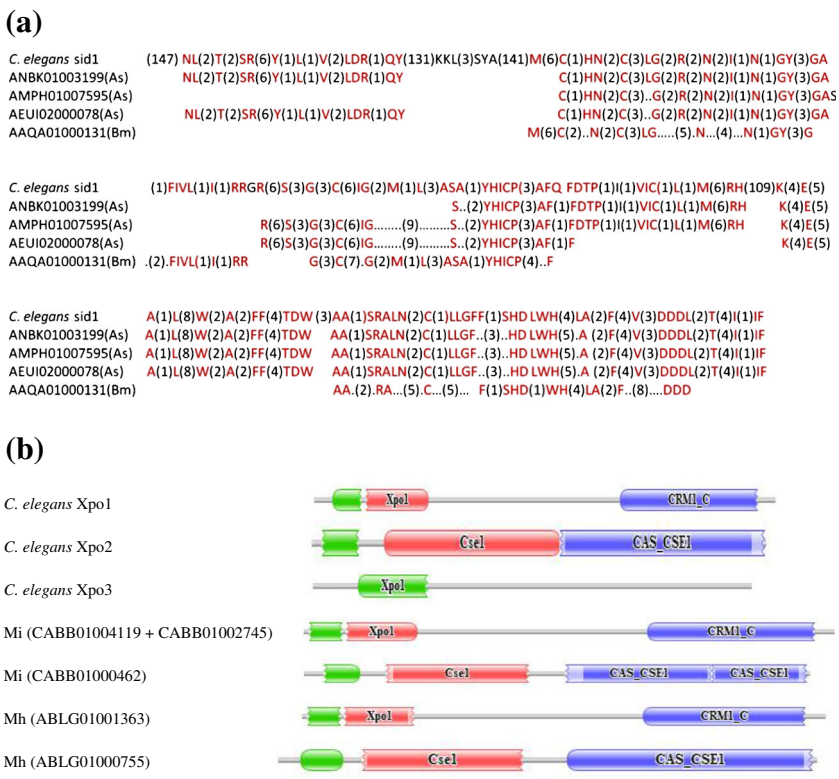

Fig. 1 **a** Conserved signature amino acids of sid-1 RNA channel in *C. elegans* with mapped contigs of *A. suum* (*As*) and *B. malayi* (*Bm*). **b** Protein domain architecture of the three exportins of *C. elegans* (*Ce*) compared to two contigs each of the *M. incognita* (*Mi*) and *M. hapla* (*Mh*) revealing that they are *xpo-1* and *xpo-2*

at NCBI that were significantly similar to the *sid-1* sequence. Similarly, no contig of any of the four parasitic nematodes matched significantly to the *sid-2* gene of *C. elegans*. The *C. elegans* gene *sid-3* encodes a protein tyrosine kinase (PTK) domain together with a Src homology 3 (SH3) and a GTPase binding domain responsible for efficiently importing dsRNA into cells (Jose et al. 2012). Protein tyrosine kinases are the second largest family of proteins in *C. elegans*, with 411 identified homologues (Plowman et al. 1999). It was therefore not surprising that a large number of genomic contigs of all the four parasitic nematodes mapped significantly to this gene, i.e. 100 for *A. suum*, 59 for *B. malayi*, 26 for *M. hapla* and 30 for *M. incognita*. Protein domain analysis on these contigs confirmed the absence of the SH3 domain from all of those contigs. However, the GTPase binding domain, together with the PTK domain, was found in three whole genome sequences for *A. suum* (ANBK01007000; AMPH01017683; AEUI02000893), two for *B. malayi* (AAQA01000282; CAPY01003672) and one each for the two *Meloidogyne spp*. (ABLG01000030; CABB01000892). As was the case for *sid-2*, no orthologues were found in the genomic contigs of the four parasitic nematodes for *sid-5*.

Of the three RNAi spreading defective genes in *C. elegans*, sequences matching only *rsd-3* were identified for all four parasitic nematodes. Identified contigs contained the epsin amino-terminal homology (ENTH) motif typical of the *rsd-3* gene of *C. elegans* and cytosolic proteins of plants which are involved in vesicle trafficking (Holstein and Oliviusson 2005). No orthologues of *rsd-2* and *rsd-6* were identified in the EST and genomic contig databases for the four parasitic nematodes considered in this study and also all other PPNs.

Contigs of *A. suum*, *B. malayi*, *M. incognita* and *M. hapla* with significant matches to the three exportin genes of *C. elegans* were analysed for the presence of functional protein domains. They were all identified in APNs but for the two PPN species only two contigs were found. Functional domain analysis for *M. hapla* contigs ABLG01001363 and ABLG01000755 confirmed the presence of *xpo-1* and *xpo-2* as the two contigs had all the protein domains for these two genes, while *M. incognita* contig CABB01000462 coded for *xpo-2*. Interestingly, detailed analysis revealed that when two *M. incognita* contigs, CABB01002745 and CABB01004119 were re-assembled, the super contig matched completely to the full-length *C. elegans xpo-1* mRNA (Fig. 1b). No contig was found coding for the XPO-3 protein in the two PPNs.

The *haf-6* gene is a member of the ATP binding cassette (ABC) transporter gene family, and the *C. elegans* genome encodes 60 proteins with ABC transporter domains (Sheps et al. 2004). Unsurprisingly, a large number of contigs of *A. suum* (95), *B. malayi* (32), *M. hapla* (14) and *M. incognita* (17) mapped to this gene. Parasitic nematodes such as *A. suum*, *B. malayi* and *Meloidogyne* spp. are known to be susceptible to environmental RNAi, and although there are degrees of susceptibility, they seem to have a smaller but similar repertoire of genes involved in uptake and spread of dsRNA.

Dicer and associated genes

The Dicer gene plays a central role in RNAi pathways of eukaryotes. For organisms which encode only one dicer, it is responsible for processing all forms of dsRNAs into small RNAs, including siRNAs and miRNAs. The DICER *(dcr-1)* of *C. elegans* was used to identify, characterise and compare the structures of dicers in the parasitic nematodes. The lengths of the predicted pre-mRNA for the *dcr-1* of both *M. incognita* and *M. hapla* are similar to that of *C. elegans* although they have more exons (Fig. 2a).

Genomic contigs with the highest sequence identity to *dcr-1*of *C. elegans* were CABB01000157 (42,843 bp) for *M. incognita*, ABLG01001138 (50,884 bp) for *M. hapla*, CAPY01005536 (830,985 bp) for *B. malayi* and ANBK01006853 (13,709 bp), AMPH01008524 (10,740 bp) and AEUI02001038 (51,650 bp) for *A. suum*. The *drh-1* and *drh-3* genes of *C. elegans* have three similar functional domains each i.e. DEXDc (DEAD-like helicase), HELICc (Helicase C-terminal domain) and RIG-1_C-RD (C-terminal domain of RIG-1). Although the mRNA sequences of *drh-1* and *drh-3* were of high sequence identity to contigs of the four parasitic nematodes, functional analysis revealed striking differences in the protein domains of these genes. Whereas these contigs had the DEXDc and HELICc domains, the RIG-1 domain was conspicuously missing in contigs matching to *drh-1* of *C. elegans*. In DRH-1 and DRH-3 of *C. elegans*, there is only 24 % identity between the RIG domains. A detailed analysis of whole genome contigs of the parasitic nematodes as well as ESTs and with those of *H. glycines*, *B. xylophilis* and *H. contortus* indicated that this domain is absent in available sequences of parasitic nematodes.

Unlike *rde-4* which encodes two dsRNA-binding motifs and did not match significantly to whole genome contigs of any of the four parasitic nematodes, contigs mapping to *C. elegans* argonaute *rde-1*, were 14 for *A. suum*, 12 for *B. malayi* and four each for *M. hapla* and *M. incognita*: nematodes have a suite of argonaute proteins as important components of gene silencing. The only functional domain of *pir-1* that is 'Dual specificity phosphatase catalytic domain', was identified in six contigs of *A. suum*, six for *B. malayi*, two for *M. hapla* and three for *M. incognita*.

The best matching contigs of *A. suum* (AEUI02000028), *B. malayi* (AAQA01000005), *M. hapla* (ABLG01000521) and *M. incognita* (CABB01000477) to the mRNA sequence of *C. elegans* Drosha (*drsh-1*) encode three protein domains; two Ribonuclease III C-terminal domains and a double-stranded RNA binding motif (DSRM). The closest matches

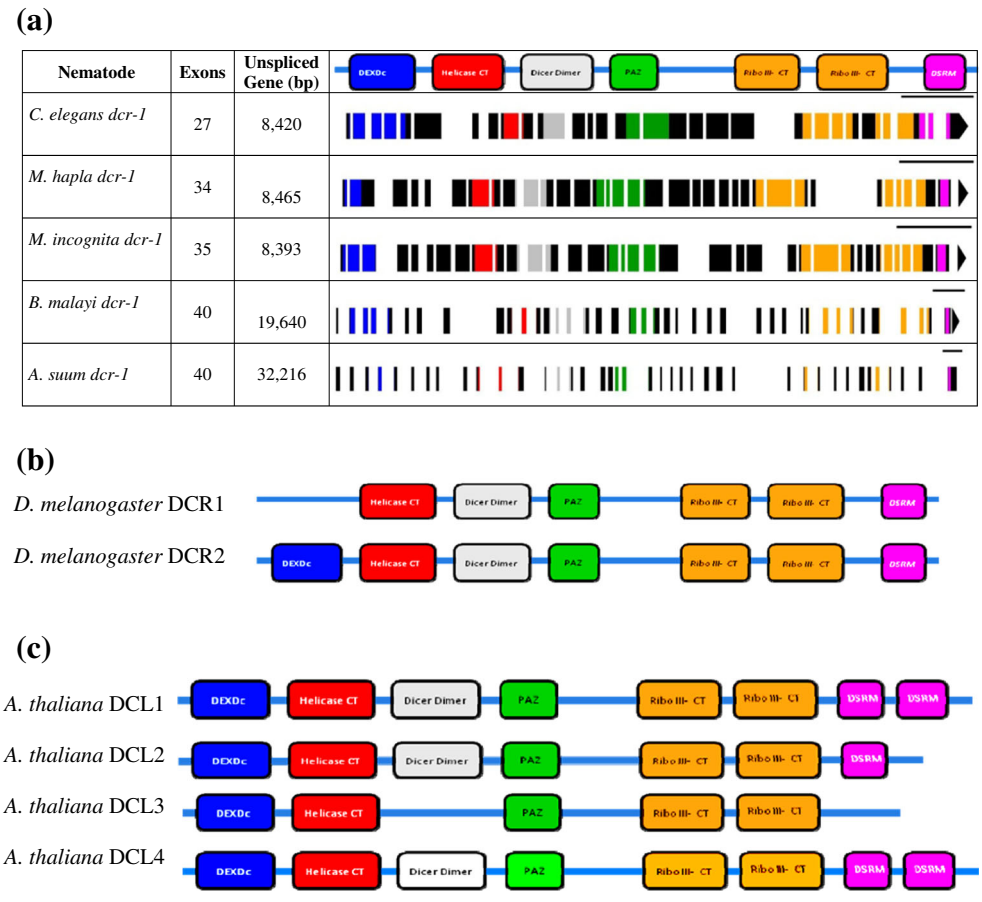

Fig. 2 **a** Graphical representation of *dcr-1* gene of *C. elegans* compared to the four parasitic nematodes with gaps indicating intron regions (scale = 1000 bp). The seven protein domains are DEXDc. Helicase C-Terminal (Helicase CT), Dicer Dimer, PAZ, two Ribonuclease III C-terminal domains (Ribo III-CT) and double-stranded RNA binding motif (DSRM). Whole genomic contigs mapped to *C. elegans dcr-1* were used to predict protein domain coding regions (coloured) in the contigs of *M. hapla* (ABLG01001138), *M. incognita* (CABB01000157), *B. malayi* (CAPY01005536) and *A. suum* (AEUI02001038; AMPH01008524; ANBK01006853). Number of exons and the unspliced sequence length (bp) are also indicated. **b** *Drosophila melanogaster* protein domain architecture for the two Dicers. **c** Differences in the protein domain architecture between the four *Arabidopsis thaliana* Dicer-like (DCL) gene products

for the *drsh-1* cofactor Pasha (*pash-1*) in the four parasitic nematodes had low total bit scores and coverages: 154 (15 %) for *A. suum*, 155 (12 %) for *B. malayi*, 161 (15 %) for *M. hapla* and 161 (15 %) for *M. incognita* contigs. However, when the characterised *pash*-1 gene of *A. suum* (HQ611976) was used as query to identify this gene in *B. malayi*, *M. hapla* and *M. incognita*, it mapped to the same contigs but with better identity scores indicating they are more closely related to each other than to *C. elegans*.

RNA-induced silencing complex

The four known proteins that interact to form the RNA-induced silencing complex (RISC) in *C. elegans* are AIN-1, AIN-2, TSN-1 and VIG-1. Both of the ALG-1 interacting proteins AIN-1 and AIN-2 carry an M domain (M domain of the protein GW182). Apart from *B. malayi* contig (AAQA01000363) coding for AIN-1, there were no significant matches for these two genes in any contigs or ESTs of the four parasitic nematodes when the *ain-1* sequence of *B. malayi* was used as query to search for matches in *A. suum*, *M. hapla* and *M. incognita*. Significant matches were identified only for contigs of *A. suum* but protein domain analysis revealed that those sequences did not code for the M domain.

The *C. elegans tsn*-1 gene encodes six functional protein domains (Fig. 3); four SNase (Staphylococcal nuclease) domains arranged in tandem followed by a Tudor domain fused with a fifth SNase domain at the C-terminus. These domains are not significantly identical to each other: the most identical are the second and fourth SNase domains with a total score of only 27 %. Contigs with high identities to the mRNA sequence of *tsn-1* of *C. elegans* are present amongst contigs of all the four parasitic nematodes. Protein domain analysis revealed the presence of both types of functional domains in all the contigs but with a lower identity score for the Tudor domain of *M. hapla* and *M. incognita* compared to that of *C. elegans*. There seems to be structural differences in proteins of the different nematodes where *C. elegans* and *M. incognita* have five SNase domains all arranged similarly. But all contigs analysed for *M. hapla*, *B. malayi* and *A. suum* indicate the presence of only four SNase domains, each missing particularly the fifth domain fused to the Tudor domain which is present in *C. elegans* and *M. incognita* (Fig. 3).

The functional significance of these differences, if indeed it is the case, is not known and like many other differences, need to be investigated further. Interestingly, the *M. incognita* whole genome contig CABB01000055 is 63,544 bp long and encodes the functional domains of the *tsn-1* gene as well as *mut*-7, nuclear RNAi effector, which in *C. elegans* is located on a different chromosome.

The *vig-1* gene was identified in whole genome sequences of the APNs *A. suum* (AMPH01002339) and *B. malayi* (AAQA01001685), but not for PPNs. However, when the identified *vig-1* domain of this *B. malayi* contig was used as a query, contig ABLG01000254 of *M. hapla* and contig CABB01000081 of *M. incognita* were identified, but with very low identity scores. Detailed analysis of these contigs confirmed the presence of the mRNA binding protein domain HABP4_PAI-RBP1 similar to that of *C. elegans* and the animal parasitic nematodes *Schistosoma japonicum* (Q5DA16) and *Schistosoma mansoni* (Q9N2M6).

RNAi amplification

Seven genes encoding proteins involved in the siRNA amplification process have been described in *C. elegans.* These are: *ego-1*, *rrf-1*, *smg-2*, *smg-5*, *smg-6*, *rde-10* and *rde-11.* The genes *ego-1* and *rrf-1* of *C. elegans* have only one RdRp domain (77 % nucleotide similarity and 59 % amino acid identity between them) are both present on chromosome I, only 0.9 kb apart (Sijen et al. 2001). Consequently, the same genomic contigs of the parasitic nematodes matched significantly to both *C. elegans rrf-1* and *ego-1* (total bit scores between 497 and1760); three each for *A. suum* and *B. malayi*, two for *M. hapla* and four for *M. incognita.* All of these sequences had the RdRp domain with the signature motif 'DbDGD'. The full-length mRNA sequences of both genes were covered in most of contigs and scaffolds of the parasitic nematodes.

Contigs encoding genes similar to the *smg-2* were found in sequences of all the four parasitic nematodes and contained all three functional domains (AAA_30, AAA_12 and UPF1_Zn_bind) typical of the *C. elegans smg-2* gene. The *smg-6* gene of *C. elegans* matched with significant identities to *A. suum* and *B. malayi* sequences. The best matching contigs for *M. hapla* (ABLG01000285) and *M. incognita* (CABB01000011) had insignificant e-values of 0.029 and 0.012, respectively. However, when the SMG-6 coding

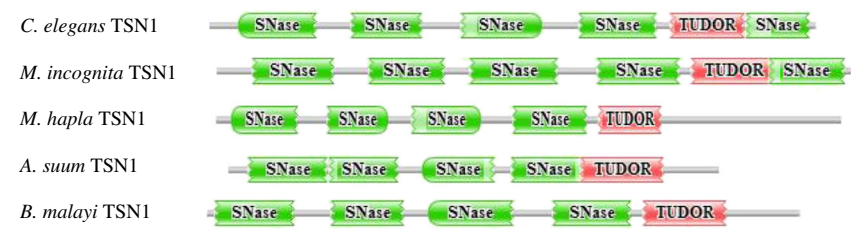

Fig. 3 Protein domain analysis of nematode TSN1 indicating the missing Staphylococcal nuclease (*SNase*) domain after the Tudor domain in *M. hapla*, *A. suum* and *B. malayi*

sequence of *B. malayi* was used as a query, significant matches were identified in contigs of *M. incognita* and *M. hapla*. These had the signature functional domains present in *C. elegans* SMG-6 namely EST1, EST1 DNA-binding domain and PINsmg6 domain. No genomic contig, scaffold or EST of any of the parasitic nematodes was similar or identical to the full-length mRNA sequences of *C. elegans smg-5* (or its endcoded domain PIN smg5) or two other genes involved in RNAi amplification, *rde-10* and *rde-11*.

RNAi inhibitors

Although their function is not exclusive to inhibition of RNAi, 14 such genes for which loss of function increases the sensitivity of RNAi, have been investigated in *C. elegans*. These are enhanced RNAi phenotype genes (*eri-1*, *eri-3*, *eri-5*, *eri-6/7*, *eri-9*), adenosine deaminase acting on RNA genes (*adr-1*, *adr-2*), XRN (mouse/*S. cerevisiae*) ribonuclease related genes (*xrn-1*, *xrn-2*), the *RdRp* containing genes *rrf-3*, *lin15-b*, *gfl-1*, *somi-1* and *zfp-2*.

The *eri-1* gene in *C. elegans* which removes the 2 nt overhangs at 3′ ends of siRNAs thereby restricting uptake by the RISC, has two functional domains i.e. SAP which is a putative DNA/RNA binding domain and ERI-1, a DEDDh 3′–5′ exonuclease domain. Spliced mRNA sequence of *eri-1* mapped significantly to three contigs each for *A. suum* and *B. malayi*, one of *M. hapla* and two of *M. incognita*. Also it appeared that contigs of *B. malayi*, *M. hapla* and *M. incognita* do not encode the SAP domains (Fig. 4a). Whether this represents a functional difference or divergence in the gene is not known.

Only one and two contigs respectively for *A. suum* and *B. malayi* were significantly identical to *eri-5* of *C. elegans*: all encoded the Tudor domain typical of this gene. The Tudor domain of *eri-5* is completely different in terms of amino acid sequence from the Tudor domain of *ekl-1* which is a nuclear RNAi effector and *tsn-1* gene of the RISC complex. However, no contigs or ESTs of *M. hapla* or *M. incognita* were found to encode a Tudor protein domain. In *C. elegans*, the *eri-6/7* mRNA is formed by trans-splicing of two pre-mRNAs *eri-6* and *eri-7* (Fischer et al. 2008) and encodes two P loop motifs designated as AAA_11 and AAA_12. These P loop motifs are also present in the *smg-2* gene designated as AAA_30 and AAA_12 but have different amino acid sequences than the *eri-6/7* domains. The *eri-6/7* mRNA mapped significantly to *A. suum* and *B. malayi* contigs. Out of the 84 effectors of RNAi analysed in this study, the *eri-6/7* gene was the only gene not present in both *Meloidogyne* spp., although it was in *M. hapla*. The genes *eri-3* and *eri-9* of *C. elegans* had no significant matches to any of the four parasitic nematodes.

The *rrf-3* gene has been reported as an inhibitor of RNAi in *C. elegans* and *rrf-3* mutants with enhanced sensitivity to RNAi have been used in several experiments to assess effects of knockdown of several genes. One hypothesis is that it competes with *ego-1* and *rrf-1* for components of the RNAi machinery (Simmer et al. 2002). Although *C. elegans rrf-3* is quite different from *ego-1* and *rrf-1* (58 and 57 % identity respectively), the same contigs of the parasitic nematodes (4 for *M. incognita*, 2 for *M. hapla*, 10 for *A. suum* and 7 for *B. malayi*) matched to all three genes. This must be because both the genes and the contigs code for RdRp domains. To explore the identity of the RdRp domains of these genes and the contigs, a phylogenetic tree was constructed using translated amino acid sequences of the domains. Contigs coding for 100 % identical RdRp domains were excluded, and RdRp domains of the same genes in *C. briggsae* and *C. remanei* were included in the analysis (Fig. 4b). The tree indicates close relationships of *ego-1* and *rrf-1* both of which are distantly related to *rrf-3*. One RdRp each for *A. suum* (ANBK01005062) and *B. malayi* (CAPY01003132) clustered with *rrf-3* of *C. elegans* whereas two each for these species clustered with the *rrf-1* and *ego-1* RdRps of *Caenorhabditis* spp. It appears the RdRps of the PPNs are distantly related to those of the free-living nematodes. While it may be safe to suggest the presence of *ego-1* and *rrf-1* in the genome of the APNs, it was not possible to distinguish the RdRps of the PPNs.

The effector *gfl-1* was conserved in all four parasitic nematodes. The genes *xrn-1* and *xrn-2* are 5′–3′ exoribonucleases similar in their protein functional domain architecture. They code for the same Xrn_N domains which in *C. elegans* has 52 % similar amino acids. Both of these inhibitor sequences mapped to significantly matching contigs of four parasitic nematodes. Two contigs for *M. incognita* (CABB01001503; CABB01003205) mapped to different parts of the *C. elegans xrn-2* completing the full-length gene. Further analysis suggested that APNs have both of these RNAi inhibitors but PPNs possess one of them, which based on the e-value and total scores was *xrn-2*.

The *adr-1* gene of *C. elegans* has three functional domains i.e. two DSRM and one Adenosine deaminase (A_deamin) domain while *adr-2* has one DSRM and one A_deamin domain which suppresses RNAi by deaminating transgenic dsRNA. *A. suum* contig (AMPH01003945) that mapped to *adr-1* codes for one DSRM domain with low identity to *C. elegans* DSRM while the whole genome scaffold AMPH01015223 mapped for *adr-2* codes for the A_deamin domain only. These results were similar for *B. malayi* contigs mapping to *adr-1* and *adr*-2. There was no significant match of any contig of *Meloidogyne spp.* to the *C. elegans adr-1* or *adr-2* (Fig. 4c).

The suppressor of over expressed miRNAs *somi-1* did not significantly match to any contig coding the NADH dehydrogenase subunit typical of this gene in the four parasitic nematodes. *C. elegans* mutants of *lin-15b* are more sensitive to RNAi. This gene encodes a protein that contains the THAP functional domain, which is a putative DNA-binding domain.

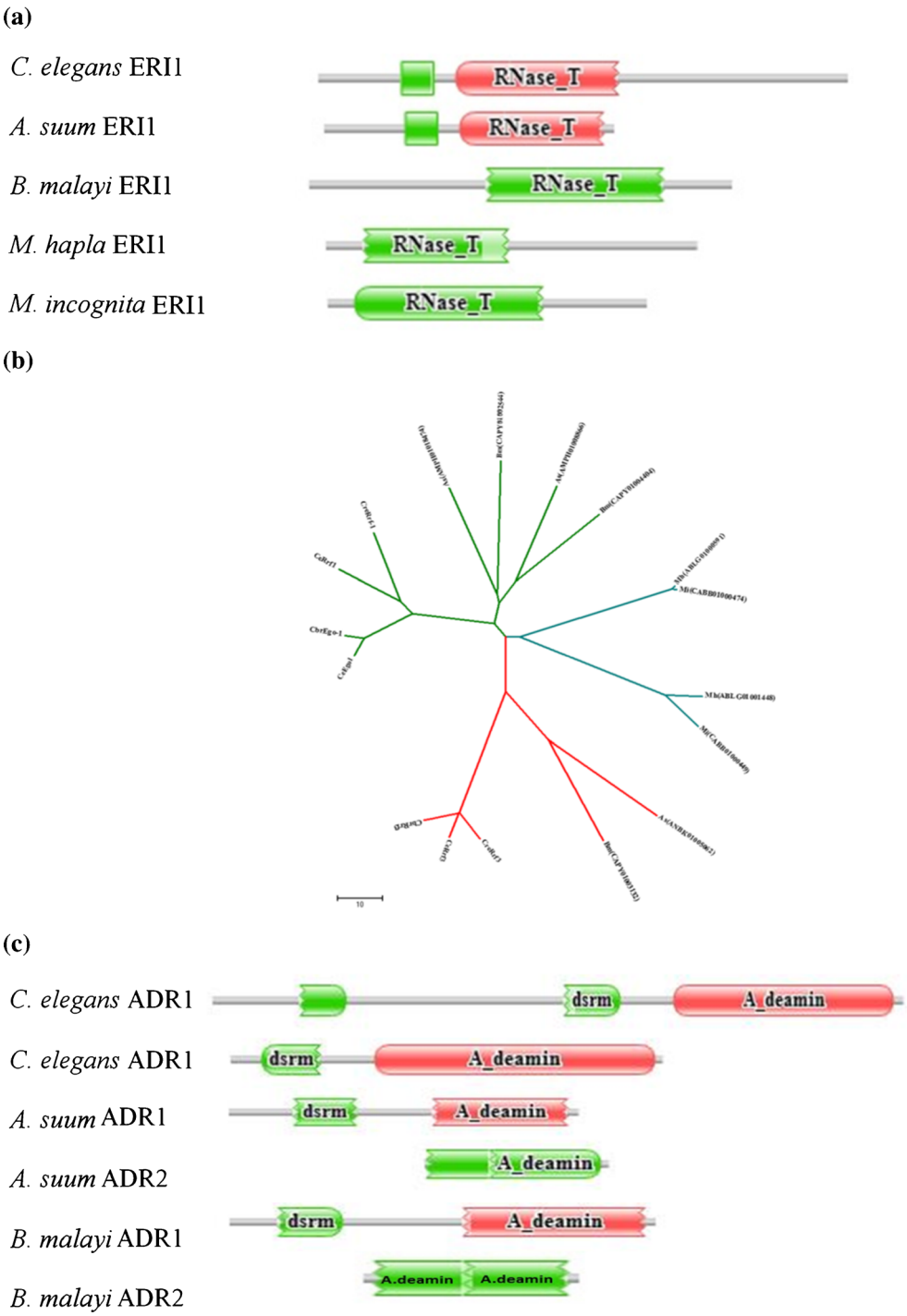

Fig. 4 **a** Protein domain composition of the RNAi inhibitor ERI1 of *C. elegans* compared to that of parasitic nematodes. **b** Phylogenetic analysis of the RNA-dependent RNA-polymerase genes of *C. elegans*, *A. suum* (*As*), *B. malayi* (*Bm*), *M. hapla* (*Mh*) and *M. incognita* (*Mi*). The Neighbour-joining tree was constructed based on ClustalW alignment of RdRp domain sequences. **c** Protein domain architecture of the inhibitor gene *adr-1* and *adr-2* of *C. elegans* compared to that of the two animal parasitic nematodes

Neither the gene sequence nor the THAP domain matched significantly to any contig or EST of the four parasitic nematodes. The full-length mRNA of *C. elegans zfp-2* mapped significantly to 40 genomic contigs of *A. suum*, 100 contigs of *B. malayi*, 17 of *M. hapla* and 20 of *M. incognita*. The reason for this large number of matching contigs is that the zinc finger proteins are amongst the most abundant proteins in eukaryotic genomes and are needed for stabilising other protein structures and as part of several transcriptional factors (Haerty et al. 2008).

Nuclear RNAi effectors

Expressed in both the cytosol and the nucleus, 17 *C. elegans* genes are known to play vital roles in the nuclear RNAi process. Eight of these were not identified from genomic contigs

or ESTs of any of the parasitic nematodes. These were the nuclear RNAi defective genes (*nrde-1*, *nrde-2* and *nrde-4*), *rde-2*, *mes-3*, *mut-16* and two enhancers of *ksr-1* lethality (*ekl-1* and *ekl-5*). Three contigs for *A. suum* and two for *B. malayi* and one each for *M. hapla* and *M. incognita* mapped to *C. elegans ekl-4* and they all code for the gene specific DMAP1 functional domain. For *ekl-6* gene, two contigs each for the APNs mapped to *C. elegans ekl-6* and encode for the DUF2435 protein domain. This gene was not found in the contigs of PPNs.

The gene *mut-7* was identified in contigs of all the four parasitic nematodes: the mut-7 domain characteristic of the gene was less identical to the one identified in contig CABB01000055 for *M. incognita*. For the six effectors *mut-2*, *mes-2*, *mes-6*, *cid-1*, *rha-1* and *zfp-1*, significantly matching contigs of the parasitic nematodes contained all the functional domains typical of these genes. Nuclear RNAi defective (nrde) genes in *C. elegans* have been associated with heritable RNAi. Interestingly, there is no detailed study on heritable RNAi in parasitic nematodes although there are suggestions of persistence of gene knockdown in some percentage of root knot nematodes.

Argonaute proteins

Twenty-eight argonaute proteins which function at different stages of the RNAi and miRNA pathways in *C. elegans* have been reported (Yigit et al. 2006). Since then *rde-3* has been renamed *mut-2*, a nuclear RNAi effector and *M03D4.6* and *C06A1.4* have been designated as pseudogenes: these were excluded from the analysis. Also during the analysis, it was found that *ppw-1* and *sago-2* sequences were identical, and so one was excluded leaving 24 argonaute sequences for study. These were analysed for the presence of functional protein domains: five of them (*alg-1*, *alg-2*, *alg-4/tag-76*, *T22B3.2*, *T23D8.7*) encode PAZ, PIWI and another domain known as DUF1785. The *C04F12.1*, *ZK218.8*, and *ZK1248.7* genes encode only the PIWI domain while the rest (*R06C7.1*, *F58G1.1*, *rde-1*, *C16C10.3*, *ppw-1/sago-2*, *ppw-2*, *sago-1*, *csr-1*, *T22H9.3*, *ergo-1*, *prg-1*, *prg-2*, *F55A12.1*, *nrde-3*, *Y49F6A.1*, *C14B1.7*) code for both PAZ and PIWI domains.

Using full-length sequences of the *C. elegans* argonautes, a total of 13, 10, 13 and 16 contigs respectively for *A. suum*, *B. malayi*, *M. hapla* and *M. incognita* were identified with significant alignment scores. This means 11 unique sequences for *A. suum*, 6 for *B. malayi*, 10 for *M. hapla* and 11 for *M. incognita* were used to analyse the presence of PAZ/PIWI domains and for constructing phylogenetic tree with argonautes of *C. elegans*. As with the *C. elegans* genes, all the contigs of parasitic nematodes have the conserved PIWI domain. The cladogram shows the phylogenetic relationship between *C. elegans* and both parasitic groups of nematodes based on the PIWI domain (Fig. 5).

Based on sequence homology, there are distinctly different sub clades: for example, in clade 1, where *alg-1*, *alg-2* and *T 23D8.*7 are relatively similar to sequences of each of the parasitic nematodes. The PIWI domains of *C. elegans* argonautes appear to be distantly related to those of the four parasitic nematodes. In both clades 2 and 6, generally, sequences of the parasitic nematodes appear more closely related to each other than those of *C. elegans.* There appears to be a clear distinction in clade 3 where no argonaute of parasitic nematodes clustered with the five *C. elegans* argonaute proteins (F58G1.1, PPW2, ZK1248.7, R06C7.1, F55A12.1) that are involved in transcriptional silencing and germline RNAi while only *A. suum* PIWI coded by the contig AMPH01002307 displayed homology to the argonaute ERGO1 in the sub clade 7. Argonaute proteins involved in nuclear RNAi (NRDE-3, C16C10.3, T22H9.3, Y49F6A.1) define a clade on their own but do appear to be distantly homologous to one argonaute each for the four parasitic nematodes.

Effect of soaking J2s of *M. incognita* in dsRNA

When J2s of *M. incognita* were soaked in medium containing FITC and dsRNA to target genes, uptake of external solution by the nematodes was evident from FITC fluorescence after 16 h. The activity of nematodes soaked without dsRNA or with ds*gfp* was comparable, but for the nematodes treated with dsRNA of target genes, movement was generally slow and resulted in varying number of nematodes with straight bodies. This phenotype was most prominent for J2s soaked with ds*drh-3*, ds*tsn-1* and ds*alg-1* where about 70, 80 and 90 % respectively appeared straight (normally a sign of nematode paralysis) instead of exhibiting the normal sinusoidal movement (Fig. 6). However, nematodes soaked in ds*rrf-1*, ds*xrn-2* and ds*mut-2* mostly exhibited an aberrant wavy movement compared to the controls.

Infectivity of J2s soaked in dsRNA

Infectivity of nematodes soaked in dsRNA to *tsn-1* and *rrf-1* did not appear to be affected since the levels of plant infection were not significantly different from the controls. However, possible disruption of *xrn-2* and *mut-2* genes reduced plant infection by 44 and 37 % respectively. The *drh-3* phenotype (straight and paralysed) differed from that of *xrn-2* and *mut-2* (wavy forms), but infection reduction (44 %) was not significantly different (Fig. 7). It was evident at the time when gall numbers on plant roots were counted that egg masses developed earlier for nematodes previously soaked in ds*rrf-1* compared to controls.

Adult females dissected from tomato roots infected with J2s treated with ds*gfp*, ds*rrf-1*, ds*tsn-1* or without dsRNA developed normally into saccate females typical of *M. incognita.* Significant ($p < 0.05$) differences in appearance

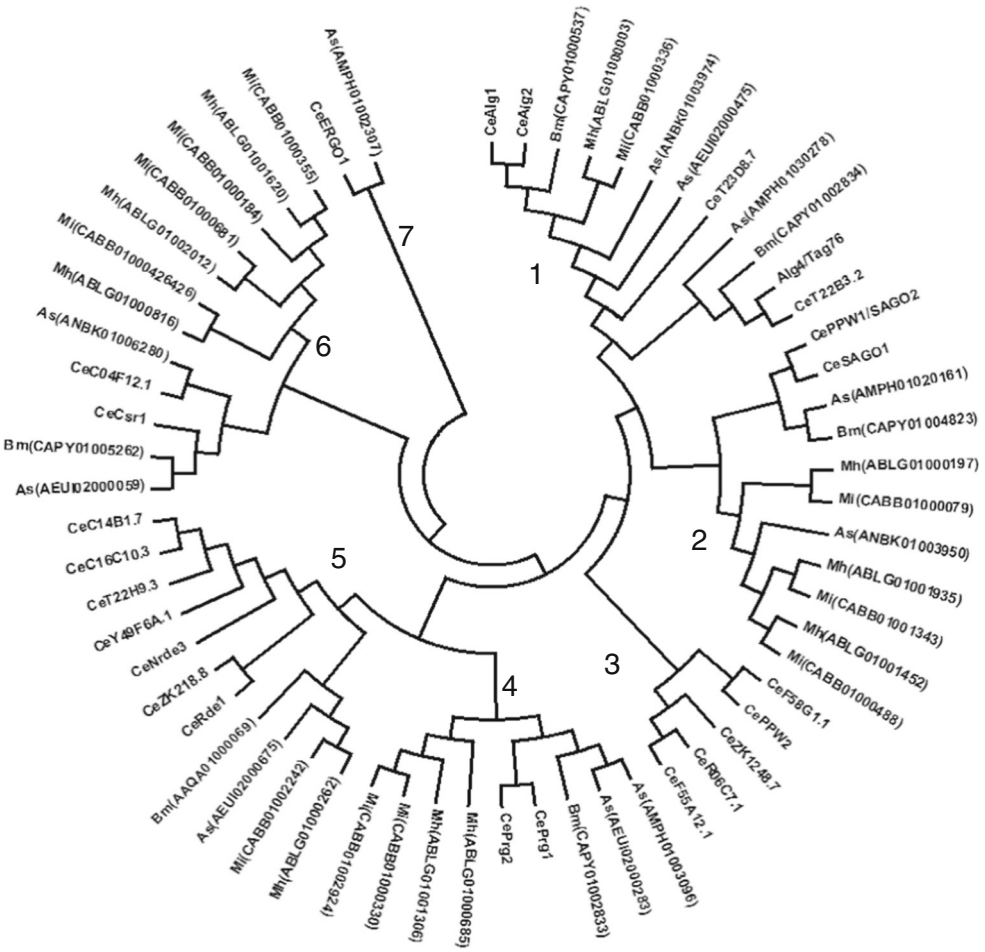

Fig. 5 Neighbour-Joining cladogram based on the conserved PIWI domain of all argonaute sequences in *C. elegans* and four parasitic nematodes *A. suum* (*As*), *B. malayi* (*Bm*), *M. hapla* (*Mh*) and *M. incognita* (*Mi*). Contig IDs of the sequences coding for the argonaute proteins are indicated in *parenthesis*

were found between controls and nematodes treated with ds*drh-3*, ds*xrn-2* and ds*mut-2*. However, 63 and 71 % of adult females developed from J2s soaked with ds*drh-3* and ds*mut-2* respectively were abnormal and had a fusiform body shape. For those soaked with ds*xrn-2*, nematodes with translucent bodies were observed for 75 % of the extracted adult females respectively. About 20 % of the females for ds*alg-1* fed nematodes had translucent bodies but this was not significantly different than controls. This translucency was also observed for adult female that developed from J2s previously soaked with ds*mut-2* (Fig. 8).

Discussion

The aims of this research were (i) to study the components of the small RNA pathway of PPNs and APNs and to compare them with those reported for the model nematode *C. elegans*, (ii) to evaluate the effect of knockdown of target genes from representatives of different classes of effectors in the RNAi pathway by in vitro RNAi treatment of J2s of the PPN *M. incognita* and to discuss the suitability of these for host-induced gene silencing (HIGS) as a method of PPN control.

We have taken into account the possibility that some functional homologues of *C. elegans* genes of the small RNA pathway might have diverged sufficiently in the parasitic nematodes for their presence to have been missed. Nevertheless, the bioinformatics analyses indicated that out of the 84 genes identified in the RNAi and miRNA pathways of *C. elegans*, only 49 homologous sequences were found in the available genomic sequences of *A. suum*, 45 in *B. malayi contigs*, and 40 each in *M. hapla* and *M. incognita*. Significantly, most of the homologues identified displayed conserved protein domain architecture. For fully sequenced genes, the protein domain analyses, based on similarity and identity scores for common genes, indicated that such sequences for PPNs are more similar to those for APNs than to those for *C. elegans*. The clear conclusion is that the genomes of parasitic nematodes, as represented by the PPNs *M. hapla*, *M. incognita* and the APNs *A. suum* and *B. malayi* have a reduced complement of small RNA pathway genes compared to *C. elegans*. This conclusion is entirely consistent with other comparative genomic studies in which it is evident that there is a trend towards a

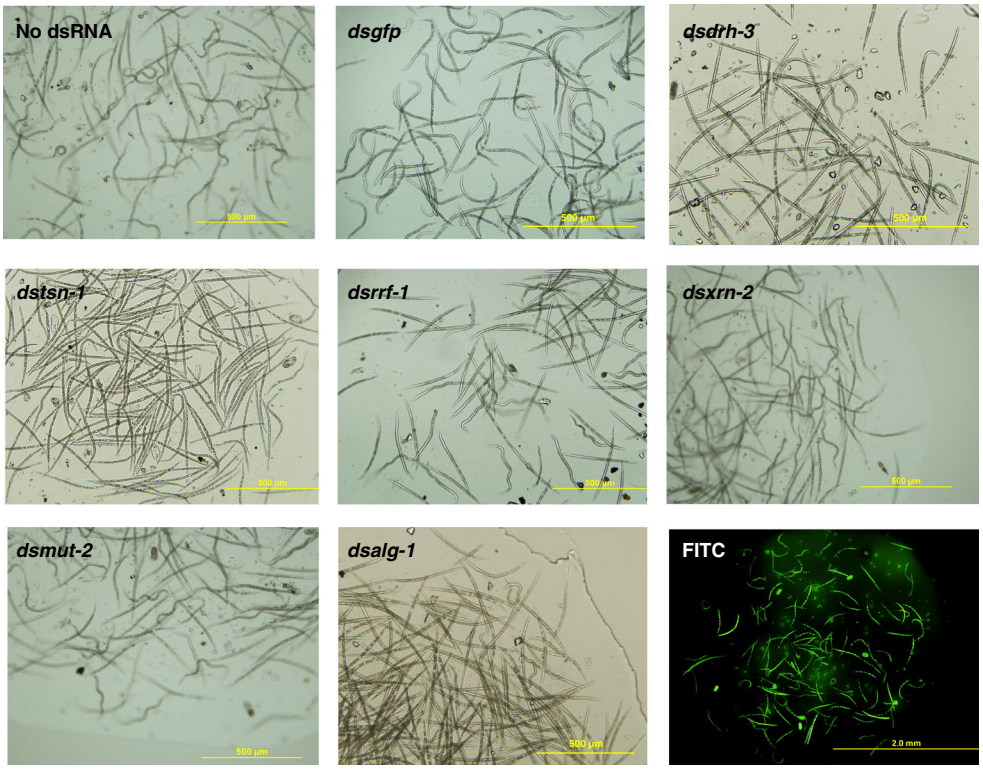

Fig. 6 Phenotypes observed after 16 h of dsRNA soaking of *M. incognita* J2s. Controls were No dsRNA and dsgfp fed J2s. Phenotypes presented after feeding dsRNA to different target genes (ds'gene' refers to dsRNA to the target gene i.e. no dsRNA, dsgfp, dsdrh-3, dstsn-1, dsrrf-1, dsxrn-2, dsmut-2 and dsalg-1). FITC uptake was also recorded (Labelled FITC) in J2s

reduction in genome size of parasites compared to free-living forms (Bird et al. 2015).

A comparison of small RNA pathway genes of PPNs

All eukaryotes displaying RNAi ability have Dicer-like genes, with four classes in *Arabidopsis*, two in insects and fungi and one characterised for mouse and humans. Amongst the sequences analysed, the Dicer-1 was the most conserved protein, with seven domains present in all the nematodes studied. Homology studies nevertheless identified differences in the functional domains of Dicer-like genes of the five nematodes compared to those of other organisms. It is possible that the distance between the PAZ and ribonuclease III domains of the dicer protein, which reflects the size of siRNAs produced (MacRae et al. 2006), may be responsible for the different sizes of siRNAs generated by *C. elegans* (~22–23 bp, Ketting et al. 2001) and those produced by plants (e.g. 21, 22, 23 and 24 bp for Arabidopsis). The length of siRNAs produced by PPNs and which are responsible for RNA silencing has not yet been investigated, and the identification and characterisation of sequences potentially encoding the dicer of PPNs as undertaken in this study, is the first step in characterising the detailed functions of Dicer in development of PPNs.

The *C. elegans* DICER has been associated with RDE-1 and RDE-4, the genetic mutants of which demonstrate a complete absence of RNAi in response to foreign dsRNA (Tabara

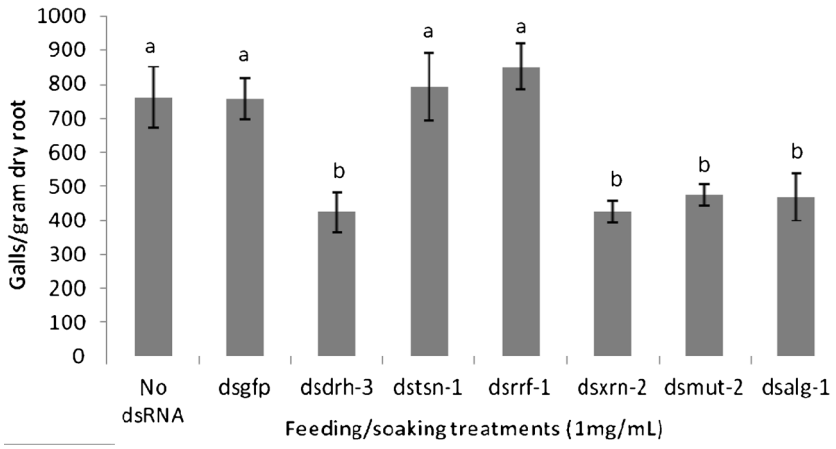

Fig. 7 Infection on tomato plants (Galls/g of dry root weight) compared with No dsRNA and dsgfp fed *M. incognita* J2s. Average number of galls developed per gram of dry root by nematodes soaked in complementary dsRNA for genes *drh-3*, *tsn-1*, *rrf-1*, *xrn-2*, *mut-2* and *alg-1* along with controls. *Different letters* indicate significant differences between means ($p < 0.05$). *Error bars* represent standard error

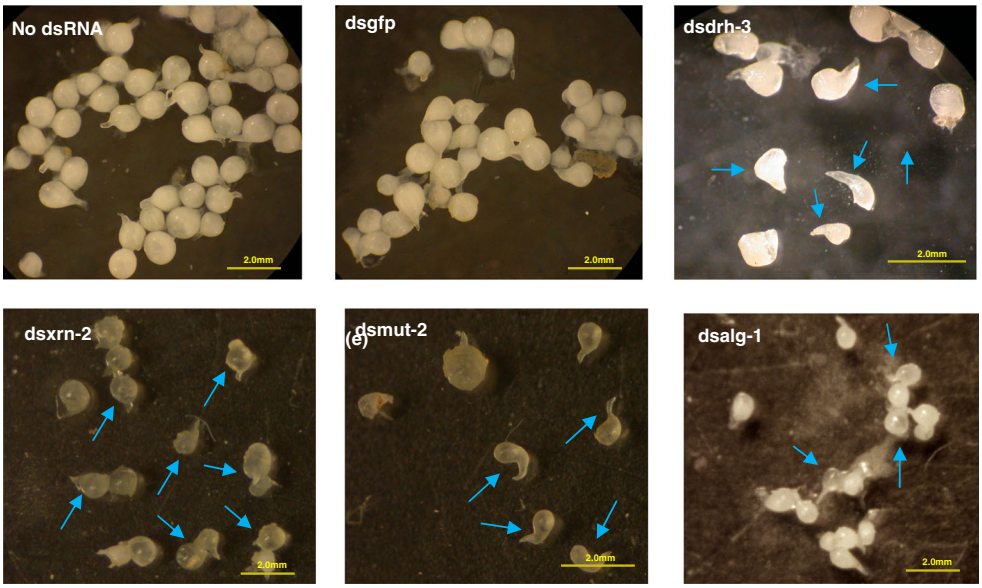

Fig. 8 *M. incognita* females fed with no dsRNA, dsgfp, dsdrh-3, dsxrn-2, dsmut-2 and dsalg-1 7 weeks after plant infection. *Arrows* indicate abnormal females in different treatments compared to controls. *Scale bar* 2 mm

et al. 1999; Tabara et al. 2002). However, similar sequences of these two essential genes were not identified in the genomic contigs of any of the parasitic nematodes. This result suggests that there may be an alternative mechanism for dsRNA retention in these nematodes as is the case for *Drosophila melanogaster* where the RISC contains DCR-2 protein as part of the complex and appears to play a role in retaining the dsRNA that enters the cell, thereby maintaining an unprocessed pool of dsRNA (Kim et al. 2007; Shih and Hunter 2011).

In plants, insects and *C. elegans*, the RNAi pathway delivers a robust antiviral response. In *C. elegans*, the DRH-1 protein has a RIG-I domain which senses and binds viral single-stranded RNA (Lu et al. 2009). This domain in vertebrates also binds to viral RNA bearing 5′-phosphates triggering antiviral responses (Rehwinkel et al. 2010). The parasitic nematodes studied here all lacked a detectable RIG-I domain in the *drh-1* sequence. Mutation in the *drh-1* in *C. elegans* leads to loss of antiviral response and it is suggested that this protein may act at the start of the antiviral response cascade triggering an immune response (Ashe et al. 2013). Such a disparity points towards an alternate mechanism for recognition of viral RNA and resulting RNAi-based antiviral response mechanisms for this group of parasitic nematodes.

Mutants of *rde-1* and *rde-4* in *C. elegans* are more susceptible to virus replication while *rrf-3* and *eri-1* mutants have increased antiviral response (Lu et al. 2005; Wilkins et al. 2005). The orthologues of *rde-1*, *rde-4* and *rrf-3* appear to be missing in the genomic sequences of all four parasitic nematodes. These nematodes also lacked the SAP domain in the sequence coding for the gene *eri-1*. Yeast assays show that the SAP domain is responsible for binding dsRNA and that ERI-1 and the SAP domain when expressed separately does not result in dsRNA degradation (Iida et al. 2006). This perplexing difference in the proteins affecting antiviral RNAi response in the parasitic nematodes requires further investigation. Another possible explanation could be that the parasitic nematodes live in a *controlled* environment for most life stages that is, inside a host, and so are less exposed to hostile external environments, and therefore have a reduced need for the most robust antiviral defences.

In *C. elegans*, RNAi can persist in the next progeny, and this effect is proposed to be dependent on amplification of a trigger (Alcazar et al. 2008). The germline amplification effector (EGO-1) is present in all the four parasitic nematodes, but the nuclear RNAi effectors NRDE-1, NRDE-2 and NRDE-4, which are the core components responsible for heritable RNAi in *C. elegans*, were not found. The *nrde-3* gene, which encodes the argonaute responsible for localising secondary siRNAs to the nucleus, was also one of the argonautes that had low significant matches to the argonautes of parasitic nematodes. This suggests a large divergence or the complete absence of this effector and the possibility of absence of heritable RNAi in these species, although this aspect has not been investigated experimentally.

Argonaute proteins show a striking contrast in numbers, with 24 in *C. elegans* and fewer than half of this number in the parasitic nematodes. Overall, there were fewer argonaute proteins and less conservation in nuclear, germline and transcription-related argonautes, which suggests some differences exist in their mechanisms/biological processes. It is possible that argonautes of parasitic nematodes act as convergent points for the various processes involved, or carry out more than one function in the small RNA pathways with the aid of other associated proteins.

Table 2 summarises the small RNA effector repertoire in *C. elegans* compared to the four parasitic nematodes studied here with already published data of *Pratylenchus coffeae*,

Table 2 Comparison of the RNAi pathway genes of the four parasitic nematodes with the published data of *G. pallida*, *B. xylophilus* and *P. coffeae* (Cotton et al. 2014; Kikuchi et al. 2011; Burke et al. 2015)

C. elegans	*A. suum*	*B. malayi*	*M. hapla*	*M. incognita*	*P. coffeae*	*G. pallida*	*B. xylophilus*
Transport proteins							
xpo-1, *xpo-2*, *xpo-3*, *rsd-2*, *rsd-3*, *rsd-6*, *sid-1*, *sid-2*, *sid-3*^{gpb}, *sid-5*^{gpb}, *haf-6*^{gpb}	*xpo-1*, *xpo-2*, *xpo-3*, *rsd-3*, *sid-1*, *sid-3*, *haf-6*	*xpo-1*, *xpo-2*, *xpo-3*, *rsd-3*, *sid-1*, *sid-3*, *haf-6*	*xpo-1*, *xpo-2*, *rsd-2*, *rsd-3*, *sid-3*, *haf-6*	*xpo-1*, *xpo-2*, *rsd-2*, *rsd-3*, *sid-3*, *haf-6*	*xpo-1*, *xpo-2*, *rsd-3*	*xpo-1*, *xpo-2*, *rsd-3*,	*xpo-1*, *xpo-2*, *rsd-6*
Dicer and associated proteins							
dcr-1, *drh-1*, *drh-3*, *pir-1*^{gpb}, *drsh-1*, *pash-1*, *rde-4*	*dcr-1*, *drh-1*, *drh-3*, *pir-1*, *drsh-1*, *pash-1*	*dcr-1*, *drh-1*, *drh-3*, *pir-1*, *drsh-1*, *pash-1*	*dcr-1*, *drh-1*, *drh-3*, *pir-1*, *drsh-1*, *pash-1*	*dcr-1*, *drh-1*, *drh-3*, *pir-1*, *drsh-1*, *pash-1*	*dcr-1*, *drh-3*, *drsh-1*	*dcr-1*, *drh-3*, *drsh-1*, *pash-1*	*dcr-1*, *drh-1*, *drh-3*, *drsh-1*, *pash-1*, *rde-4*
RNA-induced silencing complex (RISC)							
ain-1, *ain-2*, *tsn-1*, *vig-1*	*tsn-1*, *vig-1*	*tsn-1*, *vig-1*, *ain-1*,	*tsn-1*, *vig-1*	*tsn-1*, *vig-1*		*tsn-1*	*tsn-1*, *vig-1*, *ain-1*
RNAi Amplification							
ego-1, *rrf-1*, *smg-2*, *smg-5*, *smg-6*, *rde-10*^{gpb}, *rde-11*^{gpb}	*ego-1*, *rrf-1*, *smg-2*, *smg-6*	*ego-1*, *rrf-1*, *smg-2*, *smg-6*	*ego-1*, *rrf-1*, *smg-2*, *smg-6*	*ego-1*, *rrf-1*, *smg-2*, *smg-6*	*ego-1*, *rrf-1*, *smg-2*, *smg-6*	*ego-1*, *smg-2*, *smg-6*,	*ego-1*, *rrf-1*, *smg-2*, *smg-6*
RNAi Inhibitors							
eri-1, *eri-3*, *eri-5*, *eri-6/7*, *eri-9*^{gpb}, *adr-1*, *adr-2*, *xrn-1*, *xrn-2*, *rrf-3*, *lin15-b*, *gfl-1*, *zfp-2*^{gpb}, *somi-1*^{gp}	*eri-1*, *eri-5*, *eri-6/7*, *adr-1*, *adr-2*, *xrn-1*, *xrn-2*, *xrn-3*, *gfl-1*, *zfp-2*	*eri-1*, *eri-5*, *eri-6/7*, *adr-1*, *adr-2*, *xrn-1*, *xrn-2*, *xrn-3*, *gfl-1*, *zfp-2*	*eri-1*, *eri-6/7*, *xrn-2*, *gfl-1*, *zfp-2*	*eri-1*, *xrn-2*, *gfl-1*, *zfp-2*	*eri-6/7*, *rrf-3*, *gfl-1*	*eri-1*, *xrn-2*, *gfl-1*	*Adr-1*, *xrn-2*, *rrf-3*, *gfl-1*, *somi-1*
Nuclear RNAi effectors							
nrde-1^{gpb}, *nrde-2* ^{gpb}, *nrde-4* ^{gpb}, *rde-2*, *mut-2*^{p}, *mut-7*, *mes-2*, *mes-3*, *mes-6*^{p}, *ekl-1*, *ekl-4*, *ekl-5*, *ekl-6*, *cid-1*, *rha-1*, *zfp-1*^{g}	*mut-2*, *mut-7*, *mes-2*, *mes-6*, *ekl-4*, *ekl-6*, *cid-1*, *rha-1*, *zfp-1*	*mut-2*, *mut-7*, *mes-2*, *mes-6*, *ekl-4*, *ekl-6*, *cid-1*, *rha-1*, *zfp-1*	*mut-2*, *mut-7*, *mes-2*, *mes-6*, *ekl-4*, *cid-1*, *rha-1*, *zfp-1*	*mut-2*, *mut-7*, *mes-2*, *mes-6*, *ekl-4*, *cid-1*, *rha-1*, *zfp-1*	*cid-1*, *ekl-1*, *ekl-4*, *mes-2*, *rha-1*	*cid-1*, *mes-2*, *ekl-4*, *rha-1*	*Mut-7*, *mes-2*, *mes-6*, *ekl-1*, *ekl-4*, *ekl-6*, *cid-1*, *rha-1*
Argonautes							
alg-1, *alg-2*, *alg-4/tag-76*^{g}, *T22B3.2*^{b}, *T23D8.7*, *R06C7.1*, *F58G1.1*, *rde-1*, *C16C10.3*, *ppw-1/sago-2*, *ppw-2*, *sago-1*, *csr-1*, *T22H9.3*, *ergo-1*, *prg-1*, *prg-2*, *F55A12.1*, *nrde-3*, *Y49F6A.1*, *C14B1.7*^{gb}, *C04F12.1*, *ZK218.8*^{gp}, *ZK1248.7*	*alg-1*, *alg-2*, *T22B3.2*, *R06C7.1*, *rde-1*, *ergo-1*, *F55A12.1*, *Y49F6A.1*, *C04F12.1*, *ZK1248.7*	*alg-1*, *alg-4/tag-76*, *R06C7.1*, *C16C10.3*, *ergo-1*, *C04F12.1*,	*alg-1*, *alg-4/tag-76*, *R06C7.1*, *F58G1.1*, *csr-1*, *T22H9.3*, *F55A12.1*, *Y49F6A.1*, *C04F12.1*	*alg-1*, *alg-4/tag-76*, *R06C7.1*, *F58G1.1*, *C16C10.3*, *F55A12.1*, *C14B1.7*, *C04F12.1*, *ZK1248.7*	*alg-1*, *alg-2*, *alg-4/tag-76*, *T22B3.2*, *T23D8.7*, *R06C7.1*, *F58G1.1*, *rde-1*, *C16C10.3*, *ppw-1/sago-2*, *ppw-2*, *sago-1*, *csr-1*, *ergo-1*, *prg-1*, *prg-2*, *F55A12.1*, *nrde-3*, *Y49F6A.1*, *C04F12.1*, *ZK1248.7*	*alg-1*, *R06C7.1*, *F58G1.1*, *C16C10.3*, *T22H9.3*, *F55A12.1*, *Y49F6A.1*, *ZK1248.7*	*alg-1*, *alg-2*, *T23D8.7*, *R06C7.1*, *rde-1*, *csr-1*, *Y49F6A.1*, *ZK1248.7*

g not determined in *G. pallida*, *p* not determined in *P. coffeae*, *b* not determined in *B. xylophilus*

Globodera pallida and Bursaphelenchus xylophilus. Despite their morphological similarities, and more than 40 % highly homologous genes to *C. elegans*, parasitic nematodes have a completely different lifestyle (Hashmi et al. 2001). Some of the remaining 60 % of the predicted genes may well have diverged to varying extents compared to those in *C. elegans*, or belong to a completely different set of genes that reflect their parasitic nature. Examples of such genes include those required for parasitism, for example to counteract host defences, invade the host and for PPNs the development of specific feeding cells (e.g. giant cells for *Meloidogyne spp*., Jones 1981; Jones and Goto 2011).

The absence of 35 to 44 RNAi effectors in the genomic sequences of the parasitic nematodes compared to those present in *C. elegans* requires explanation through further functional studies. The presence of sequences similar to *eri-6/7* in *M. incognita* genome and its apparent absence in sequences of the closely related *M. hapla* is striking if in fact this is the case, since RNAi is a conserved mechanism. This could be related to the fact that *M. incognita* and *M. hapla* belong to different phylogenetic clades and have different modes of reproduction (Bert et al. 2011).

RNAi of selected target genes

Six genes with different roles in the RNAi pathway were chosen for functional analysis after knockdown in J2s of *M. incognita*. Following soaking for 16 h with dsRNA, the J2s exhibited different phenotypic effects depending on the target gene, ranging from complete paralysis to little or no obvious effect. When treated J2s were subsequently used to infect tomato plants, there were variable levels of infection and gall formation, accompanied by the presence of adults with various developmental deformities. Downregulation of the orthologous genes to *tsn-1* and *alg-1* in J2s may have resulted in an imbalance of miRNA-regulated gene, resulting in a lower success rate of establishment of feeding sites. Although after soaking in dsRNA corresponding to *tsn-1* and *alg-1* there was apparent paralysis in J2s, they successfully infected tomato plants resulting in some gall formation. This result suggests that some J2s recovered after initial dsRNA treatment, but that the recovery rates differed for the two genes as there was no significant infection reduction for dstsn-1-soaked nematodes but there was a reduction of 39 % in galls for nematodes previously soaked in ds*alg-1*. Perhaps this difference reflects the relative importance of the genes in the RNAi pathway in that the genes are somewhat resistant to knockdown, or that higher amounts of dsRNA are required for transcript knockdown. However, differences in transcript reduction of different genes after soaking J2s of *M. incognita* in the same amount of dsRNA has been reported and such differences could be attributed to several reasons including the turnover of genes and base composition and position of dsRNA construct in relation to the target gene (Tan et al. 2013; Papolu et al. 2013).

When *xrn-2*, *drh-3* and *mut-2* genes of the J2s of *M. incognita* were knocked down and the nematodes used for plant inoculation, there was a substantial reduction in the level of infection of tomato roots 4 weeks after inoculation. Also, the nematodes that did develop to adults were abnormal, reflecting a longer term effect of dsRNA treatments associated with knockdown of these genes. The mechanism responsible for the abnormal development may include regulation feedback mechanisms and effects on genes downstream in the pathway, or it is possible that secondary siRNAs can persist through moults and contribute to abnormal development, resulting for example in an abnormal development of translucent adults. The latter clearly reflect severe metabolic or developmental dysfunction, and a failure to accumulate lipid reserve droplets normally present in healthy adult females. Similar observations, that is failure to develop the typical saccate body shape by adult *M. incognita* after in vitro dsRNA treatment has been reported previously, for example after knockdown of the cathepsin L cysteine proteinase gene (Shingles et al. 2007), as has adult translucency of nematodes infecting plants producing dsRNA complementary to nematode splicing factor and integrase genes (Yadav et al. 2006).

In conclusion, our results show that there are substantial differences in the number of genes involved in the small RNA pathway encoded by genomes of nematodes, in particular that the genomes of the plant and animal parasitic nematodes (*M. hapla*, *M. incognita*, *A. suum* and *B. malayi*) studied have a reduced complement of these genes compared to that of *C. elegans*. Nevertheless, the correct functioning of the small RNA pathways of these nematodes, including the miRNA pathway, is required for normal growth and development. Disturbing the RNAi pathway can reduce nematode reproductive ability, and this suggests that RNAi pathway genes are possible targets for nematode control using HIGS.

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