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Host-mediated RNAi of a Notch-like receptor gene in *Meloidogyne incognita* induces nematode resistance

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Research Article

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Abstract

GLP-1 (abnormal germline proliferation) is a Notch-like receptor protein that plays an essential role in pharyngeal development. In this study, an orthologue of *Caenorhabditis elegans glp-1* was identified in *Meloidogyne incognita*. A computational analysis revealed that the orthologue contained almost all the domains present in the *C. elegans* gene: specifically, the LIN-12/Notch repeat, the ankyrin repeat, a transmembrane domain and different ligand-binding motifs were present in orthologue, but the epidermal growth factor-like motif was not observed. An expression analysis showed differential expression of *glp-1* throughout the life cycle of *M. incognita*, with relatively higher expression in the egg stage. To evaluate the silencing efficacy of *Mi-glp-1*, transgenic *Arabidopsis* plants carrying double-stranded RNA constructs of *glp-1* were generated, and infection of these plants with *M. incognita* resulted in a 47–50% reduction in the numbers of galls, females and egg masses. Females obtained from the transgenic RNAi lines exhibited 40–60% reductions in the transcript levels of the targeted *glp-1* gene compared with females isolated from the control plants. Second-generation juveniles (J2s), which were descendants of the infected females from the transgenic lines, showed aberrant phenotypes. These J2s exhibited a significant decrease in the overall distance from the stylet to the metacorpus region, and this effect was accompanied by disruption around the metacorporeal bulb of the pharynx. The present study suggests a role for this gene in organ (pharynx) development during embryogenesis in *M. incognita* and its potential use as a target in the management of nematode infestations in plants.

Introduction

Meloidogyne incognita, the root-knot nematode, is a member of the group of sedentary plant-parasitic nematodes (PPNs) that cause serious damage (to the tune of hundreds of billions of dollars) to crop production worldwide (Elling, 2013). As an obligate endoparasite, *M. incognita* resides permanently inside the roots of its host throughout its lifecycle, and to derive nutrients, this organism has developed a specialized organ called the stylet. The pharynx of *M. incognita* is continuous with the stylet lumen and aids with the ingestion of food material into the intestine (Eisenback and Hunt, 2009). Pharyngeal development occurs during embryogenesis, and several genes responsible for its development, most of which belong to the Notch-like receptor family, have been identified in *Caenorhabditis elegans*. Notch pathways are known to regulate the aspects of growth and patterning in metazoans (Rudel and Kimble, 2001). Pharyngeal cells are produced by two distinct molecular pathways, ABa and EMS. The ABa pathway is dependent on the Notch receptor orthologue *glp-1* (abnormal germline proliferation) (Mango, 2007), which is a homologue of the *Drosophila* Notch gene (Roehl *et al.* 1996) and induces germline proliferation during pharyngeal embryonic development. *Glp-1* is structurally and functionally similar to another Notch-related receptor, *lin-12*, and these two proteins have similar conserved motifs that contribute to the functional roles of these proteins as membrane-bound receptors involved in Notch signalling (Yochem and Greenwald, 1989).

With recent advances in the generation of genomic datasets for nematodes, many studies have focused on identifying and characterizing genes with varied functional roles and determining their conservation throughout evolution. Various secretory and effector proteins have been characterized over the past few years (Banerjee *et al.* 2017). *Misp12* is a potential root-knot nematode effector expressed in the dorsal oesophageal gland (Xie *et al.* 2016). The SXP/RAL-2 secretory protein was identified in *M. incognita* and is expressed in the subventral pharyngeal glands (Tytgat *et al.* 2004). However, there are limited reports concerning the identification and characterization of genes involved in nematode development. Among the genes involved in organ development in nematodes, collagen genes are the most studied, and the *M. incognita* collagen genes, *Mi-col-1* and *lemmi-5*, and the *Globodera pallida* collagen

genes, *col-1* and *col-2*, have been identified and characterized (Wang *et al.* 1998; Gray *et al.* 2001). Chitin synthase, which functions in the production of eggshells, has been reported in *C. elegans* and *Meloidogyne artiellia* (Veronico *et al.* 2001; Fanelli *et al.* 2005). However, with the exception of the study conducted by Calderón-Urrea *et al.* (2016), who studied and compared the early development of *M. incognita* with that of *C. elegans*, almost no detailed studies have investigated genes associated with the early development of *M. incognita*.

Several reports detail the RNAi-mediated knockdown of specific secretory genes that affect parasitism in PPNs (Rehman *et al.* 2016). However, only some of the known developmental genes have been targeted by host-mediated gene silencing in the management of PPNs. The *Rpn7* gene, which is essential for the integrity of the 26S proteasome, has been targeted in attempts to control the root-knot nematode *M. incognita*, and the silencing of this gene resulted in a reduction in nematode motility (Niu *et al.* 2012). Two genes essential for body wall formation in *Meloidogyne graminicola*, namely the *Mg-pat-10* and *Mg-unc-87* genes, which function in muscle contraction and the maintenance of structural myofilaments, have been identified and suppressed *in vitro* through soaking, resulting in 91% and 87% reductions in infectivity, respectively (Nsengimana *et al.* 2013). *nhr-48*, a nuclear receptor gene that regulates various developmental, reproduction and pathogenicity processes in nematodes, has been silenced in *M. incognita*, resulting in delayed development and reduced reproduction (Lu *et al.* 2016). Despite these efforts, few developmental genes have been targeted in nematode control efforts.

The present study reports the identification and characterization of the *glp-1* gene of *M. incognita*. A host-mediated RNAi silencing approach was adapted to determine the potential of *Mi-glp-1* as a candidate gene for the control of nematode infection.

Materials and methods

Genome-wide identification of the glp-1 *gene in* M. incognita

The *M. incognita* genome database was downloaded from http://www6.inra.fr/Meloidogy ne_incognita (Abad *et al.* 2008), and local nucleotide and protein *M. incognita* databases were created through a BioEdit local BLAST search for the identification of the *glp-1* gene in *M. incognita*. A tblastn search was conducted against these databases using the *C. elegans glp-1* (CAA79620.1) gene as the query and default parameters (expected threshold value 10; maximum number of aligned sequences displayed 100). All the sequences that met the requirements were subjected to gene predictions through GeneMark and FGENSH analyses, and the genes that did not contain the known conserved domains and motifs detailed in the Pfam database (http://pfam.janelia.org/) were removed (Finn *et al.* 2016). MiV1ctg1087 (Minc16055), the single contig obtained with the maximum possible score, was selected as the probable *Mi-glp-1* for further experimentation. In addition to Minc16055 in *M. incognita*, we identified *glp-1* orthologues in four other *Meloidogyne* species (spp.), namely *M. hapla* (Mh10g200 708_Contig1018), *M. floridensis* (contig nMf_1_1_scaf00321), *M. javanica* (MJ01378, MJ05005) and *M. chitwoodi* (MC01544, MC00257), and in *G. pallida* (GPLIN_000999900.1), a cyst nematode, using their genome, transcriptome and/or expressed sequence tag (EST) databases. The derived sequences were downloaded from their genome assembly or transcriptomic databases or EST clusters available at either NCBI, NEMBASE4 (www.nematode.org) and/or nematode.net V4.0 (Opperman *et al.* 2008; Elsworth *et al.* 2011; Cotton *et al.* 2014; Lunt *et al.* 2014; Martin *et al.* 2015).

Phylogenetic and gene structure analyses

The identified GLP-1 amino acid sequences in PPNs, including *M. incognita*, *M. hapla*, *M. floridensis*, *M. javanica*, *M. chitwoodi* and *G. pallida*, along with the reported GLP-1 sequences of free-living nematode species, e.g. *C. elegans*, *Caenorhabditis briggsae*, *Caenorhabditis japonica* and *Caenorhabditis remanei*, and the Notch-like protein of the marine worm *Priapulus caudatus* (retrieved from NCBI), were aligned in MUSCLE using the default parameters (Edgar, 2004). To analyse the clustering pattern, an unrooted phylogenetic tree was constructed based on the neighbour-joining (NJ) method using MEGA 7.0 software (http://www.megasoftware.net/), with Poisson correction, pairwise deletion and the bootstrap value set to 2000 replicates (Kumar *et al.* 2016).

The protein sequences were analysed with the Pfam (http://pfam.sanger.ac.uk/) and SMART (http://smart.emblheidelberg.de/) databases to confirm the presence of conserved motifs. SignalP3.0 and TMHMM server v. 2.0 were employed to predict the presence of signal peptide sequences and transmembrane domains (TMDs), respectively. Two other software programs (Kd and Protscale) based on different algorithms were used to determine the presence of TMDs (Kyte and Doolittle, 1982; Gasteiger *et al.* 2005).

The motif-based sequence analysis tool MEME 4.11.2 (http://meme.sdsc.edu/meme/meme.html) (Bailey *et al.* 2015) with the following parameters was used to identify conserved motifs in the *glp-1* gene of *M. incognita*: optimal width, 10–300 amino acids and maximum number of motifs 10. The gene architecture of this gene, which depicts the exon/intron arrangement, gene length and upstream/downstream region, was designed using the online Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/) (Hu *et al.* 2015) with the coding sequences and corresponding genomic sequences.

Maintenance of pure cultures of nematodes

Tomato (*Solanum lycopersicum*) seeds were sterilized by soaking 20 min in sterile distilled water, 5 min in 70% ethanol and 15 min in 5% NaOCl and 0.1% Tween 20 followed by washing four times in sterile distilled water and germinated on a mixture of cocopeat, vermiculite and sand (1:1:1) in an Indian Agricultural Research Institute (IARI) glass house. Two-week-old tomato seedlings were infected with a pure culture of *M. incognita* maintained in our laboratory (Kumar *et al.* 2017). Egg masses of *M. incognita* were hand-picked from the roots of the infested plants and then maintained until hatching at 28 °C in Petri plates containing 10–15 mL of sterile water to collect second-stage juveniles (J2s) of *M. incognita*. These J2s were then used for subsequent inoculation assays using transgenic *Arabidopsis* lines.

Quantitative real-time PCR analyses

To evaluate the expression at different developmental stages, cDNA from the samples of *M. incognita* egg masses, infective J2s and mature females as well as roots of infected plants harvested at different time points after inoculation was subjected to quantitative real-time PCR (qRT-PCR). J2s were hatched from freshly picked egg masses, mature females were excised from infected roots, and other stages, namely third-stage juveniles (J3s) and fourth-stage juveniles (J4s), were analysed from nematodes within the roots of infected plants harvested at 10 and 21 days post-inoculation (dpi), respectively. The second molt, which gives rise to J3s, occurs 10 dpi, and the third molt, which yields J4s, occurs approximately 16 dpi (Moens *et al.* 2009; Martinuz *et al.* 2013). Total RNA was isolated using a Pure

Link RNA Mini Kit (Ambion, Carlsbad, CA, USA) according to the manufacturer's instructions and then subjected to DNase treatment using the Qiagen DNase enzyme. cDNA was synthesized using the SuperScript® III First-Strand Synthesis System (Invitrogen, USA). SYBR-based chemistry was adopted for the qRT-PCR, which was performed in a StepOne Plus™ real-time PCR System. The primers for *M. incognita*-specific actin and 18S rRNA genes were used as references (Nguyễn *et al.* 2014; Ye *et al.* 2015) (Supplementary Table S1). Three biological and three technical replicates were included for each sample. The data were analysed using the 2[-Delta DeltaC(T)] method, and real-time data are reported as the means ± standard error (S.E.) of three biological replicates per sample (Livak and Schmittgen, 2001). The data obtained were statistically analysed by analysis of variance (ANOVA), and the significance of the differences between sample means was then determined through Student's *t*-tests ($P < 0.05$).

A qRT-PCR analysis was also performed to determine the silencing efficacy of *glp-1* at the transcript level in *M. incognita*. Transgenic *Arabidopsis* RNAi lines were infected with *M. incognita* J2s, and the infected roots were harvested at 45 dpi. The mature females were isolated from these infected transgenic RNAi lines and compared with females from control (wild-type) *Arabidopsis* plants using the above-described protocol for qRT-PCR analysis.

In vivo *RNAi silencing*

A 419 bp fragment of the *Mi-glp-1* gene was amplified using gene-specific primers (Supplementary Table S1) in the sense and antisense directions for double-stranded RNA (dsRNA) construction. The primers were designed based on the region flanking the conserved Lin-12/Notch repeat (LNR) domain found in three tandem copies of Notch-related proteins encoded by the *glp-1* gene. The RNAi binary vector pBC6 was used for the design of dsRNA constructs (Yadav *et al.* 2006), and the positive dsRNA constructs were confirmed by double digestion of the sense strand with the *Bam*HI and *Xho*I restriction enzymes and of the antisense strand with *Kpn*I and *Sac*I. The integrity of the products was further confirmed by Sanger sequencing. A positive dsRNA construct and an empty vector construct were transferred into *Agrobacterium tumefaciens* GV3101. *Arabidopsis thaliana* (Col0) plants were then transformed with the designed RNAi constructs driven by the CaMV35S promoter using the floral dip method (Clough and Bent, 1998). T_1 seeds were selected on antibiotic selection medium containing kanamycin (50 μg mL^{-1}). Kanamycin-resistant plants were transferred to a greenhouse maintained at 22 °C with a 16 h light/ 8 h dark photoperiod, and the presence of the nematode gene in independent transgenic RNAi lines was confirmed by PCR.

Meloidogyne incognita *infection bioassay for evaluating silencing efficacy*

All the analyses of transgenic plants were performed with the T_3 generation, and 10–15 biological replicates of each line were used. Seeds of T_3 transgenic lines expressing *glp-1* dsRNA or empty vector and of control (wild-type) *Arabidopsis* plants were surface-sterilized with 70% alcohol for 2 min and 0.1% mercuric chloride +0.1% sodium dodecyl sulphate for 7 min and then washed in sterile distilled water. The seeds were then maintained on Murashige and Skoog media at 4 °C for vernalization prior to germination, and after germination, the plates were maintained at ±22 °C under a 16 h light/8 h dark photoperiod. Twelve days after germination, the control seedlings and the transgenic seedlings expressing *glp-1* dsRNA or empty vector were uprooted from the medium, transferred to a suitable mixture of vermiculite, cocopeat and sand (1:1:1), with one plant per slot (3 inches) in a 24-slot tray, and maintained in a growth chamber with a 16 h light/8 h dark photoperiod. After 1 week, each plant was inoculated with 1000 freshly hatched J2s to evaluate the silencing efficacy *in vivo*. Nematode infection bioassays were performed using mature females isolated from the root samples collected 45 dpi. The isolated females were used for qRT-PCR and morphometric studies. The number of galls per plant, the number of females and the egg mass per gram of root fresh weight were determined and used as measures for determining the degree of nematode infection. Specifically, the nematode multiplication factor (MF) of *M. incognita* was calculated as follows: (number of egg masses × number of eggs per egg mass) ÷ nematode inoculum level. The statistical significance of the differences between the means of replicates of transgenic lines and wild-type control plants was determined by ANOVA and Student's *t*-tests ($P < 0.05$ or $P < 0.01$).

The nematodes within all the samples of infected roots were stained with acid fuchsin (Bybd *et al.* 1983) and photographed using a Nikon microscope. The size of the stained females isolated from the infected plant samples was measured using the 10× objective of a Nikon microscope equipped with the NIS-Elements D measurement software (Eisenback and Hunt, 2009; Kaur and Attri, 2013). The stylet and pharyngeal structures of the J2 progeny of females isolated from transgenic RNAi and control plants were also observed with an Zeiss AxioImager.M2m microscope coupled to an AxioCam with a 40× objective, and the data were subjected to statistical analyses as described above.

Detection of glp-1 *by Northern hybridization*

The small RNA fraction was isolated from the RNAi line, empty vector line and control plants using the LiCl method (Verwoerd *et al.* 1989). Twenty micrograms of the small RNA fractions were resolved on 15% polyacrylamide/1X MOPS urea gels and blotted onto a Hybond-N$^+$ membrane (Amersham). The Block-It™ RNAi designer (https://rnaidesigner.thermofisher.com/rnaiexpress), siRNA wizard (http://www.invivogen.com/sirnawizard/) and siDesign centre (http://dharmacon.gelifesciences.com/design-center/) tools were used to identify the most suitable regions for siRNA target design, and primers for probe preparation were designed based on the region that was deemed most suitable for siRNA targeting (Supplementary Table S1). To generate specific DNA probes (183 bp in size), ^{32}P-α-dCTP labelling was performed with a Megaprime DNA Labeling kit (GE Healthcare, Amersham) using gene-specific antisense fragments. Hybridization was performed using PerfectHyb™ Plus according to the manufacturer's instructions (Sigma). The signals were detected by autoradiography.

Results

glp-1 *orthologues among nematode species*

The tblastn analysis performed against the *M. incognita* genome using the *C. elegans glp-1* protein as the query (retrieved from WormBase) and the subsequent Pfam analysis yielded various hits. Among these hits, MiV1ctg1087, the only contig with a high bit score, showed 100% similarity with Minc16055, which was identified through an OrthoMCL analysis of *M. incognita* as a *Ce-glp-1* orthologue by Abad *et al.* (2008). Thus, functional annotation studies of MiV1ctg1087 involving a Pfam analysis and computer simulation predictions were performed to determine its gene and protein structures. Mi-GLP-1 (Minc16055 or MiV1ctg1087) is a member of the LIN-12/Notch family of receptors and is composed of a series of motifs that are conserved among all Notch receptors. The *Ce-glp-1* and *Mi-glp-1* genes were nearly identical in size, 7.458 and 7.449 kb, respectively.

However, a computational analysis showed differences in the numbers of introns and exons in these orthologous genes: *Ce-glp-1* has only nine exons and eight introns (Rudel and Kimble, 2001), whereas *Mi-glp-1* has 18 exons and 17 introns. This finding indicates that the exon/intron structures of these orthologous genes are not well conserved among nematodes. At the mRNA level, *Mi-glp-1* is 3.252 kb in length, whereas *Ce-glp-1* comprises 4.326 kb (Fig. 1). The *Mi-glp-1* gene encodes a 1083-amino-acid-long truncated protein, as determined through the Pfam analysis, whereas *Ce-glp-1* is composed of 1295 amino acids. The gene encoding GLP-4, which is considered a member of the Notch GLP protein family, was recently identified in *C. elegans* (Rastogi *et al.* 2015), and to date, this new member has only been identified in *C. elegans* and *C. briggsae*. The genome-wide search performed in this study identified only a single *glp-1* contig, which is similar to the findings obtained for *C. japonica* and *C. remanei*, each of which has a single *glp*.

Because *glp-1* is a vital gene involved in pharyngeal development during embryogenesis, we also performed a comprehensive species-wide tblastn search for this gene in the genomes and ESTs of various other *Meloidogyne* species available in NCBI and/or Nematode.net. A *glp-1* homologue was identified in various *Meloidogyne* spp., namely, *M. hapla*, *M. floridensis*, *M. javanica* and *M. chitwoodi* and in *G. pallida*, another PPN (details in Supplementary Table S2). We also considered free-living nematodes, including *C. elegans*, *C. briggsae*, *C. japonica* and *C. remanei* and a marine worm (*P. caudatus*) Notch receptor gene in our phylogenetic study (Supplementary Fig. S1). The findings revealed that among the homologues identified in *Meloidogyne* spp., the *glp-1* homologue in *M. hapla* contains all the domains that are present in the *Ce-glp-1* gene, whereas *Mi-glp-1* does not have an epidermal growth factor (EGF) domain. A motif search including all the species analysed in this study (Supplementary Fig. S2) revealed a conserved ankyrin repeat (ANK).

Protein sequence analysis and phylogenetic analysis of Notch receptor orthologues in selected nematode species

An amino acid sequence analysis of the *M. incognita* GLP-1 protein revealed a single TMD, an extracellular domain containing the LNRs, the ANK, which is an intracellular domain, and a Pro-Glu-Ser-Thr (PEST) domain. The molecular and biological functions of the LNR and ANK in *C. elegans* have been described (Austin and Kimble, 1989; Roehl *et al.* 1996). Thus, the presence of these motifs in Mi-GLP-1 indicates its probable role in embryogenesis. Specific primers for the LNR were designed, and the region was successfully amplified, confirming the presence of the LNR in *M. incognita* (data not shown). Interestingly, the presence of a conserved cysteine ('C') as the first residue of the LNR was identified in the *glp-1* proteins of all nematode species evaluated in this study. Mi-GLP-1 contains one ANK with three copies, three LNR domains at the 5′end, a TMD and an RAM domain but does not have an EGF domain (Fig. 2). GLP-1 induces downstream transcriptional regulators and interacts with them to mediate signalling. Our sequence analysis revealed an 'RTGGGAA' DNA-binding site and an RAM domain in *Mi-glp-1*. These sites have been proposed as binding sites for the LAG-1 (Lin-12 and Glp-1) protein, which is required during embryogenesis to regulate pharyngeal development (Christensen *et al.* 1996).

All identified GLP-1 protein sequences belonging to the different nematode species were aligned using MUSCLE (Fig. 2). This alignment revealed the presence of several conserved motifs in these parasitic and free-living nematodes, and the LNR and ANK motifs showed maximal amino acid conservation. A phylogenetic analysis using the amino acid sequences was performed to understand the patterns of relatedness among the *glp-1* genes of the nematodes analysed in this study. The phylogenetic analysis of GLP-1 in *Meloidogyne* and other species, which was conducted using the NJ method, revealed a separate cluster comprised of Mi-GLP-1, Gp-GLP-1, Mh-GLP-1 and Mf-Notch-like protein. As observed in the phylogenetic tree, Mi-GLP-1 appeared to be most closely related to the Mf-Notch-like protein, which lacks an ANK (as demonstrated in the Pfam analysis). However, a higher degree of similarity in the LNR, RAM domain and ANK was found between Mi-GLP-1 and Gp-GLP-1, conclusively demonstrating the close relationship between these two genes (Fig. 3).

Transcript abundance of Notch-receptor genes in the developmental stages of M. incognita

To study the expression pattern of *glp-1* in *M. incognita* development, five different stages were selected: egg masses, J2s, mature females, J3s and J4s (the last two were obtained from the samples of infected plant roots harvested 10 and 21 dpi). Interestingly, *glp-1* showed higher expression during early development in *M. incognita*, i.e. in egg masses (Fig. 4). This finding is consistent with those of previous studies that investigated *glp-1* abundance at early developmental stages in *C. elegans* embryos (Austin and Kimble, 1989; Crittenden *et al.* 1997). A qRT-PCR analysis also revealed that the *glp-1* transcript levels were increased in mature females, indicating a possible role for this protein at this developmental stage in *M. incognita*.

Determination of the efficacy of glp-1 *gene silencing*

Nematode infection assay on Arabidopsis *RNAi lines*

A dsRNA expression construct of the *glp-1* gene was designed in the pBC vector (Yadav *et al.* 2006). The constructs were mobilized into *Arabidopsis* plants for the host-mediated delivery of RNAi of the *glp-1* gene. To evaluate the silencing efficacy of *Mi-glp-1*, T_3 seedlings of two independent transgenic lines were infected by

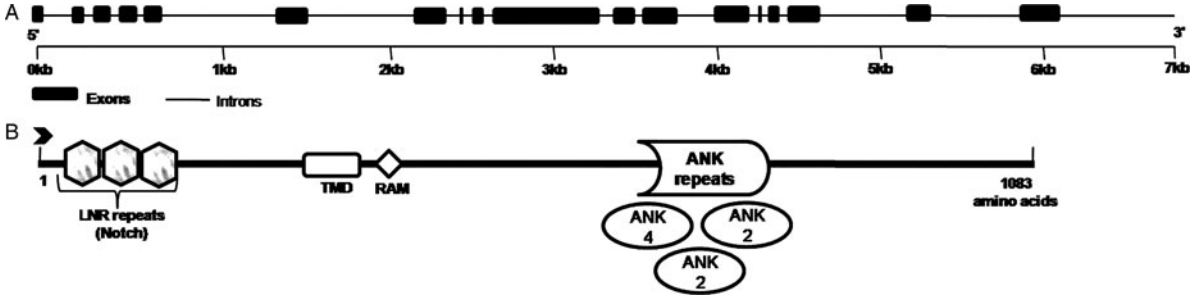

Fig. 1. Diagrammatic representation of the predicted structures of the *glp-1* gene and its encoded protein. (A) Exon/intron structure of *Meloidogyne incognita glp-1*. The boxes represent exons, and the black lines represent introns. (B) Architecture of the *M. incognita* GLP-1 protein. The functional domains are indicated as follows: LNR, TMD, RAM domain and ANK.

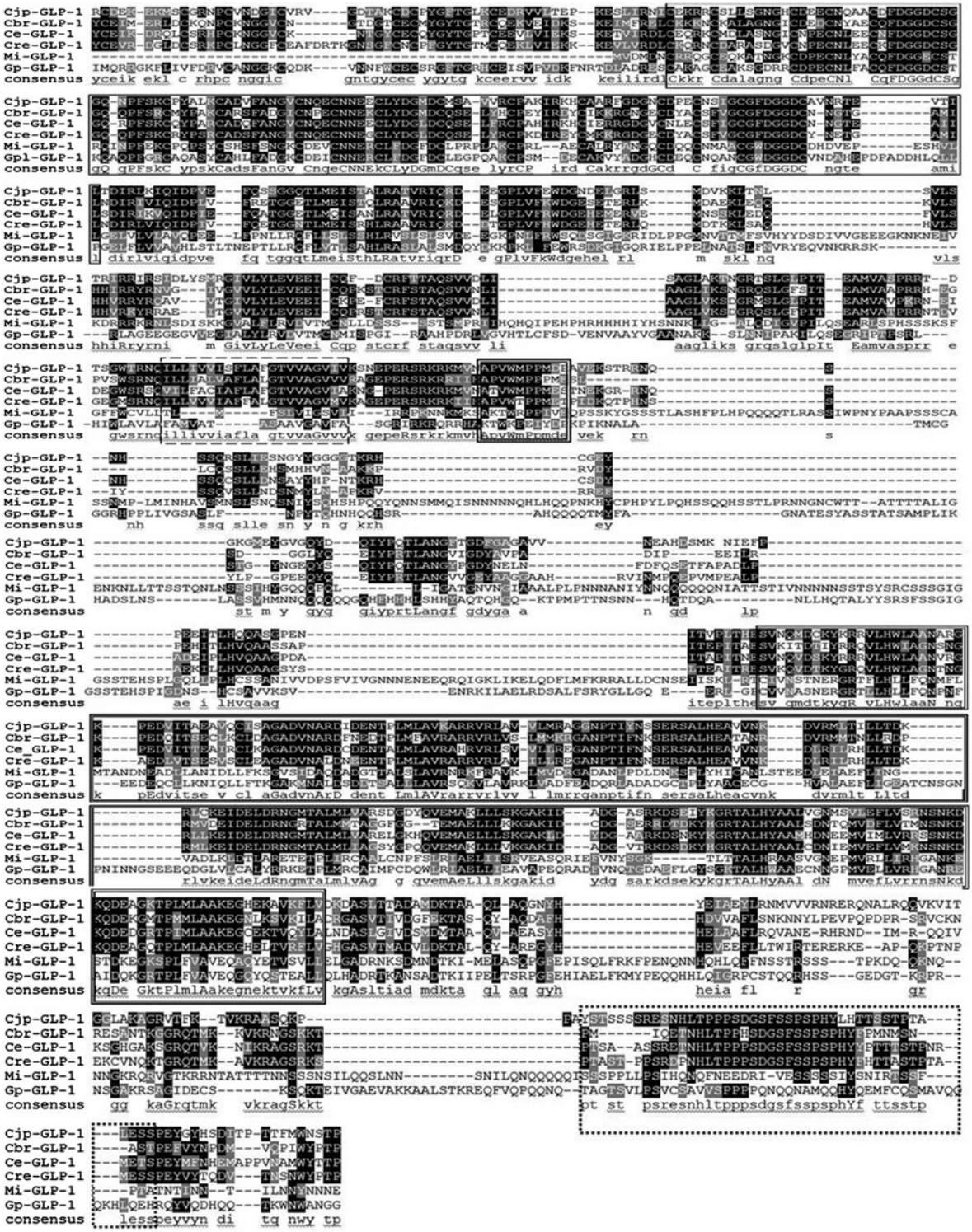

Fig. 2. Multiple sequence alignment of GLP-1 in free-living and plant-parasitic nematodes. Black shading indicates conserved amino acids. Rectangular boxes represent different motifs conserved in GLP-1 in these species (▭ LNR, ⌜⌝ TMD, ▭ RAM domain, ▭ ANK and ⋯ PEST domain). Ce, *Caenorhabditis elegans*; Cbr, *Caenorhabditis briggsae*; Cjp, *Caenorhabditis japonica*; Cre, *Caenorhabditis remanei*; Mi, *Meloidogyne incognita*; Gp, *Globodera pallida*.

parasitic juveniles. Two *glp-1* RNAi lines (RNAi lines 1 and 2) were evaluated by staining the nematodes inside their roots and determining the infection level (Fig. 5A). Both *glp-1* RNAi lines exhibited reductions in the number of galls (47.8% and 51.3%), females (51.5% and 47.79%) and egg masses (49.3% and 59.4%) compared with the control plants and transgenic plants harbouring an empty vector (Fig. 5A–C). Altogether, both transgenic RNAi lines presented significant reductions in the number of galls, females and egg masses, indicating that the silencing of this gene has a deleterious effect on the growth and development of nematodes. The adult female nematodes were isolated, and their sizes were analysed to determine any phenotypic effects, if any, on *M. incognita*. The length and width of randomly selected females were measured using a scale on a Nikon microscope (Supplementary Fig. S3). The average length and width of the females dissected from control plants were 396.27 and 248.18 μm, respectively, whereas the average length and width of the females isolated from the RNAi lines were significantly

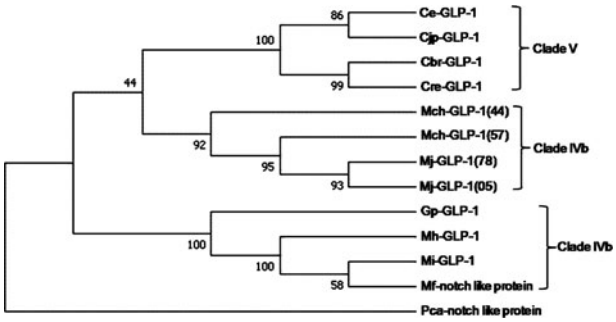

Fig. 3. Phylogenetic analysis of Notch-like receptors. Neighbour-joining phylogenetic tree of Notch-like receptors from *Caenorhabditis elegans* (Ce), *Caenorhabditis japonica* (Cjp), *Caenorhabditis briggsae* (Cbr), *Caenorhabditis remanei* (Cre), *Meloidogyne chitwoodi* (Mch), *Meloidogyne javanica* (Mj), *Globodera pallida* (Gp), *Meloidogyne incognita* (Mi), *Meloidogyne floridensis* (Mf), *Meloidogyne hapla* (Mhp) and *Priapulus caudatus* (Pca, as an outgroup). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) is shown next to the branches. The phylogenetic analysis was conducted using MEGA7.

decreased, with values of 231.9 and 140.36 μm, respectively (Supplementary Table S3). Therefore, the nematodes obtained from the RNAi lines showed reductions of 41.4% and 43.4% in their length and width, respectively.

Molecular analyses of RNAi lines through qRT-PCR and Northern blotting

The transcript abundance of the target gene in the females isolated from both RNAi lines showed an approximately 60% reduction compared with that in the females isolated from control plants (Fig. 6A). This decrease in the transcript levels of *glp-1* in females obtained from the RNAi lines demonstrated the effect of its silencing through host-delivered dsRNA. A Northern analysis was also performed, and the results confirmed the presence of *glp-1* dsRNA in RNAi line 1 (Fig. 6B). One of the key components of host-mediated RNAi was the 419 bp dsRNA of *glp-1*, which was detected in the transgenic RNAi line.

Effect of silencing on M. incognita *fecundity and next-generation J2s*

The females that fed on RNAi lines produced fewer numbers of egg masses compared with those that fed on wild-type plants. To further investigate the deleterious effects, if any, of the silencing of the *glp-1* gene, the number of eggs present in these egg masses was evaluated, and these eggs were maintained for subsequent hatching. A 26% reduction in the number of eggs was noted in the RNAi lines, and the corresponding MF value was estimated to determine the nematode population. The estimated MF value for the infection of *M. incognita* in transgenic RNAi lines was calculated to equal 11.57, whereas that for wild-type plants was 31.1 (Supplementary Table S4A). Although J2s with well-developed pharynxes and stylet structures were observed in the samples isolated from the RNAi lines, several J2s showed aberrations around the metacorpus region as well as a shorter distance from the stylet to the metacorpus region of the pharyngeal structures (Fig. 7 and Supplementary Table S4B). Thus, it can be deduced that this effect on J2s is due to the nature in which RNAi is inherited from one generation to the next. Studies in *C. elegans* have shown inherited effects of silencing even in the absence of the original trigger (Bird *et al.* 2009), and the persistence of silencing effects has also been observed after suppression of the *Mi-1* gene of tomato (Gleason *et al.* 2008).

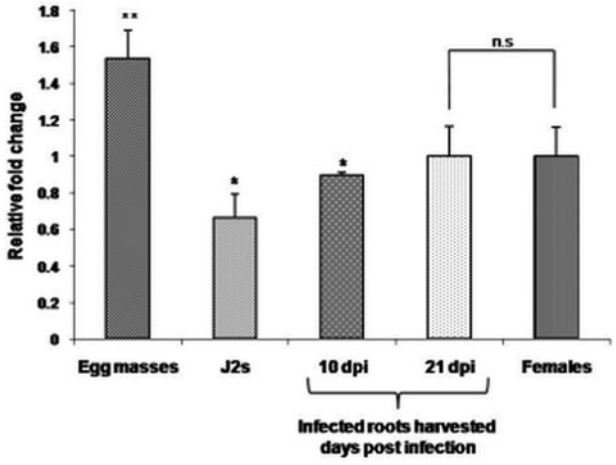

Fig. 4. Expression analysis of *Mi-glp-1* at different developmental stages in *Meloidogyne incognita*. The histogram indicates the relative fold change ($2^{-\Delta\Delta CT}$ value) normalized based on the actin gene as an endogenous gene and the female ΔCt value as a calibrator. The asterisks * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively, and 'n.s.' indicates no significance.

Discussion

Although several genes related to neuropeptides and secretory pathways involved in the parasitism of PPNs have been reported (Rehman *et al.* 2016), detailed information regarding the structural and functional roles of genes with functions in organ development in PPNs, including *M. incognita*, is lacking. Very few studies have identified genes that play a crucial role in organ development in a PPN. *Mi-glp-1*, a Notch family member, was identified in this study as an orthologue of *Ce-glp-1*. In our study, a genome-wide computational approach revealed MiV1ctg1087, a single contig, as *Mi-glp-1* in *M. incognita*. Interestingly, Minc16055 was also annotated as a Notch receptor protein in an identification of microsatellite loci in *M. incognita* (Castagnone-Sereno *et al.* 2010). A phylogenetic tree showed 12 orthologues originating from three clusters, all of which were branched, as well as one separate unbranched cluster (*P. caudatus*) as an outgroup. Notably, the branching of these three clusters originated from a common node. Thus, it could be hypothesized that the Mi-GLP-1 product has putative functions closely related to those of the Ce-GLP-1 protein. Nevertheless, further studies on the function of each conserved domain in Mi-GLP-1 will support its predicted role.

Mi-glp-1 is composed of 7.449 kb, which is similar to the size of *Ce-glp-1*. However, the corresponding mRNA sizes showed a difference of approximately 1.07 kb, which resulted from differences in the overall gene organization and structure. A similar size difference at the mRNA level was also observed with *G. pallida glp-1* (Supplementary Table S2). The observed difference in the exon/intron structure could be due to the scaffold form of the *M. incognita* genome, which resulted in the formation of a truncated protein. A genome-wide search to identify more members of *glp-1* in the *M. incognita* genome was attempted using *Ce-glp-1* as the query sequence, but only a single member was identified. To date, only one other member of the GLP protein family, named GLP-4, has been characterized in *C. elegans* and *C. briggsae* (Mango, 2007; Rastogi *et al.* 2015). At the protein level, Mi-GLP-1 has several conserved motifs that are also present in the corresponding protein in *C. elegans*. A Pfam analysis showed the presence of a TMD, LNR, an RAM domain and an ANK. The LNR mediates ligand-binding functions, and the ANK and RAM domain are needed for signalling responses (Kelley *et al.* 1987; Rebay *et al.* 1991; Heitzler and Simpson, 1993; Artavanis-Tsakonas *et al.* 1999). Based on previous experimental evidence, the ANK is thought to be critical for the function of Notch receptors (Roehl and Kimble, 1993; Diederich *et al.* 1994). In addition, three different software programs predicted

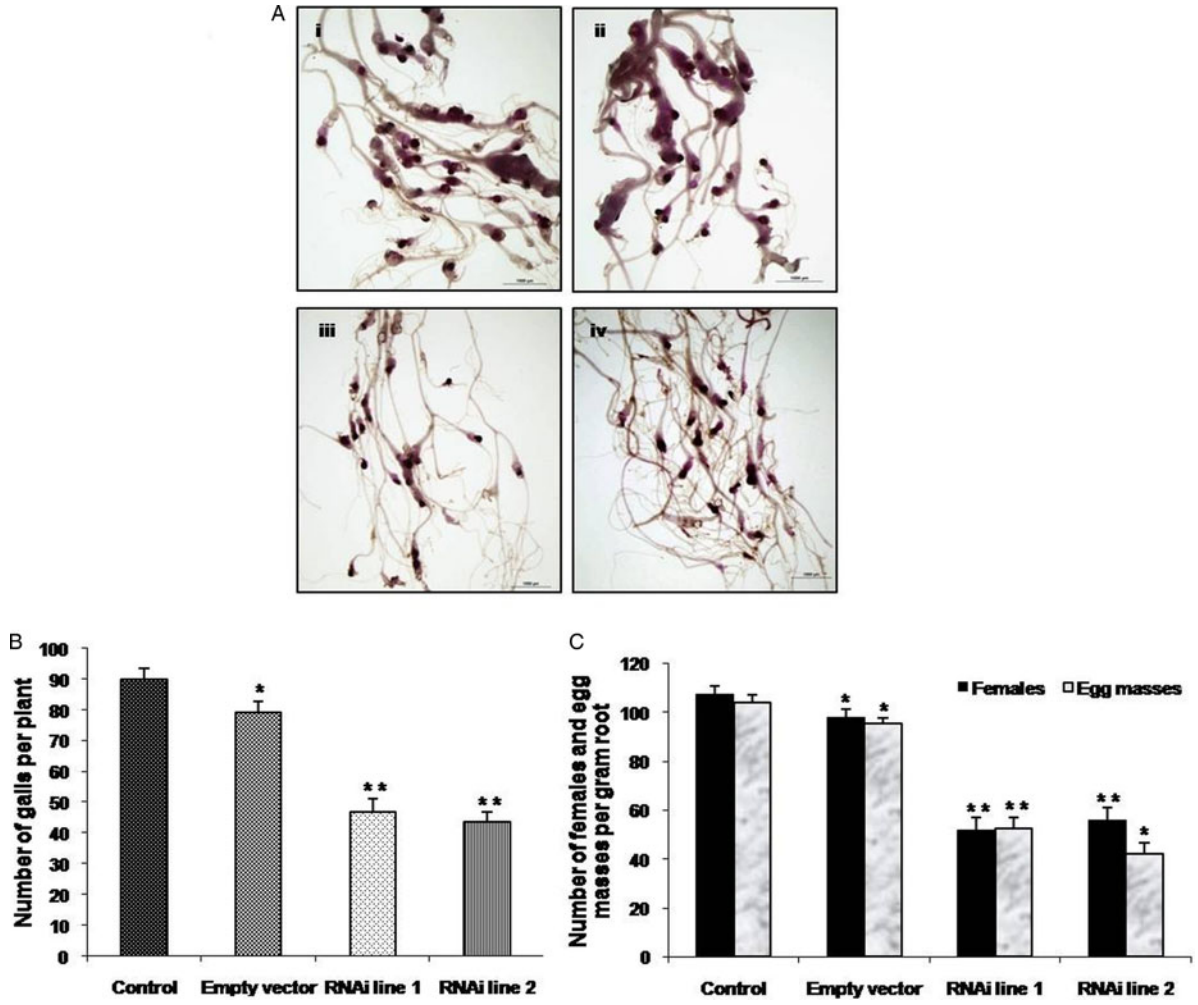

Fig. 5. Nematode infection assay. (A) Representative photograph depicting *Meloidogyne incognita* in infected roots stained with acid fuchsin: (i) wild-type plants, (ii) empty vector line, (iii) RNAi line 1 and (iv) RNAi line 2. (B) and (C) Histogram depicting the number of galls, females and egg masses in the RNAi lines, empty vector transgenic lines and control (wild-type) plants. The asterisks * and ** indicate a significant difference at $P < 0.05$ and $P < 0.01$, respectively.

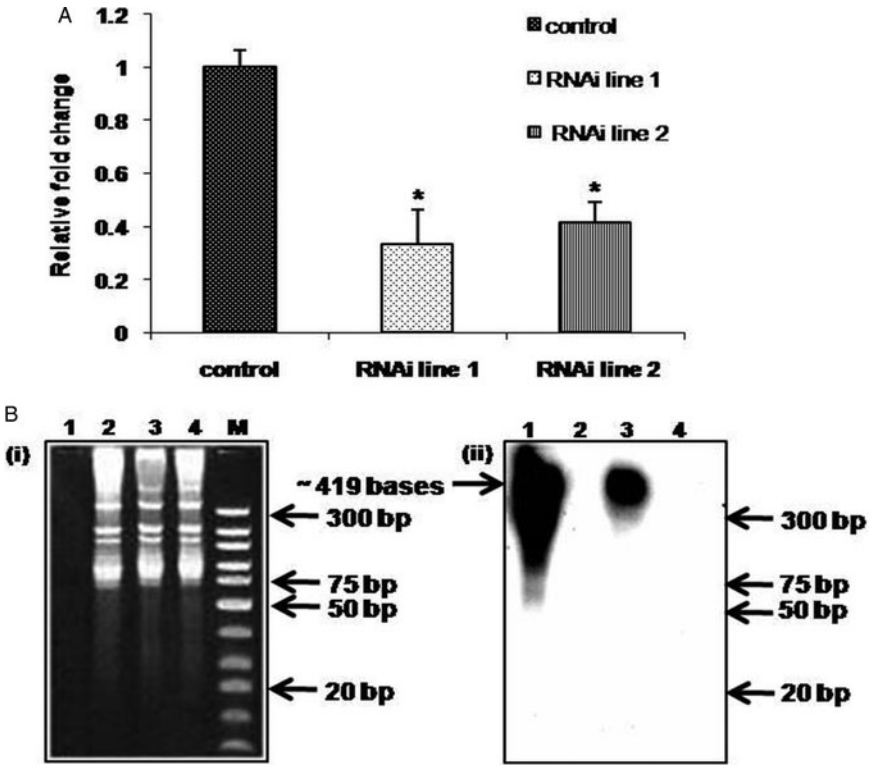

Fig. 6. Molecular analyses of *Meloidogyne incognita* isolated from transgenic *Arabidopsis* plants. (A) RT-qPCR-based *glp-1* expression analysis of *M. incognita* females isolated from infected roots of RNAi lines 1 and 2 and females isolated from control plants. (B) Ethidium bromide-stained PAGE gel (before transfer) showing the low-molecular-weight RNA fraction (i) and the corresponding Northern blot for detecting the presence of *glp-1* dsRNA (ii). The 183 bp probe corresponding to *glp-1* was designed from the LNR domain. In (i) and (ii), lane 1 – gene-specific PCR product as a positive control, lane 2 – empty vector line, lane 3 – RNAi line 1, lane 4 – wild type as a control and lane M – molecular marker.

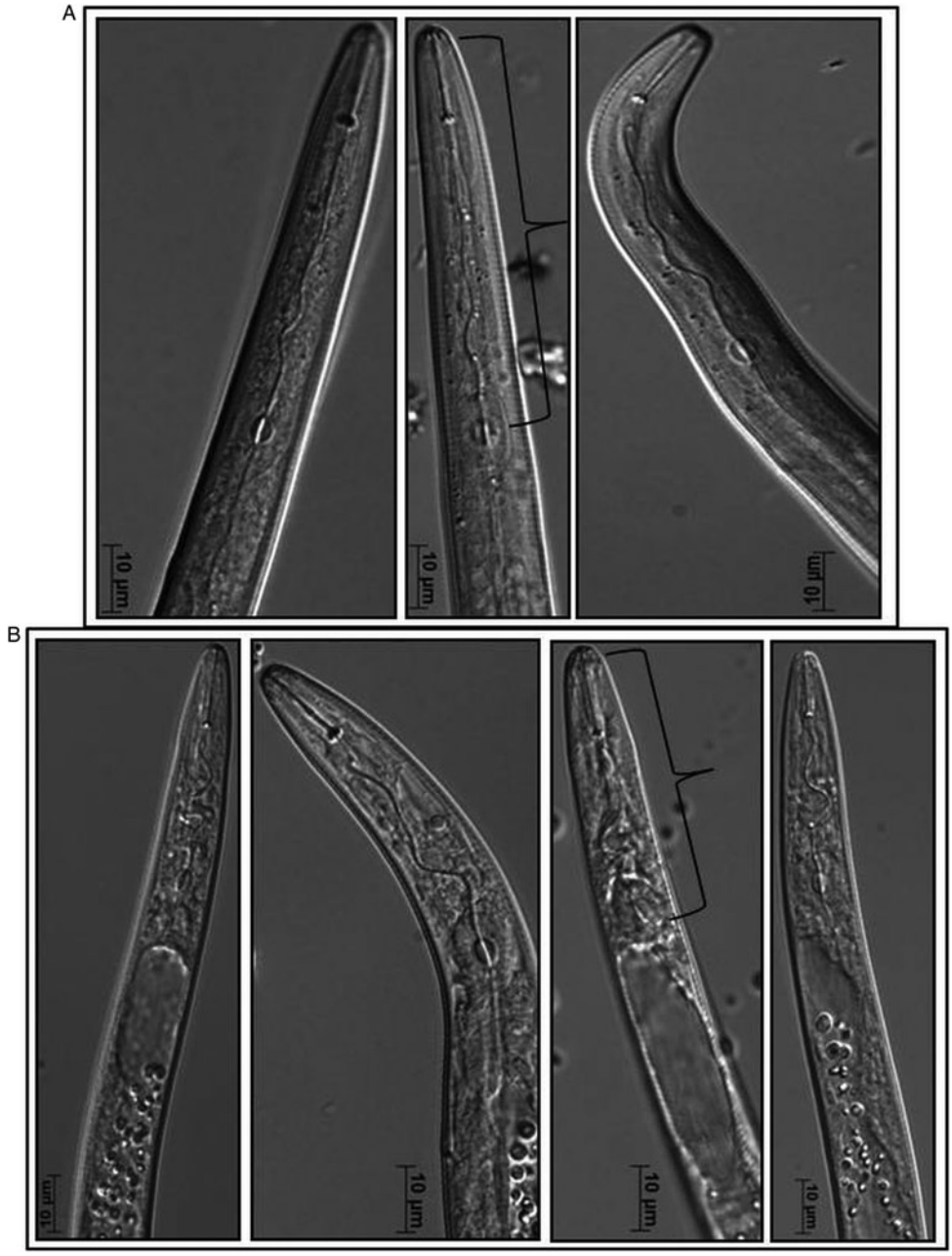

Fig. 7. Phenotypes of stylets and pharynxes of J2s descended from *Meloidogyne incognita* females isolated from infected plants: (A) wild-type (control) plants and (B) RNAi lines. Curly brackets indicate the length from the mouth to the metacorpus bulb in J2s. Please note shortening of length in few J2s from RNAi lines as compared to control plants.

the presence of a TMD, further indicating that Mi-GLP-1 most likely functions as a receptor protein (Supplementary Fig. S5A and B), although a clear mechanism of interaction has not been determined. A noticeable difference between Ce-GLP-1 and Mi-GLP-1 is the absence of an EGF domain at the 5′ end of the Mi-GLP-1 amino acid sequence, suggesting certain structural and functional differences between the *glp-1* gene of *C. elegans* and that of *M. incognita.* Hence, the putative GLP-1 protein identified in this study has properties and structures similar to those of a receptor protein with varied domains, each with a specific function. In addition to these conserved domains, DNA-binding sites for ligands such as LAG-1 proteins were also found to be present in Mi-GLP-1. The presence of this type of motif at more than one site on the Mi-GLP-1 Notch receptor suggests that a vital function of GLP-1 is mediated through LAG-1 ligand binding to induce downstream reactions in the signalling pathway. Based on previous studies on Ce-LAG-1 DNA-binding sites, the potential function of this protein in LIN-12/GLP-1 signalling pathways has been proposed (Christensen *et al.* 1996). Experiments including yeast, two-hybrid assays and *in vitro* protein co-precipitation have demonstrated direct interactions between LAG-1 and the GLP-1 receptor *via* these domains in nematodes (Roehl *et al.* 1996). Further studies are needed to determine the role of these domains in signalling processes. Thus, based on *in silico* analyses and the available literature, it can be concluded that the gene identified in this study is a Notch receptor protein present in *M. incognita.*

Our study revealed that *Mi-glp-1* is differentially expressed during different developmental stages of *M. incognita*. The increased transcript level observed in egg masses suggests an important role during embryogenesis. This observation was consistent with previous studies that suggested a possible role for this protein during embryogenesis in *C. elegans* (Priess *et al.* 1987; Austin and Kimble, 1989). However, a slight increase in the transcript level was detected in mature females, suggesting another

possible role for this protein during later stages of RKN development. Interestingly, similar reports regarding a possible role for *glp-1* in the adult stage of *C. elegans* have not been reported. Future studies examining the localization of this gene product will confirm the role of GLP-1 during female development in *M. incognita*.

Host-delivered RNAi-mediated root-knot nematode resistance is an established technology, but the identification of better candidate genes remains necessary. In addition, the identification of genes involved in different developmental pathways will enable the targeting of several vital genes. Parasitic and secretory genes have traditionally been the genes of choice in silencing studies. However, there are very few candidate genes from the perspective of nematode development. A few developmental genes, such as Rpn7 (essential for the integrity of the 26S proteasome), *nhr-48*, FAR-1, splicing factor and integrase genes, have been targeted using RNAi in efforts aiming to control infection with the root-knot nematode *M. incognita* (Yadav *et al.* 2006; Niu *et al.* 2012; Iberkleid *et al.* 2015; Lu *et al.* 2016). The *glp-1* gene, which was identified in this study, appears to be involved in the development, and a dsRNA/siRNA strategy was used for the host-mediated RNAi-based suppression of *Mi-glp-1* in *M. incognita*. Transgenic *Arabidopsis* lines expressing dsRNA against *Mi-glp-1* and infected with *M. incognita* did not show any phenotypic changes compared with wild-type plants. An approximately 47% reduction in the infection level was recorded in two independent *glp-1* RNAi lines, and decreases in the gall formation, female infestation and egg mass production were also observed in these RNAi lines. In addition, an overall decrease in the number of females feeding on RNAi lines was recorded compared with females isolated from control plants. These results unequivocally demonstrated that *glp-1* was suppressed in females that fed on the established RNAi lines. The nematode females isolated from control plants had an average length and width similar to the reported body length (ranging from 510 to 690 μm) and width (300 to 430 μm) of a healthy mature *M. incognita* female (Whitehead, 1968; García and Sánchez-Puerta, 2012). In contrast, the females isolated from the RNAi lines had average lengths and widths of 232 and 141 μm, respectively, indicating an evident decrease in the overall body size in the females that fed on the RNAi lines. However, whether this decrease was a direct result of the low expression of *glp-1* is unclear. Direct evidence supporting a role for *glp-1* in the growth of *M. incognita* at later stages in the lifecycle has not yet been reported. However, in this study, a 60% decrease in transcript abundance was noted in the adult nematode females that fed on the RNAi lines. Additionally, to confirm the reduction in expression due to dsRNA/siRNA effects, the dsRNA molecules in the control and transgenic plants (empty vector and RNAi lines) were detected by Northern hybridization. The results confirmed the presence of *glp-1*-specific dsRNA molecules in the transgenic lines, an important factor for successful host-mediated RNAi (Bass, 2000; Agrawal *et al.* 2003), but we were unable to identify *glp-1*-specific siRNAs despite numerous attempts. Several studies have reported a similar problem with other genes, and this difficulty is most likely due to the low sensitivity of the Northern blot analysis (Dinh *et al.* 2014). The ability of *M. incognita* to ingest large molecules, which has been well established, and the presence of four dicers in *Arabidopsis*, which likely processed the *glp-1*-specific dsRNAs (as shown in our Northern analysis) into siRNAs to trigger RNAi responses in *M. incognita* females feeding on the transgenic RNAi lines might also have played a role. However, future studies comparing the transcript and protein levels in progenies are required to confirm our observations.

To investigate the suppression effect of *glp-1* on the fecundity of *M. incognita* females, egg masses were hand-picked from all females in the infected RNAi lines, control plants and empty vector lines and subsequently incubated to determine their hatching capability, and the number of J2s hatched from each sample was counted. A decrease in the population of J2s obtained from the RNAi lines was noted, and the lower estimated MF value for *M. incognita* in the transgenic RNAi plants compared with control plants suggested a reduced infectivity of *M. incognita* on the RNAi lines compared with the wild-type plants. In embryos, *glp-1* RNA is maternally donated (i.e. by the mother) in *C. elegans* (Evans *et al.* 1994); therefore, dsRNA appears to affect even the progeny produced from the affected females. In our J2s, aberrations in the structures of the metacorporeal bulbs and their stylet discs were clearly apparent. A noticeable and significant decrease in the length of the region from the stylet to the metacorpus was also evident. Our study showed an adverse effect of the suppression of the *glp-1* gene on the pharyngeal and stylet structures and thereby provides insight into the function of the *glp-1* gene in pharyngeal development in *M. incognita*. Similarly, Geng *et al.* (2016) reported a degradation of intestinal tissues of *M. incognita* and identified Sep1, a novel serine protease, as a potent bio-agent from *Bacillus firmus* for controlling PPN populations. Thus, it can be inferred that the females obtained from transgenic plants exerted effects on the foregut region of their descendants. Our results strongly suggest the transmission of dsRNA-mediated deleterious effects to the next generation of root-knot nematodes. Because a difference in the phenotype of J2s was observed, a decrease in the GLP-1 protein level is possible; however, a more detailed study is required to fully evaluate this hypothesis.

The present work identified and characterized a gene encoding a Notch receptor protein in *M. incognita* involved in the development of the pharynx during embryogenesis and investigated the evolutionary conservation of its function between free-living nematodes and PPNs. Several common features in the Mi-GLP-1, Ce-GLP-1 and Gp-GLP-1 amino acid sequences were revealed. The *in planta* production of RNAi has been shown to effectively reduce the infectivity and size of *M. incognita* females. Deleterious effects were also observed in later generations (J2s) obtained from females isolated from infected transgenic plants. Taken together, our results demonstrate that the host-mediated gene silencing of the nematode *glp-1* is an efficient method for inhibiting plant infection. The presence of *glp-1* homologues in other PPNs, such as *M. hapla*, *M. floridensis*, *M. javanica*, *M. chitwoodi* and *G. pallida*, suggests the wide potential applicability of the silencing of this gene using host-mediated gene silencing approaches for the control of these parasitic nematodes. However, several important questions remain unanswered: (1) Does the *glp-1* gene directly determine the pharyngeal development in PPNs or does it interact with other genes to define the development of the anterior region of the pharynx, as shown in free-living nematodes? (2) Does this gene have a specific role in other stages, such as the adult female stage, as indicated from our expression analysis? Providing answers to these questions requires experimental assessment of the phenotypic and molecular function(s) of the *glp-1* gene. Although further investigations are essential to fully determine the role of *glp-1* in parasitic nematodes, the observations obtained in this study provide a reasonable indication that *glp-1* plays a significant role in pharyngeal development in PPNs. The role of GLP-1 as a receptor protein has been well characterized in *C. elegans*, but its involvement in pathogenic nematodes has not yet been reported. Establishing the involvement of *glp-1* in pharyngeal development could further aid the elucidation of various pathways in PPNs, and the identification of genes downstream of the GLP-1 receptor and of its ligands will provide further insights into the various processes and pathways in which *glp-1* is involved. These ligands and associated genes can then be targeted to obtain a more in-depth

understanding of the cross-talk between different pathways and could eventually be used as targets in RNAi-mediated silencing approaches for PPN management to improve overall crop productivity.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018000641

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Conflicts of interest. None.

Ethical standards. Not applicable

References

Abad P, *et al.* (2008) Genome sequence of the metazoan plant-parasitic nematode *Meloidogyne incognita*. *Nature Biotechnology* **26**, 909–915.

Agrawal N, *et al.* (2003) RNA interference: biology, mechanism, and applications. *Microbiology and Molecular Biology Reviews* **67**, 657–685.

Artavanis-Tsakonas S, Rand MD and Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770–776.

Austin J and Kimble J (1989) Transcript analysis of *glp*-1and *lin*-12, homologous genes required for cell interactions during development of *C. elegans*. *Cell* **58**, 565–571.

Bailey TL, *et al.* (2015) The MEME Suite. *Nucleic Acids Research* **43**, W39–W49.

Banerjee S, *et al.* (2017) RNA interference: a novel source of resistance to combat plant parasitic nematodes. *Frontiers in Plant Science* **8**, 834.

Bass BL (2000) Double-stranded RNA minireview as a template for gene silencing. *Cell* **101**, 235–238.

Bird DM, *et al.* (2009) The genomes of root-knot nematodes. *Annual Review of Phytopathology* **47**, 333–351.

Bybd Jr. DW, Kirkpatrick T and Barker KR (1983) An improved technique for clearing and staining plant tissues for detection of nematodes. *Journal of Nematology* **15**, 142–143.

Calderón-Urrea A, *et al.* (2016) Early development of the root-knot nematode *Meloidogyne incognita*. *BMC Developmental Biology* **16**, 10.

Castagnone-Sereno P, *et al.* (2010) Genome-wide survey and analysis of microsatellites in nematodes, with a focus on the plant-parasitic species *Meloidogyne incognita*. *BMC Genomics* **11**, 598.

Christensen S, *et al.* (1996) lag-1, a gene required for lin-12 and glp-1 signaling in *Caenorhabditis elegans*, is homologous to human CBF1 and Drosophila Su(H). *Development* **122**, 1373–1383.

Clough SJ and Bent AF (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16**, 735–743.

Cotton JA, *et al.* (2014) The genome and life-stage specific transcriptomes of *Globodera pallida* elucidate key aspects of plant parasitism by a cyst nematode. *Genome Biology* **15**, R43.

Crittenden SL, *et al.* (1997) Genes required for GLP-1 asymmetry in the early *Caenorhabditis elegans* embryo. *Developmental Biology* **181**, 36–46.

Diederich RJ, *et al.* (1994) Cytosolic interaction between deltex and Notch ankyrin repeats implicates deltex in the Notch signaling pathway. *Development* **120**, 473–481.

Dinh PTY, Brown CR and Elling AA (2014) RNA interference of effector gene *Mc*16*D*10*L* confers resistance against *Meloidogyne chitwoodi* in *Arabidopsis* and Potato. *Phytopathology* **104**, 1098–1106.

Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* **32**, 1792–1797.

Eisenback JD and Hunt DJ (2009) General morphology. In Perry RN, Moens M and Starr JL (eds). *Root Knot Nematode*. Oxfordshire, UK: CAB International 2, pp. 18–50.

Elling AA (2013) Major emerging problems with minor *Meloidogyne* species. *Phytopathology* **103**, 1092–1102.

Elsworth B, Wasmuth J and Blaxter M (2011) NEMBASE4: the nematode transcriptome resource. *International Journal of Parasitology* **41**, 881–894.

Evans TC, *et al.* (1994) Translational control of maternal *glp*-1 mRNA establishes an asymmetry in the *C. elegans* embryo. *Cell* **77**, 183–194.

Fanelli E, *et al.* (2005) Analysis of chitin synthase function in a plant parasitic nematode, *Meloidogyne artiellia*, using RNAi. *Gene* **349**, 87–95.

Finn RD, *et al.* (2016) The Pfam protein families database: towards a more sustainable future. *Nucleic Acids Research* **44**, D279–D285.

García LE and Sánchez-Puerta MV (2012) Characterization of a root-knot nematode population of *Meloidogyne arenaria* from Tupungato (Mendoza, Argentina). *Journal of Nematology* **44**(3), 291–301.

Gasteiger E, *et al.* (2005) Protein identification and analysis tools on the ExPASy server. In Walker JM (ed). *The Proteomics Protocols Handbook*. Totowa, NJ: Humana Press, pp. 571–607.

Geng C, *et al.* (2016) A novel serine protease, Sep1, from *Bacillus firmus* DS-1 has nematicidal activity and degrades multiple intestinal-associated nematode proteins. *Scientific Reports* **6**, 25012.

Gleason CA, Liu QL and Williamson VM (2008) Silencing a candidate nematode effector gene corresponding to the tomato resistance gene Mi-1 leads to acquisition of virulence. *Molecular Plant-Microbe Interactions* **21**(5), 576–585.

Gray LJ, Curtis RH and Jones JT (2001) Characterisation of a collagen gene subfamily from the potato cyst nematode *Globodera pallida*. *Gene* **263**, 67–75.

Heitzler P and Simpson P (1993) Altered epidermal growth factor-like sequences provide evidence for a role of Notch as a receptor in cell fate decisions. *Development* **117**, 1113–1123.

Hu B, *et al.* (2015) GSDS 2.0: an upgraded gene feature visualization server. *Bioinformatics (Oxford, England)* **31**(8), 1296–1297.

Iberkleid I, Sela N and Brown Miyara S (2015) *Meloidogyne javanica* fatty acid- and retinol-binding protein (Mj-FAR-1) regulates expression of lipid-, cell wall-, stress- and phenyl propanoid-related genes during nematode infection of tomato. *BMC Genomics* **16**, 272.

Kaur H and Attri R (2013) Morphological and morphometrical characterization of *Meloidogyne incognita* from different host plants in four districts of Punjab, India. *The Journal of Nematology* **45**, 122–127.

Kelley MR, *et al.* (1987) Mutations altering the structure of epidermal growth factor-like coding sequences at the Drosophila Notch locus. *Cell* **57**, 539–548.

Kumar A, *et al.* (2017) Host-delivered RNAi-mediated root-knot nematode resistance in *Arabidopsis* by targeting *splicing factor* and *integrase* genes. *Journal of General Plant Pathology* **83**, 91–97.

Kumar S, Stecher G and Tamura K (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* **33**, 1870–1874.

Kyte J and Doolittle R (1982) A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.

Livak KJ and Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta DeltaC(T)) method. *Methods* **25**(4), 402–408.

Lu CJ, *et al.* (2016) Nuclear receptor *nhr*-48 is required for pathogenicity of the second stage (J2) of the plant parasite *Meloidogyne incognita*. *Scientific Reports* **6**, 34959.

Lunt DH, *et al.* (2014) The complex hybrid origins of the root knot nematodes revealed through comparative genomics. *PeerJ* **2**, e356.

Mango SE (2007) The *C. elegans* pharynx: a model for organogenesis. In *Wormbook*, The *C. elegans* Research community. WormBook. doi/10.1895/wormbook.1.129.1.

Martin J, *et al.* (2015) Helminth.net: expansions to Nematode.net and an introduction to Trematode.net. *Nucleic Acids Research* **43**, D698–D706.

Martinuz A, Schouten A and Sikora RA (2013) Post-infection development of *Meloidogyne incognita* on tomato treated with the endophytes *Fusarium oxysporum* strain Fo162 and *Rhizobium etli* strain G12. *BioControl* **58**, 95–104.

Moens M, Perry RN and Starr JL (2009) *Meloidogyne* species: a diverse group of novel and important plant parasites. In Perry RN, Moens M and Starr JL (eds). *Root-knot nematodes*. Wallingford, UK: CABI Publishing, pp. 1–17.

Niu J, *et al.* (2012) RNAi silencing of the *Meloidogyne incognita Rpn7* gene reduces nematode parasitic success. *European Journal of Plant Pathology* **134**, 131–144.

Nguyễn PV, *et al.* (2014) *Meloidogyne incognita* – rice (*Oryza sativa*) interaction: a new model system to study plant-root-knot nematode interactions in monocotyledons. *Rice* **7**, 23.

Nsengimana J, *et al.* (2013) Silencing of Mg-pat-10 and Mg-unc-87 in the plant parasitic nematode *Meloidogyne graminicola* using siRNAs. *Agriculture* **3**, 567–678.

Opperman CH, *et al.* (2008) Sequence and genetic map of *Meloidogyne hapla*: a compact nematode genome for plant parasitism. *Proceedings of the National Academy of Sciences USA* **105**, 14802–14807.

Priess JR, Schnabel H and Schnabel R (1987) The glp-1 locus and cellular interactions in early *C. elegans* embryos. *Cell* **51**, 601–611.

Rastogi S, *et al.* (2015) *Caenorhabditis elegans* glp-4 encodes a valylaminoacyl tRNA synthetase. *Genes Genomes Genetics* **5**, 719–728.

Rebay I, *et al.* (1991) Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687–699.

Rehman S, Gupta VK and Goyal AK (2016) Identification and functional analysis of secreted effectors from phytoparasitic nematodes. *BMC Microbiology* **16**, 48.

Roehl H and Kimble J (1993) Control of cell fate in *C. elegans* by a GLP-1 peptide consisting primarily of ankyrin repeats. *Nature* **364**, 632–635.

Roehl H, *et al.* (1996) Roles of the RAM and ANK domains in signaling by the *C. elegans* GLP-1 receptor. *The EMBO Journal* **15**, 7002–7012.

Rudel D and Kimble J (2001) Conservation of glp-1 regulation and function in nematodes. *Genetics* **157**, 639–654.

Tytgat T, *et al.* (2004) An SXP/RAL-2 protein produced by the subventral pharyngeal glands in the plant parasitic root-knot nematode *Meloidogyne incognita*. *Parasitology Research* **95**, 50–54.

Veronico P, *et al.* (2001) Nematode chitin synthases: gene structure, expression and function in *Caenorhabditis elegans* and the plant parasitic nematode *Meloidogyne artiellia*. *Molecular Genetics and Genomics* **266**, 28–34.

Verwoerd TC, Dekker BM and Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Research* **17**(6), 2362.

Wang T, Deom CM and Hussey RS (1998) Identification of a *Meloidogyne incognita* cuticle collagen gene and characterization of the developmental expression of three collagen genes in parasitic stages. *Molecular and Biochemical Parasitology* **93**, 131–134.

Whitehead AG (1968) Taxonomy of *Meloidogyne* (Nematodea: Heteroderidae) with descriptions of four new species. *Journal of Zoology* **l31**(3), 263–401.

Xie J, *et al.* (2016) A novel *Meloidogyne incognita* effector Misp12 suppresses plant defense response at latter stages of nematode parasitism. *Frontiers in Plant Science* **7**, 964.

Yadav BC, Veluthambi K and Subramaniam K (2006) Host-generated double stranded RNA induces RNAi in plant-parasitic nematodes and protects the host from infection. *Molecular and Biochemical Parasitology* **148**, 219–222.

Ye W, Zeng Y and Kerns J (2015) Molecular characterisation and diagnosis of root-knot nematodes (*Meloidogyne* spp.) from Turf grasses in North Carolina, USA. *PLoS ONE* **10**(11), e0143556.

Yochem J and Greenwald I (1989) glp-1 and lin-12, genes implicated in distinct cell-cell interactions in *C. elegans*, encode similar transmembrane proteins. *Cell* **58**, 553–563.