Plant Pathology (2017) **66**, 1214–1224 Doi: 10.1111/ppa.12664

Characterization and silencing of the fatty acid- and retinol-binding *Pp-far-1* gene in *Pratylenchus penetrans*

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Fatty acid- and retinol-binding proteins (FARs) are unique to nematodes, and are implicated in a wide range of metabolic and parasitic related functions. Along with the *in silico* analyses performed in this study, three different FAR members of this family were identified in *Pratylenchus penetrans*. The cDNA corresponding to the fatty acid- and retinol-binding *Pp-far-1* gene was cloned and herein characterized at the molecular level for the first time for this genus. The translated 185 amino acid sequence of *P. penetrans* FAR-1 sequence, with a predicted molecular weight of 20.82 kDa and a pI of 5.49, shares highest sequence identity to FARs of other migratory nematodes of the Pratylenchidae family (90% to *P. vulnus* and 80% to *Radopholus similis*). *In situ* hybridization localizes *Pp-far-1* transcripts in the hypodermis of the nematode. RT-qPCR detected *Pp-far-1* transcripts for all nematode developmental stages, with highest expression levels found in juveniles, adult females and adult males, respectively. *Pp-far-1* is also highly expressed during infection and establishment of *P. penetrans* in roots of different host plants, such as corn, lily and soybean. The importance of *Pp-far-1* was studied by *in planta* RNA interference (RNAi) assays using stable soybean hairy root lines. Targeting *Pp-far-1* decreased expression of the nematode *Pp-far-1* and significantly reduced the reproduction of nematodes, with a total of between 44% and 70% fewer nematodes in comparison to the average number of nematodes counted for control lines. The results indicate that suppressing the expression levels of *Pp-far-1* can act as an effective target gene to control *P. penetrans*.

Keywords: gene silencing, *Lilium longiflorum*, Pratylenchidae, RNAi, soybean hairy roots, *Zea mays*

Introduction

Root lesion nematodes are considered the third most important group among plant-parasitic nematodes (Jones *et al.*, 2013). Within this group of nematodes, *Pratylenchus penetrans* is considered a cosmopolitan species, with a preferential distribution along temperate regions. This species has been recorded as being associated with more than 400 plants, and is considered to be a limiting factor for the production of important agronomic [e.g. alfalfa (*Medicago sativa*), maize (*Zea mays* subsp. *mays*), potato (*Solanum tuberosum*)], ornamental [e.g. lily (*Lilium candidum*), roses (*Rosa* spp.)] and fruit [e.g. apple (*Malus pumila*), cherry orchards (*Prunus* spp.), raspberry (*Rubus* spp.)] plants (Castillo & Vovlas, 2007). In some countries *P. penetrans* is considered as an A1 quarantine plant pest due to its potential impact on economically important crops (EPPO Global database, https://gd.eppo.int).

Pratylenchus penetrans is a migratory endoparasitic species that feeds and migrates within the root cortical tissue, causing a reduction in root growth after infection, accompanied by the formation of lesions, necrotic areas, browning and cell death (Fosu-Nyarko & Jones, 2016). As migratory endoparasites, the destruction of the root system can cause surface wounds, which allow access to a combination of other soilborne pathogens, such as fungi (Rotenberg *et al.*, 2004) and bacteria (Vrain & Copeman, 1987), leading to severe damage of the plant. Like other nematodes, the life cycle of *P. penetrans* is punctuated by six stages (eggs, four juvenile stages and adults). With the exception of egg and first juvenile stages (J1), all the remaining stages are motile and able to enter the roots and cause damage (Castillo & Vovlas, 2007).

Silencing core genes through RNA interference (RNAi) can promote lethal or inhibitory effects (Lilley *et al.*, 2012; Danchin *et al.*, 2013), making it a very promising tactic in the control of plant-parasitic nematodes. However, one imperative factor is the identification of plant-pathogen specific genes, or target sequences, that lack homologues in non-target organisms such as mammals, plants or beneficial insects (Danchin *et al.*, 2013).

Fatty acid- and retinol-binding proteins (FARs) comprise a family of unusual α-helix-rich lipid-binding proteins that have high binding affinity for fatty acids, retinol and retinoic acids, and are exclusively found within the phylum Nematoda (Kennedy *et al.*, 2013). This family of proteins occurs in several isoforms of approximately 20 kDa, which can be found in varying numbers among species of the different clades of

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Published online 19 January 2017

Plant Pathology

Nematoda (Garofalo *et al.*, 2003; Iberkleid *et al.*, 2015). In the case of free-living nematodes, eight isoforms have been found within the genome of *Caenorhabditis elegans* (Garofalo *et al.*, 2003), whereas in *Pristionchus pacificus*, 19 proteins have been identified (Dieterich *et al.*, 2008; Dillman *et al.*, 2015). In the case of animal- and plant-parasitic nematodes, a variable number of FAR isoforms have been found. In *Necator americanus* at least six FAR genes have been identified, while for a substantial portion of species studied so far, a single gene has been reported (Kennedy *et al.*, 1997; Garofalo *et al.*, 2003; Tang *et al.*, 2014; Iberkleid *et al.*, 2015). In plant-parasitic nematodes, a single FAR gene has been reported for both sedentary [e.g. *Globodera pallida* (Prior *et al.*, 2001), *Meloidogyne javanica* (Iberkleid *et al.*, 2013), *Heterodera avenae* (Le *et al.*, 2016) and *Heterodera filipjevi* (Qiao *et al.*, 2016)] and migratory [e.g. *Aphelenchoides besseyi* (Cheng *et al.*, 2013), *Radopholus similis* (Zhang *et al.*, 2015)] species. More recently, comparative transcriptome and genomic data suggested that plant-parasitic species (e.g. *Bursaphelenchus xylophilus* and *H. avenae*) have additional members of this FAR family (Dillman *et al.*, 2015; Espada *et al.*, 2016; Qiao *et al.*, 2016). In entomopathogenic nematode species of the genus *Steinernema*, a wide expansion of the number of genes within the FAR family has been reported, ranging between 38 and 54 gene members of this family (Dillman *et al.*, 2015).

FARs seem to play a relevant role in the binding of lipids from their environment or host, as nematodes are unable to synthesize *de novo* fatty acids (Kennedy *et al.*, 2013). A wide range of functions has been implicated for nematode FARs, such as scavenging, transport and metabolism of hydrophobic lipophilic molecules like fatty acids, eicosanoids, retinoids and steroids (McDermott *et al.*, 1999; Kennedy, 2000). These proteins have important functions as energy sources and are used in metabolic and developmental processes such as embryogenesis, glycoprotein synthesis, growth and cellular differentiation (McDermott *et al.*, 1999; Kennedy, 2000). In addition to their role in the nematode's physiological activity, FARs have raised strong interest because of their implication in the parasitism process of both animal- and plant-parasitic nematodes (Kennedy *et al.*, 1997; Bradley *et al.*, 2001; Prior *et al.*, 2001; Iberkleid *et al.*, 2013).

Previously, Vieira *et al.* (2015a) identified in the transcriptome of *P. penetrans* a highly abundant transcript encoding for a FAR gene. Along with the *in silico* analyses performed in this study, three different FAR members of this family were identified in *P. penetrans*, and a molecular characterization of the *Pp-far-1* gene is provided herein. This study sets out to confirm the expression levels of *Pp-far-1* in different nematode developmental stages, and at earlier time points of nematode infection in different host plants. *Pp-far-1* was then targeted using plant-mediated RNAi silencing assays to evaluate the importance of this gene during parasitism.

Materials and methods

Nematode collection and extraction

A single isolate of *P. penetrans* (NL 10p RH), initially collected in Beltsville (MD, USA) and provided by the Nematology Laboratory (USDA-ARS, USA), was maintained and multiplied *in vitro* in roots of corn (*Z. mays* 'Iochief') growing in Murashige and Skoog (MS) medium agar plates. Nematodes were recultured every 2 months onto new roots of maize and maintained in the dark at 28 °C. For nematode extraction, infected roots were chopped into small pieces and both roots and media were placed into sterile glass bowls filled with sterile water containing 50 mg L^{-1} carbenicillin and 50 mg L^{-1} kanamycin. Nematodes were collected after 5 days using a 500 μm mesh sieve, washed with sterile distilled water and collected into a 50 mL Falcon tube. Nematodes were then used for the experiments described below.

Cloning and sequencing of *Pp-far-1* coding sequence

Total RNA was extracted from mixed life stages (eggs, juveniles, adult females and males) of *P. penetrans* using the RNeasy Plant Mini kit (QIAGEN) following the manufacturer's instructions. RNA was then treated with RNase-free DNase (QIAGEN) before reverse transcription. The quantity and quality of the extracted RNA was assessed by a ND-1000 NanoDrop spectrophotometer (Thermo Scientific). The first strand cDNA was synthesized using the iScript first-strand synthesis kit (Bio-Rad) following the manufacturer's instructions. The full length *Pp-far-1* coding sequence was amplified using primers (Table S1) designed based on the predicted mRNA sequence obtained from the *de novo* assembly performed for this species (Vieira *et al.*, 2015a). A CACC sequence was added to the 5′ end of the forward primer to allow insertion of the amplified sequence into the pENTR Directional TOPO Cloning kit (Invitrogen). The amplified *Pp-far-1* cDNA fragment was gel purified and subsequently ligated into the pENTR vector, and used to transform One Shot TOP10 (Invitrogen) chemically competent *Escherichia coli* cells, which were spread on LB-agar plates with kanamycin, and grown overnight at 37 °C. Three insert-positive clones were grown in 3 mL of LB, overnight at 37 °C, followed by plasmid DNA extraction (QIAprep Spin Miniprep kit; QIAGEN), and sequenced by Macrogen USA (MD, USA).

Sequence analysis

The nucleotide and translated amino acid sequences were analysed for similarity to other genes and proteins using BLAST analyses against the NCBI non-redundant nucleotide and protein databases (http://www.ncbi.nlm.nih.gov/). In addition, protein sequence analyses were conducted through the following programs: SIGNALP v. 4.0 was used to predict protein signal peptide (Petersen *et al.*, 2011), the protein molecular mass and the theoretical isoelectric point using PROTPARAM (Wilkins *et al.*, 1999), and secondary structure prediction of the protein sequence was performed using CLC MAIN WORKBENCH v. 7 software.

The obtained coding sequence and predicted Pp-FAR-1 protein were used to perform BLAST searches to different transcriptome datasets of *P. penetrans* (Vieira *et al.*, 2015a) in order to detect additional members of this family. Protein sequences obtained were then aligned with other representative nematode FARs using MUSCLE (Edgar, 2004). Aligned protein sequences were then evaluated by distance analysis using CLC MAIN

WORKBENCH v. 7 software's neighbour-joining package. To evaluate the branch strength of the phylogenetic tree, a bootstrap analysis was evaluated with 1000 replications.

Nematode developmental stage expression analyses

RNA was extracted from approximately 150 nematodes of each nematode developmental stage as previously stated. RNA was then treated with RNase-free DNase before reverse transcription (RT). RNA was added to the RT reaction using the iScript first-strand synthesis kit to produce cDNA, which was posteriorly used for semiquantitative RT-PCR and RT-qPCR analyses. Specific primers were design to amplify a *Pp-far-1* fragment of 133 bp, and a 148 bp fragment of *P. penetrans 18S rRNA* gene that was used as reference gene (Table S1). Real-time qPCRs included 3.5 μL of SYBR green mix (Roche), 1 μL of 5 μM primers and 100 ng cDNA. Reactions were performed on a CFX96 Real-time system machine (Bio-Rad). The amplification reactions were run using the following programme: a hot start of 95 °C for 3 min; then 40 cycles of 95 °C for 10 s and 60 °C for 30 s. After 40 cycles a melt curve analysis or dissociation programme (95 °C for 15 s, 60 °C for 15 s, followed by a slow ramp from 60 to 95 °C) was performed to ensure the specificity (above 90%) of amplification. Three independent biological experiments were conducted by RT-qPCR, using three technical replicates for each independent experiment. Data analysis of *Pp-far-1* was performed using the CFX MANAGER v. 3 software (Bio-Rad). The values of the relative normalized expression of *Pp-far-1* were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), relative to the expression levels of *P. penetrans 18S rRNA* gene, and using the transcript expression levels of the eggs as baseline. The statistical significance was analysed by analysis of variance (ANOVA), using Tukey's test ($P < 0.05$).

In situ hybridization of *Pp-far-1* transcripts

To assess localization of *Pp-far-1* transcripts, whole mount *in situ* hybridizations were performed in all stages of *P. penetrans* following the protocol of de Boer *et al.* (1998). Specific primers were designed to amplify a 232 bp product (Table S1) from cDNA collected from a nematode population of mixed stages. The resulting PCR product was used as template for generation of sense and antisense DIG-labelled *Pp-far-1* probes, using a DIG-nucleotide labelling kit (Roche). Hybridized probes within the nematode tissues were detected using anti-DIG antibody conjugated to alkaline phosphatase and its substrate. Nematode sections were then observed using an Eclipse *5i* light microscope (Nikon).

Differential expression analysis of *Pp-far-1* in different hosts

To quantify the expression levels of *Pp-far-1* during nematode–plant interaction, infected roots of corn, lily and soybean hairy roots were used, and total RNA extraction was performed using a pool of six infected roots at 1, 3 and 7 days after nematode infection (DAI). Nematode infections of soybean hairy roots and lily plants followed the protocol described in Vieira *et al.* (2015b). In the case of maize, maize seeds were initially sterilized using 70% ethanol for 10 min, followed by one wash in a bleach solution (20% bleach and 4 drops of Tween 20) for 15 min, four consecutive washes in sterile water, and dried under a laminar flow hood for at least 30 min. Seeds were then placed in Petri dishes containing MS medium, and kept in a growth chamber with a 16 h light/8 h dark photoperiod at 25 °C. Five days later, each germinating plant was transferred to individual Petri dishes containing MS medium. Ten-day-old seedling roots were individually inoculated with *c.* 400 nematodes (mixed population containing juveniles, adult females and males), collected from *in vitro* maize roots as described above, with the nematode pellet resuspended in water containing 50 mg L^{-1} carbenicillin and 50 mg L^{-1} kanamycin. Infected roots were collected at 1, 3 and 7 DAI, and immediately frozen in liquid nitrogen and stored at −80 °C until used. RNA and cDNA preparation, and RT-qPCR were performed as described above. Three independent biological experiments were performed per time point and host plant by RT-qPCR, each consisting of three technical replicates. Data analysis of *Pp-far-1* was performed using the CFX MANAGER v. 3. The values of the relative normalized expression of *Pp-far-1* were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), relative to the expression levels of *P. penetrans 18S rRNA* gene, and using the transcript expression levels of nematodes prior to infection as baseline. The statistical significance was analysed by ANOVA, using Tukey's test ($P < 0.05$).

Plant-mediated *Pp-far-1* gene silencing and generation of transgenic hairy roots

The template for the production of a *Pp-far-1* dsRNA construct was amplified using nematode cDNA of mixed nematode stages. A fragment of the coding DNA sequence (CDS) of *Pp-far-1* (291 bp) was amplified with specific primers containing the CACC adapter through PCR (Table S1). The nematode gene fragment was cloned into the pENTR vector, following the same steps as described above for DNA sequencing. The *Pp-far-1* fragment was then transferred to the pRAP17 vector (Ibrahim *et al.*, 2011), which is designed to express dsRNA of the target sequence. The cloning reaction was mediated using the Gateway LR Clonase Enzyme Mix (Invitrogen). Transformation of *Pp-far-1* fragment into the pRAP17 vector was confirmed by PCR using two primer pairs (Table S1). The first primer pair was used to confirm the presence of the RNAi gene in the forward direction using the gene-specific forward primer and intron reverse primer, while a second pair was used to confirm the presence of the *Figwort mosaic virus* (FMV) promoter subgenomic transcript and the RNAi gene in the reverse direction, using a forward primer for the FMV region and, as reverse primer, a gene-specific region (Ibrahim *et al.*, 2011). The pRAP17 construct was then transferred to competent *Rhizobium rhizogenes* (K559) and transformations were confirmed by PCR using the same set of primers described above, and a pair of primers to amplify a fragment of 812 bp of the Ri plasmid (Ibrahim *et al.*, 2011). Soybean hairy root lines were then generated using the *Pp-far-1* dsRNA construct, while control hairy root lines were generated using *R. rhizogenes* harbouring an empty vector (pBIN-JIT) with kanamycin resistance to both bacteria and plants (Ferrandiz *et al.*, 2000). Generation of soybean hairy roots was carried out according to Cho *et al.* (2000).

For confirmation of the nematode gene fragment in the transformed hairy roots, genomic DNA was isolated for PCR amplification using the FastDNA kit (MP Biomedicals). Afterwards, total RNA was isolated from 100 mg fresh soybean hairy roots using the RNeasy Plant Mini kit (QIAGEN) following the manufacturer's instructions. The RNA was treated with RNase-free DNase before reverse transcription. One microgram of treated RNA was added to the RT reaction using the iScript Select cDNA synthesis kit as described previously. The oligonucleotide

primer specific for the intron of the pRAP17 vector was used to synthesize the first cDNA strand for each transformed soybean hairy root line, and the corresponding cDNAs were used as a template for amplification of a 241 bp fragment. After confirming the presence of the transgene by PCR and expression levels of the intron that separates each fragment by semiquantitative RT-PCR, four *Pp-far-1* dsRNA independent RNAi lines were selected for nematode resistance assays.

Nematode RNAi gene silencing assays

For nematode resistance assays, soybean hairy roots 3–5 cm in length were excised from stock cultures and transferred to fresh MS plates without antibiotics. Four independent lines were challenged with nematodes using nine hairy root systems per line. Three control lines (containing the pBIN-JIT empty vector) were selected using nine hairy roots per line. Two weeks later, each hairy root system was inoculated with a mixed population of approximately 300 sterile *P. penetrans* and maintained in the dark at 28 °C. Approximately 3 months after nematode inoculation, infected soybean hairy roots were chopped into small pieces and both roots and media were placed into sterile glass bowls filled with sterile water containing 50 mg L^{-1} carbenicillin and 50 mg L^{-1} kanamycin. Nematodes were collected 5 days later using a sieve of 500 μm mesh. Two independent nematode challenge assays were performed using the same lines. Data are expressed as the total mean number of nematodes ± standard error of the mean (SEM) collected for each line per challenge nematode assay ($n = 3$). All data were analysed using ANOVA, and means were compared using Tukey's honestly significant difference (HSD) test at the 5% probability level.

For each nematode resistance test, a set of the extracted nematodes growing in each corresponding RNAi soybean hairy roots (lines L4, L7, L8 and L13) were frozen immediately in liquid nitrogen and stored at −80 °C. Nematodes collected from control line L1 were used as control. Total RNA was isolated and reverse transcribed to cDNA as described above. For each line RT-qPCRs were run in triplicate, and using three technical replicates. Data analysis of *Pp-far-1* was performed using the CFX MANAGER v. 3. The values of the relative normalized expression of *Pp-far-1* were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001), relative to the expression levels of *P. penetrans 18S rRNA* gene, and using the transcript expression levels of nematodes collected from the control line L1 as baseline. The statistical significance was analysed by ANOVA, using Tukey's test ($P < 0.05$).

Results

Cloning of *Pp-far-1* full-length coding region

Previously, Vieira *et al.* (2015a) identified a transcript coding for a FAR gene highly abundant in the transcriptome dataset of *P. penetrans*. *In silico* analysis revealed a predicted mRNA sequence of 825 nucleotides with a 558 bp open reading frame (ORF), a 5′ UTR region of 117 nucleotides before the ATG initiation codon and a 3′ terminal region of 128 nucleotides composed of the 3′ UTR and the polyA tail sequence (Fig. S1). Based on this sequence, specific primers flanking the full-length coding sequence and partial sequences of both UTR regions were designed, resulting in the amplification of a PCR product of 665 bp, with a corresponding coding sequence of 558 bp (Fig. S1). Sequencing results revealed a nucleotide sequence showing 100% similarity to the generated *de novo* assembly, with highest similarity with the *R. similis far-1* gene (77%, accession number JN968974), and deposited at NCBI as *Pp-far-1* with the accession number KY312539.

Characterization and sequence analysis of Pp-FAR-1

Translation of the *Pp-far-1* ORF revealed 185 amino acids with a predicted molecular weight of 20.82 kDa and a pI of 5.49. A signal peptide for secretion predicted by SIGNALP v. 4.0 locates between the amino acids Ala16 and Ala17 (Fig. 1a), and no predicted transmembrane domains (TMHMM server), suggesting that this protein is secreted by the nematode. As in other nematode FARs, the secondary structure of Pp-FAR-1 presents a mostly alpha-helical conformation (Fig. 1a).

Protein BLAST searches of Pp-FAR-1 (minimum *E*-value cutoff $<1e^{-5}$) in different public databases showed different ranges of similarities among other nematode FARs. The alignment of the deduced Pp-FAR-1 protein with FARs of other plant-parasitic nematodes showed significant sequence conservation (Fig. 1b), with highest sequence identity ranging from 90% to 80% for FAR-1 of *Pratylenchus vulnus* and *R. similis* (Family Pratylenchidae), 75% to 70% for *H. filipjevi* and *G. pallida* (Family Heteroderidae), 68% for FAR-1 of *M. javanica* (Family Meloidogynidae), and 55% to 51% for *A. besseyi* and *B. xylophilus* (Family Aphelenchoididae), while similarity to animal-parasitic nematodes ranged from 54% to 51% between *Onchocerca volvulus* and *Brugia malayi* (Family Onchocerdidae), and 41% to FAR-1 of the free-living nematode *C. elegans*. In addition, a TBLASTX of Pp-FAR-1 against different transcriptome sets of *P. penetrans* (Vieira *et al.*, 2015a) revealed two additional putative FAR members for this species, showing 36% and 31% (E-values $7.37e^{-35}$ and $9.52e^{-26}$, respectively) sequence identity to Pp-FAR-1 (Fig. S2), suggesting the presence of other FAR isoforms for *P. penetrans*.

Phylogenetic analyses revealed the relationship of Pp-FAR-1 among other nematode FARs. Representative species per genus of different clades among the phylum Nematoda were selected for comparison. The Pp-FAR-1 clustered together with FAR-1 of other plant-parasitic nematodes, closest to Pratylenchidae species (*P. vulnus* and *R. similis*), and separated from other clusters holding FARs of animal-parasitic and free-living nematode species (Fig. 2). The position of the two other predicted *P. penetrans* FARs re-enforces the idea of the presence of different isoforms for this species.

Expression pattern profile of *Pp-far-1* within different nematode development stages

The expression of *Pp-far-1* was quantified for differential nematode developmental stages [eggs, juveniles (J2–J4), adult males and females] using RT-qPCR (Fig. 3). A successful amplification of *Pp-far-1* was verified for all

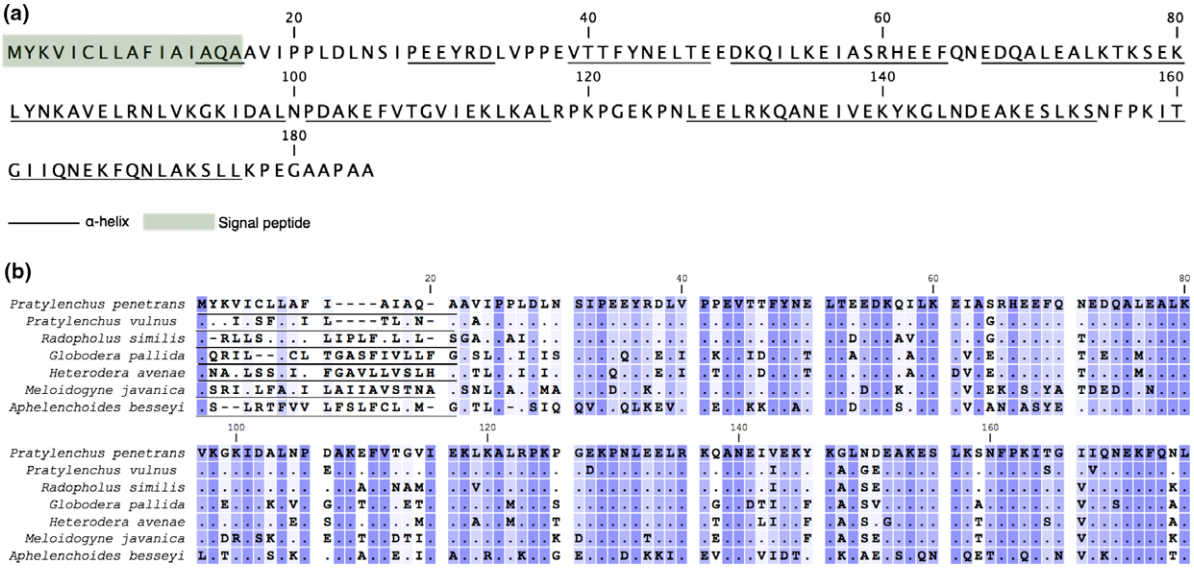

Figure 1 Molecular characterization of Pp-FAR-1 protein. (a) A predicted signal peptide (SignalP program) is shown at the N-terminus, with a cleavage site between two alanine residues at positions 16 and 17. As in other nematode FARs the secondary structure of Pp-FAR-1 presents an α-helical conformation. (b) Multiple sequence alignment of the predicted Pp-FAR-1 protein with FARs from other plant-parasitic nematodes. The corresponding predicted signal peptide for secretion is underlined for each protein. Conserved residues among species are indicated by dark blue shading and dots, whereas similar residues are represented in light blue, using a threshold for shading of 50% similarity. [Colour figure can be viewed at wileyonlinelibrary.com].

developmental stages, as well as for the *18S rRNA* gene that was used as a reference gene. The lowest level of *Pp-far-1* transcripts was found in eggs, which was set as baseline to calculate the relative expression changes in other stages. A marked increase of *Pp-far-1* transcripts could be observed for the remaining stages, with a successive increase in transcript accumulation in juveniles, adult females and males, respectively (Fig. 3a). Tissue localization of the *Pp-far-1* transcripts was evaluated using whole mount *in situ* hybridization. A positive signal was detected along different areas of the nematode body consistent with the localization of the nematode hypodermis (Fig. 3b–d). In the case of the control, no signal could be detected using the sense probe (Fig. 3e).

Pp-far-1 transcript expression upon nematode root infection

To evaluate whether the expression of the *Pp-far-1* gene was host–related or consistently expressed in different nematode–host interactions, the expression levels of *Pp-far-1* were studied at early time points of nematode infection on corn, lily and soybean hairy roots. In this case, RNA of roots infected with *P. penetrans* was collected at 1, 3 and 7 DAI for each host. The expression profile of *Pp-far-1* in the different hosts is presented in Figure 4. In general, an up-regulation of the *Pp-far-1* gene could be observed as nematodes penetrate (1 DAI), and become established within the host (3 and 7 DAI). A substantial increase for *Pp-far-1* expression was registered in the host–nematode interaction and at all time points tested (Fig. 4), highlighting the continuous expression of this gene during parasitism.

Plant-mediated RNAi silencing of *Pp-far-1*

Soybean hairy roots-mediated RNAi was used to silence the expression of *Pp-far-1* gene using the pRAP17 vector (Ibrahim *et al.*, 2011), using a 291 bp fragment located at the C-terminal region. A total of 15 independent transgenic dsRNA hairy root lines were initially generated and used for molecular characterization (Fig. 5). The presence of the *Pp-far-1* fragment was validated by PCR, using a forward primer located in the FMV promoter region and a reverse primer located in the nematode gene fragment, resulting in a product of 420 bp (Fig. 5a). The expression levels of the dsRNA constructs were evaluated by semiquantitative RT-PCR using the amplification of a fragment of 241 bp of the pRAP17 intron (Fig. 5b). For nematode challenge assays, four lines (L4, L7, L8 and L13) were chosen based on the intron expression and growth performance (hairy root lines growing weakly were not selected for nematode challenge assays). Lines transformed with pBIN-JIT empty vector (L1, L3 and L4) were used as control (Fig. S3). In comparison to control lines, no apparent phenotypic variation could be observed between the selected lines for nematode challenge assays (data not shown).

To evaluate whether the *in planta* expression of *Pp-far-1* dsRNA fragment had any effect on nematode development/reproduction, the total number of

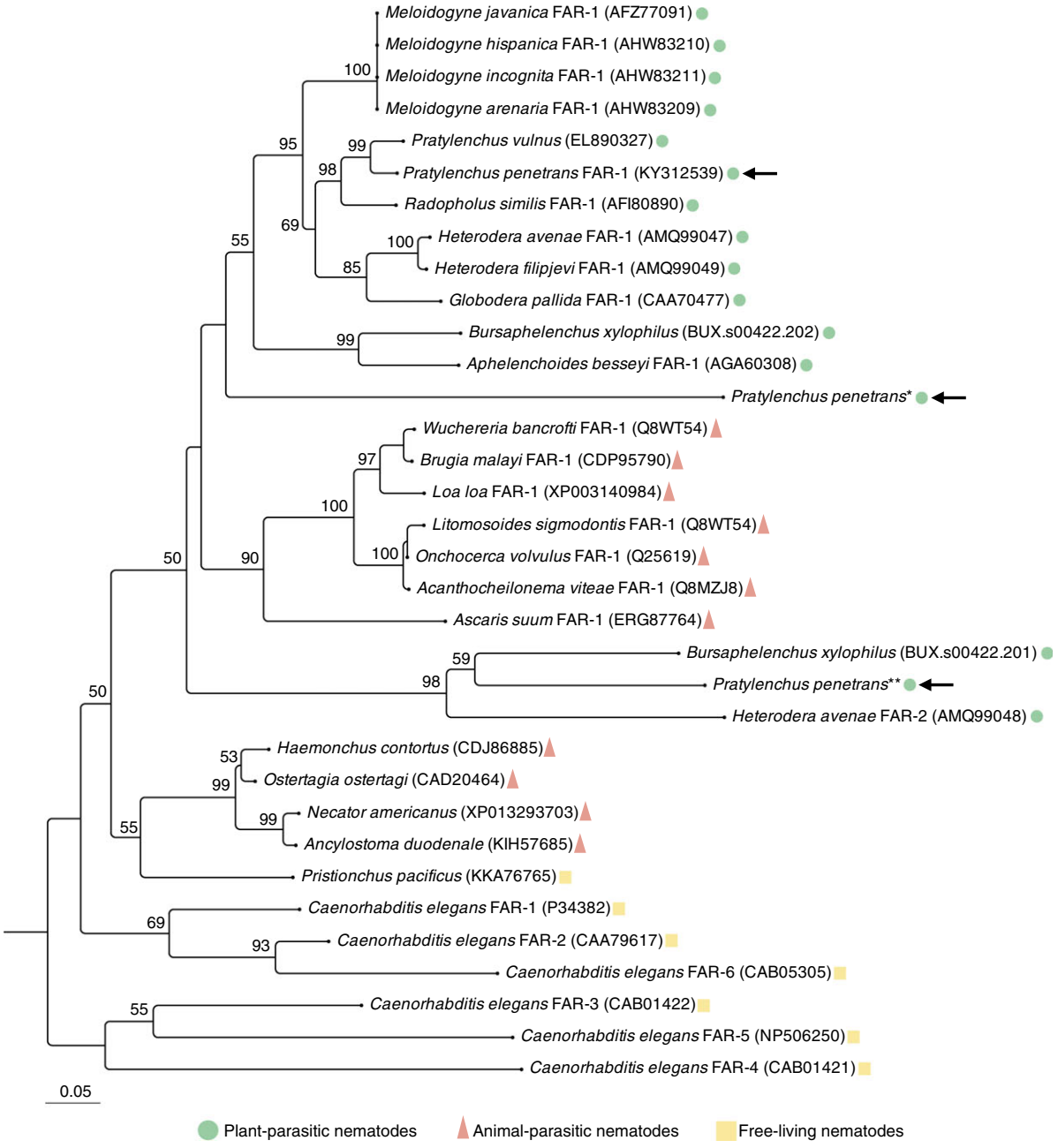

Figure 2 Phylogenetic relationship of Pp-FAR-1 to other representative nematode fatty acid- and retinol-binding proteins (FARs). Protein sequences of *Meloidogyne hispanica*, *M. incognita* and *M. arenaria* are partial sequences. All sequences were obtained from NCBI, with the exception of the sequences for *Bursaphelenchus xylophilus*, which were obtained at http://www.genedb.org/Homepage/Bxylophilus; and *Pratylenchus penetrans*, which were obtained in this study and indicated with an arrow. The two additional FAR members of *P. penetrans* were identified and represented as Pp-FAR* and Pp-FAR**. The phylogenetic tree was generated by neighbour-joining analysis and rooted against the most distant FARs of *Caenorhabditis elegans*, with 1000 bootstrap replications. Bootstrap values (above 50) are indicated at the nodes. Scale bar = substitutions per site. [Colour figure can be viewed at wileyonlinelibrary.com].

nematodes associated with each soybean hairy root line was quantified three months after nematode inoculation. Two independent nematode challenge assays were performed using the same lines ($n = 3$), and data is presented as the total average number of nematodes recovered for each individual line (Fig. 6). A significant reduction ($P < 0.05$) in the number of nematodes developing in hairy roots expressing *Pp-far-1* dsRNA was observed in both nematode challenge assays, with a total of between 44% and 70% fewer nematodes in comparison to the number of nematodes counted for the control lines (Fig. 6a).

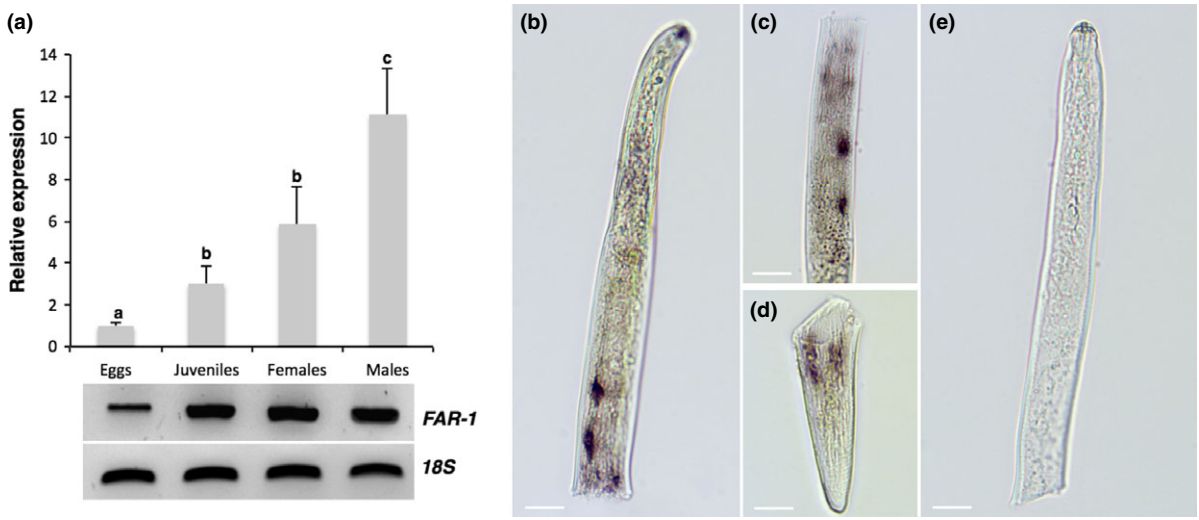

Figure 3 Determination of *Pp-far-1* expression levels during different nematode developmental stages, and *in situ* tissue localization of *Pp-far-1* in *Pratylenchus penetrans*. (a) *Pp-far-1* gene expression was initially detected by semiquantitative RT-PCR to different developmental stages (gel images), using cDNA of one biological independent experiment. Relative transcript expression values (graphic) were quantified by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method, relative to the expression levels of *P. penetrans 18S rRNA* gene, and using the transcription expression levels of the eggs as baseline. Data presented are averages of three independent experiments ± standard error, each consisting of three technical replicates. Statistically significant differences were calculated by ANOVA, using Tukey's test, with different letters indicating significant differences ($P < 0.05$). (b–e) Whole mount *in situ* hybridization of *Pp-far-1*, and sense probe (e), showing the detection of the digoxigenin-labelled probe hybridized to *Pp-far-1* transcripts along the hypodermis (brown colour) in different sections of the nematode body using the antisense probe (b–d), while in the control sections no signal was detected with the sense probe (e). Scale bars = 10 μm. [Colour figure can be viewed at wileyonlinelibrary.com].

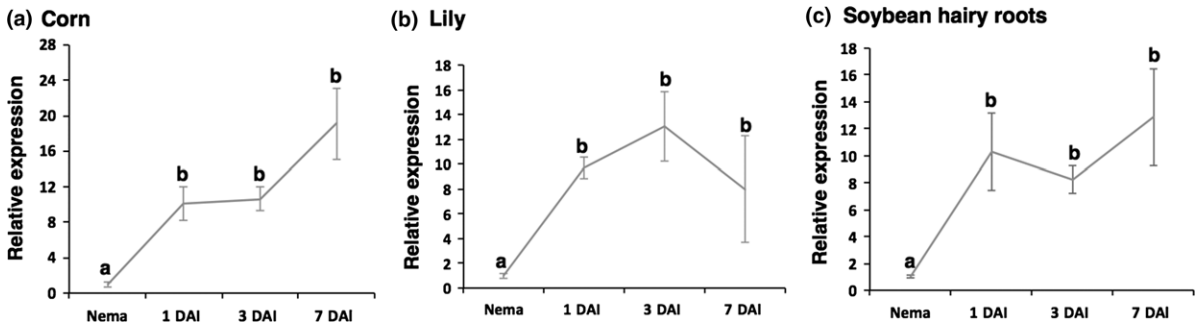

Figure 4 Relative transcript expression values of *Pp-far-1* quantified at early time points of infection (1 to 7 days after infection (DAI)) within different host plants [maize (*Zea mays*), lily (*Lilium longiflorum*) and soybean (*Glycines max*) hairy roots]. Relative transcript expression values were quantified by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method, relative to the expression levels of *Pratylenchus penetrans 18S rRNA* gene, and using the transcription expression levels of nematodes prior to infection (Nema) as baseline. Data presented are averages of three independent experiments ± standard error, each consisting of three technical replicates. Statistically significant differences were calculated by ANOVA, using Tukey's test, with different letters indicating significant differences ($P < 0.05$).

To confirm the silencing effect on the nematode *Pp-far-1* transcript levels, total RNA was extracted from a mixture of nematode stages (eggs, juveniles and adults) developing on individually transformed *Pp-far-1* RNAi hairy roots lines (L4, L7, L8 and L13) and compared to control line L1. The results showed that the silencing effect among the different lines showed some variability, although when compared to the control, a reduction of the *Pp-far-1* transcripts could be observed, suggesting partial knockdown of *Pp-far-1* nematode gene (Fig. 6b).

Discussion

This study identified and characterized for the first time a FAR protein for *P. penetrans*. *Pp-FAR-1* encodes an 185 amino acid peptide, possessing an N-terminal secreted signal peptide and no transmembrane domain. Among plant-parasitic nematodes, FAR-1 homologues have been identified for both sedentary nematodes, such as cyst nematodes [*G. pallida* (Prior *et al.*, 2001), *H. avenae* (Le *et al.*, 2016; Qiao *et al.*, 2016), *H. filipjei* (Qiao *et al.*, 2016)] and root-

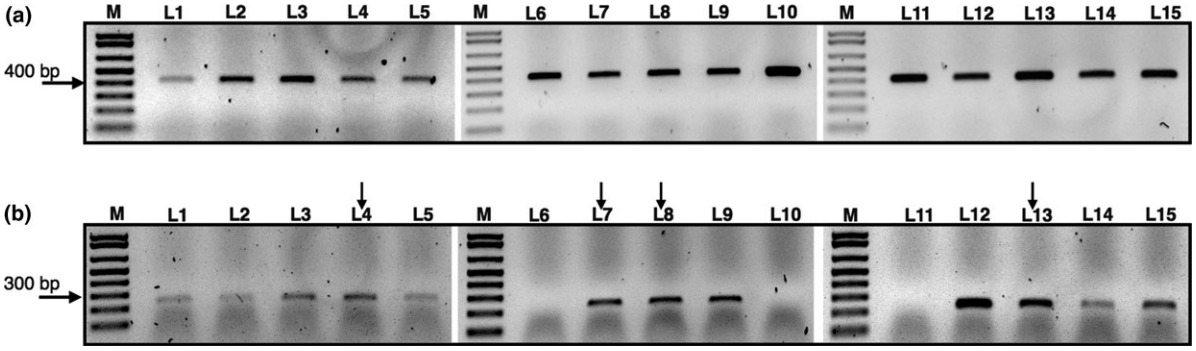

Figure 5 Molecular characterization of soybean (*Glycines max*) hairy root lines expressing a *Pp-far-1* dsRNA construct. (a) For selection of transformed hairy roots, PCR detection was used for the presence of the *Pp-far-1* gene fragment in the soybean hairy roots. (b) Semiquantitative RT-PCR of the pRAP17 intron of the hairpin dsRNA was used to confirm the expression levels of *Pp-far-1* dsRNA in soybean hairy root lines 1 to 15. Lines L4, L7, L8 and L13 (arrows) were selected for nematode challenge assays due to their growth performance and intron expression levels.

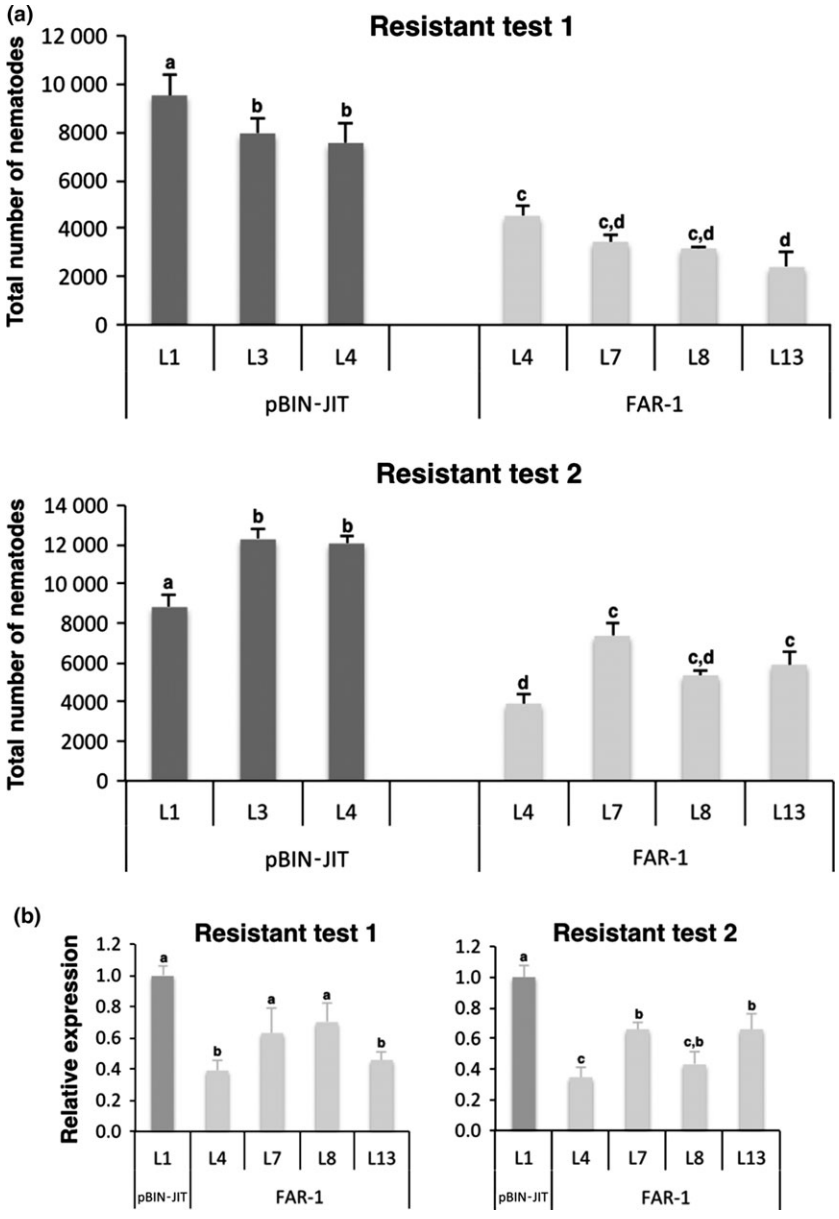

Figure 6 Effect of root RNAi-mediated silencing of *Pp-far-1* on the development and propagation of *Pratylenchus penetrans*. (a) Nematode challenge assays in transgenic soybean (*Glycines max*) dsRNA hairy root lines 3 months after infection. Data shown represent the total mean number ± SEM of nematodes recovered from roots, using a pool of nine soybean hairy roots for each line ($n = 3$). Resistant tests 1 and 2 correspond to two independent biological assays, using the same hairy root lines. As control, hairy roots transformed with an empty vector (pBIN-JIT) were used. Statistical significant differences were calculated by ANOVA, using Tukey's test, with different letters indicating significant differences ($P < 0.05$). (b) Relative normalized expression of *Pp-far-1* of *P. penetrans* collected from transgenic soybean hairy root lines and analysed by RT-qPCR. Relative transcript expression values were quantified by RT-qPCR using the $2^{-\Delta\Delta Ct}$ method, relative to the expression levels of *P. penetrans 18S rRNA* gene, and using the transcription expression levels of nematodes collected from control line L1 as baseline. Data presented are averages of RT-qPCR reactions run in triplicate ± standard error, each consisting of three technical replicates. Statistically significant differences were calculated by ANOVA, using Tukey's test, with different letters indicating significant differences ($P < 0.05$).

knot nematodes [*M. javanica* (Iberkleid *et al.*, 2013) and *Meloidogyne hispanica* (Duarte *et al.*, 2014)], and for migratory species such as *R. similis* (Zhang *et al.*, 2015), *A. besseyi* (Cheng *et al.*, 2013) and *B. xylophilus* (Espada *et al.*, 2016). More recently, two FAR genes (*Ha-far-1* and *Ha-far-2*) have been identified in the cyst nematode *H. avenae* (Qiao *et al.*, 2016). When compared with other nematode FARs, Pp-FAR-1 showed higher sequence identity to other Pratylenchidae species, followed by cyst (Heteroderidae) and root-knot (Meloidogynidae) nematodes. The *in silico* and phylogenetic analyses indicate that the FARs of *P. penetrans* belong to distinct clusters, supporting the idea of additional members of this family in *P. penetrans* besides FAR-1, with *Pp-far-1* being highly expressed for this species. The development of genomic and transcriptome resources for species, belonging to different nematode clades and distinct living strategies, not only revealed an expansion of distinct FAR members for specific groups of species (Tang *et al.*, 2014; Dillman *et al.*, 2015; Espada *et al.*, 2016), but also leads to questions about the evolutionary path of this unique family of proteins within the Nematoda.

The expression of *Pp-far-1* could be detected among all developmental stages, showing higher levels of expression for motile nematode stages. In both cyst and root-knot nematodes, the *far-1* gene is expressed among all nematode stages but is particularly highly expressed in infective juveniles (Prior *et al.*, 2001; Iberkleid *et al.*, 2013), which corresponds to the migratory stage of these species. Different stage-specific expression patterns than the one detected for *Pp-far-1* were observed for the *far-1* gene of other animal- (Kuang *et al.*, 2009; Dillman *et al.*, 2015) or plant- (Cheng *et al.*, 2013; Le *et al.*, 2016) parasitic species. The relative abundance of *far-1* homologues varied between different species and individual nematodes stages, perhaps reflecting the array of life cycle or parasitism strategies. Whereas the differential expression exhibited among the stages of *P. penetrans* could be correlated with their activity, the mode of action is still preliminary.

In situ hybridization of *Pp-far-1*, a widely used approach for studying mRNA localization in nematode tissues, detected transcripts in the hypodermis region along different areas of the nematode body. Other studies have shown a similar localization pattern for *far-1* homologues in plant-parasitic nematodes (Cheng *et al.*, 2013; Zhang *et al.*, 2015; Le *et al.*, 2016), while immunolocalization of FAR-1 protein has been detected along the nematode cuticle surface of *G. pallida* (Prior *et al.*, 2001), *M. javanica* (Iberkleid *et al.*, 2013), and *H. avenae* (Le *et al.*, 2016). More recently, the mRNA transcripts of an additional putative FAR gene were detected in the oesophageal glands of the pinewood nematode, *B. xylophilus* (Espada *et al.*, 2016). The oesophageal glands are considered one of the main producers of key parasitism proteins of plant-parasitic nematodes, with parasitism proteins secreted through the nematode stylet into the host. FARs of animal-parasitic nematodes have been detected within the excretory/secretory products (Mei *et al.*, 1997; Basavaraju *et al.*, 2003; Kennedy *et al.*, 2013) showing that these secreted FARs may interact with the host tissues. The localization of *Pp-far-1* transcripts, together with the presence of an N-terminal signal peptide for secretion, supports the idea that Pp-FAR-1 might be secreted by the hypodermis onto the nematode cuticle surface, ultimately interacting with the plant cells and molecules, such as the epidermal and cortical root cells. Although the *in silico* analyses here revealed the presence of other FAR members of *P. penetrans*, their localization within the nematode tissues and expression profiles remains to be evaluated.

Once interacting with the plant, the expression profiles of *Pp-far-1* showed a significant transcript up-regulation in all the hosts tested, highlighting the importance of *far-1* during interaction of *P. penetrans* with the plant. In this work, host-mediated silencing by overexpressing a *Pp-far-1* dsRNA fragment in hairy roots of soybean was used to knockdown the *far-1* gene of *P. penetrans*. The RNAi assays validated the importance of Pp-FAR-1 during this plant host interaction, as silencing of *Pp-far-1* resulted in up to 70% reduction of the total number of nematodes in comparison to control roots. Host-mediated silencing of the root-knot *M. javanica far-1* gene using tomato hairy roots provided a significant reduction of nematode and gall development, whereas constitutive expression of *Mj-far-1 in planta* increased nematode susceptibility (Iberkleid *et al.*, 2013). In the case of *R. similis* isolates, a positive correlation could be observed among the expression levels of *far-1* gene, as isolates expressing higher levels of *Rs-far-1* showed higher levels of reproduction and pathogenicity (Zhang *et al.*, 2015).

Nematode lipid-binding proteins play key roles in the transport of fatty acids and retinols, which are ultimately consumed for important metabolic and developmental processes of the nematode, such as embryogenesis, glycoprotein synthesis, growth and cellular differentiation (McDermott *et al.*, 1999; Kennedy, 2000). Fluorescence-based ligand binding assays have shown that recombinant FARs of parasitic nematode species possess a strong binding activity to fatty acids and retinol (Rey-Burusco *et al.*, 2015; Zhang *et al.*, 2015; Qiao *et al.*, 2016), and recent studies suggest that FARs are also capable of binding to a wide range of lipidic compounds such as diacylglycerol and phospholipids (Franchini *et al.*, 2015). In animal-parasitic nematodes, FARs have been found within the excretory/secretory secretome, together with an extensive series of proteins that can interact with a range of activities of the nematode, to facilitate invasion and contribute to immune modulation of the host (Garofalo *et al.*, 2002; Kennedy *et al.*, 2013). In plant-parasitic species, the recombinant Gp-FAR-1 of the cyst nematode (*G. pallida*) presented the capacity of binding to a broad range of fatty acids, including linolenic and linoleic acids, which are precursors of jasmonic acid (JA) signalling pathway components and plant defence compounds (Prior *et al.*, 2001). The root-knot nematode *M. javanica* secreting FAR-1 showed a high

accumulation of FAR-1 at the nematode cuticle surface and at the extracellular space of the adjacent root cells (Iberkleid *et al.*, 2013). Predictions of the 3D structure of Mj-FAR-1 also reveal new features for this protein when compared to the 3D crystal structure of Ce-FAR-7, such as a bigger electrostatically charged P2 pocket that could accommodate larger isoprenoid chains of retinol and other compounds with charged characteristics (Iberkleid *et al.*, 2015). These differences are in agreement with the 3D structure of the Na-FAR-1 of *N. americanus* determined by nuclear magnetic resonance and X-ray crystallography, probably reflecting differences in their ligand selectivity (Rey-Burusco *et al.*, 2015). One route to determine the function of FAR-1 of *M. javanica* has been recently performed by overexpressing *Mj-far-1* in stable hairy root lines of tomato, and studying the consequential gene expression changes by transcriptome data analyses (Iberkleid *et al.*, 2015). A complex manipulation, either direct or indirectly, of genes involved in the host defence-related responses seems to be mediated by Mj-FAR-1, comprising differential regulation of plant cell wall-, hormone- and fatty acid-related genes, suppression of JA-responsive genes, and genes belonging to the phenylpropanoid pathway (Iberkleid *et al.*, 2013, 2015).

These findings are in line with the growing idea that FARs might have multitask functions across all nematode lineages of the phylum, rather than a basic supply acquisition role only, as they have been linked to the manipulation of the lipid-based signalling pathway in plants (Prior *et al.*, 2001; Iberkleid *et al.*, 2013, 2015; Le *et al.*, 2016), and contributing to immune evasion or suppression of the signalling defences in animals (Garofalo *et al.*, 2002; Kennedy *et al.*, 2013). Given that FARs are confined to Nematoda, playing key roles either in core metabolic pathways and/or parasitism, they constitute an attractive target to use to control nematode parasitic species. The understanding of their structures and mode of action of the different individual isoforms within a host–nematode interaction will elucidate their effective role in the nematode's activities. Studies of animal FARs have provided relevant data for the potential development of new drugs or vaccines against animal-parasitic nematodes, as targeting fatty acid uptake by these nematodes showed a reduction on nematode infection (Fairfax *et al.*, 2009; Bath & Ferris, 2012). In this study, the efficacy of *Pp-far-1* RNAi against *P. penetrans* was evaluated using a host-mediated silencing RNAi assay. The results demonstrated that silencing the *Pp-far-1* gene had a substantial detrimental effect on nematode reproduction. Thus inhibition of *Pp-far-1* expression through RNAi could be seen as an effective approach to target and control root lesion nematodes.

Acknowledgements

The authors acknowledge the technical assistance of Sarah Shih. This material is based upon work that is supported by the National Institute of Food and Agriculture, US Department of Agriculture, under award number 11588909. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Figure S1 Complete *Pp-far-1* mRNA sequence generated by *de novo* assembly of *Pratylenchus penetrans* transcriptome, and validated by cDNA PCR sequencing analyses. The cloned and resulting sequenced CDS is highlighted in yellow, showing 100% similarity to the transcript generated by *de novo* assembly.

Figure S2 Protein alignment of FARs identified in the transcriptome of *Pratylenchus penetrans*. A tblastx search was performed using Pp-FAR-1 against the transcriptome of *P. penetrans* (Vieira *et al.*, 2015b), using a minimum *E*-value cut-off $<1e^{-5}$. Two additional FAR members were identified and represented herein as Pp-FAR* and Pp-FAR**.

Figure S3 Transformation and validation of control soybean hairy root lines expressing an empty vector (pBIN-JIT). (a) For selection of transformed hairy roots, PCR detection was used for the presence of the *NTPII* fragment. (b) Expression of the *NPTII* gene was confirmed by semiquantitative RT-PCR of the hairy root lines, and *UBQ3* gene was used as a positive reference control. Lines L1, L3 and L4 were selected for nematode challenge assays.

Table S1 List of primers.