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Characterization of the venom allergen—like protein (*vap-1*) and the fatty acid and retinol binding protein (*far-1*) genes in *Meloidogyne hispanica*

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Abstract The root-knot nematode (RKN) *Meloidogyne hispanica* has been found in all continents associated with a wide host range, including economically, important plants and can be considered a species of emerging importance. Considerable progress has been made to identify nematode effector genes as they are important targets for the development of novel control strategies. The effector genes, venom allergen-like protein (*vap-1*) and fatty acid and retinol binding protein (*far-1*), were identified, isolated and sequenced in *M. hispanica* (*Mhi-vap-1* and *Mhi-far-1*) using the genome information available for the RKNs *M. incognita* and *M. hapla*. These genes are differentially expressed during *M. hispanica* development and their amplification products were observed from cDNA of the eggs, second-stage juveniles (J2) and adult females. However, *Mhi-vap-1* showed the highest level of expression in J2. In situ hybridization analysis revealed that the *Mhi-vap-1* and *Mhi-far-1* transcripts are accumulated within the J2 subventral oesophageal glands. The specific expression in the subventral oesophagel glands and presence of the secretion signal peptide for both genes suggests that these proteins are secreted by the J2 and may play a role in the early parasitic stage of the infection process. These genes were also isolated and sequenced in *M. arenaria*, *M. incognita* and *M. javanica*; and phylogenetic analysis revealed that the predicted protein sequences belonging to *M. hispanica* and several other species of plant-parasitic nematodes have a high degree of conservation.

Keywords Nematode effectors · Root-knot nematodes · Secretions · Subventral oesophageal glands

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Introduction

The sedentary endoparasite root-knot nematode (RKN) *Meloidogyne hispanica* Hirschmann (1996), detected for the first time in Seville, Spain, from the peach rootstock, *Prunus persica silvestris* Batsch, has a worldwide distribution, being found in all continents (Europe, Africa, Asia, Australia and North, Central and South America) associated with a wide range of host plants (Hirschmann 1996; Maleita et al. 2012a). Recent studies showed that *M. hispanica* can spread in Europe and move northwards; can overcome the tomato *Mi*-1.2 gene and can

attack economically important plant species and cultivars, including commercial tomato crops. These are characteristics of a polyphagous species of emerging importance, which is difficult to control with crop rotation and with the use of resistant cultivars (Maleita et al. 2011, 2012a, b, c).

Like other plant-parasitic nematodes, RKNs are known to secrete effector proteins into the host tissues which can alter plant physiology and assist the infection process (Rosso and Grenier 2011). These molecules are secreted from the nematode oesophageal gland cells, as well as from amphids and nematode surface cuticle (Davis et al. 2008; Davis and Curtis 2011; Rosso et al. 2012). A number of candidate effector genes, from both cyst nematodes and RKNs have been identified, using cDNA libraries from RNA extracted specifically from the oesophageal gland cells of these nematodes (Wang et al. 2001; Gao et al. 2003; Huang et al. 2003; Hussey et al. 2011). So far, the functions for several potential effector proteins have been predicted and showed that various cellular processes can be targeted by the nematode for successful manipulation of the host response. These include the cell wall structure, manipulation of cell fate, protein synthesis and alteration of signaling pathways. Functional tests, using RNAi, have supported the putative role of some of these nematode effectors in pathogenesis (Bellafiore et al. 2008; Roze et al. 2008; Bellafiore and Briggs 2010).

The venom allergen-like proteins (VAPs) are homologues of the plant and animal cystein-rich secretory proteins (CRISPs). Although, the VAPs are part of a family of effectors considered to be conserved in all parasitic nematodes of plants and animals, its function is still unknown (Haegeman et al. 2012). They have been identified and characterized from the RKNs *M. incognita*, *M. hapla* and *M. chitwoodi*, the cyst nematodes *Heterodera glycines*, *H. schachtii* and *Globodera pallida*, the root-lesion nematode *Pratylenchus coffeae* and the pinewood nematode *Bursaphelenchus xylophilus* (Ding et al. 2000; Gao et al. 2001; Vanholme et al. 2006; Wang et al. 2007; Opperman et al. 2008; Roze et al. 2008; Jones et al. 2009; Kang et al. 2010, 2012; Haegeman et al. 2011, 2012). Various allergen proteins were described as being highly transcribed during plant nematode parasitism (Ding et al. 2000; Gao et al. 2001; Wang et al. 2007). Some are proposed to be involved in the defence response mediated by extracellular innate immune receptors and host invasion (Hawdon et al. 1999; Murray et al. 2001). The *vap-1 gene* is recognized as being potentially associated with the *M. incognita* infection process, with the induction of a host immune response and resistance since it triggers a Cf-2/Rcr3pim dependent programmed cell death in tomato plants (Gao et al. 2001; Haegeman et al. 2009; Chen et al. 2010; Lozano-Torres et al. 2012).

The protein FAR-1 is a member of the nematode specific fatty-acid and retinol binding (FAR) family of proteins and was detected for the first time in *G. pallida*. This protein is present in the surface coat of potato cyst nematode species and binds fatty acids, including linoleic acids that are precursor of plant defence compounds (Prior et al. 2001). These fatty acids are metabolized by lipoxygenase as part of the signalling pathway leading to the production of jasmonic acid and FAR-1 inhibited this biochemical process in vitro thus may have a role in the suppression of jasmonate synthesis and of the downstream signalling pathways, reducing host defences (Curtis 2007; Haegeman et al. 2012). A study with *M. javanica* suggests that the MJ-FAR-1 protein has an important role in the infection process. FAR-1 induces susceptibility to RKN through the manipulation of jasmonate-dependent defence response (Iberkleid et al. 2013). FAR-1 has also been identified in *H. schachtii*, *M. chitwoodi*, *M. hapla, M. incognita*, *P. coffeae* and *Radopholus similis* (Vanholme et al. 2006; Bellafiore et al. 2008; Jacob et al. 2008; Opperman et al. 2008; Roze et al. 2008; Haegeman et al. 2009, 2011).

The main goals of this research were to isolate, to characterize the expression and to localize the *vap-1* and *far-1* genes in *M. hispanica*, which may be good targets for the development of novel control strategies for this species, and also to investigate the phylogenetic relationship of *M. hispanica* with other *Meloidogyne* species.

Material and methods

Nematode isolate

The *M. hispanica* isolate used in this study was originally obtained from infected fig tree (*Ficus carica* L.) roots collected in Odeceixe, Faro, Portugal; the *M. incognita* isolate was provided by Rothamsted Research, UK; and the Portuguese isolates of *M. arenaria* and *M. javanica* were originally obtained from *Oxalis corniculata* L. and *Solanum tuberosum* L.

roots, respectively. All the isolates were maintained on tomato, *S. lycopersicum* L., cv. Tiny Tim, in pots containing sterilized sandy loam soil and sand (1:1), in a growth chamber, at 25±2ºC, with approximately 75 % relative humidity. Two months after the inoculation with 10 egg masses (EM), the eggs were extracted using 0.52 % sodium hypochlorite (NaOCl) solution (Hussey and Barker 1973), the freshly hatched J2 were obtained from the EM placed on a 25 μm mesh sieve, and the females extracted from galled roots. The species identification was confirmed by esterase phenotype analysis (Pais et al. 1986; Abrantes et al. 2008).

Bioinformatics and sequence analyses

Homolog proteins VAP-1 and FAR-1 sequences were searched in the National Center for Biotecnology information (Genbank accessions Nº. ABL61274.1 for VAP-1 in *M. arenaria* and Nº. CAA70477.2 for FAR-1 in *G. pallida*). Gene models were attributed to expressed sequence tag (EST) contigs using tblastn searches against the predicted proteins from the genome of *M. incognita* (http://www.inra.fr/meloidogyne_incognita). The same criteria were used to attribute gene models from the *M. hapla* genome (http://www.pngg.org/cbnp/index.php). Putative orthologs were searched by reciprocal best-hit comparison, using gene models from the genome of *M. incognita* (Minc17158) and *M. hapla* (Mh10g200708_contig2874) for *vap-1* and from the genome of *M. incognita* (Minc08986) and *M. hapla* (Mh10g200708_contig113) for *far-1*.

Alignments were analysed in the program Multiple Sequence Alignment by Florence Corpet (MultAlin Hosted by the Plateforme Bioinformatique Genotoul). After the alignment, the conserved regions of the DNA sequences of *vap-1* and *far-1* in the two species of RKN, *M. incognita* and *M. hapla*, were used to design primers using the program Vector NTI (Invitrogen, UK). For *vap-1* gene, the primers were MIHA-VAP-1f/MIHA-VAP-1r and for *far-1* MIHA-FAR-1f/MIHA-FAR-1r (Table 1).

DNA extraction

Genomic DNA was extracted from *M. hispanica*, *M. arenaria*, *M. incognita* and *M. javanica* J2, using an adaptation of the protocol described by Orui (1999). Nematodes were homogenized in nitrogen liquid with 400 μl of extraction buffer (200 mM Tris–HCl pH 8; 250 mM NaCl and 25 mM EDTA) and centrifuged at 20,000×g for 5 min. The supernatant was transferred to a new tube and equal volume of isopropanol was added. After swirling the tube, the mixture was incubated at room temperature for 30 min and centrifuged at 20,000×g for 15 min. The supernatant was removed and the pellet washed with 500 μL of 70 % ethanol. After centrifugation for 5 min at 20,000×g, the supernatant was removed and the pellet dried. The DNA was then resuspended in 30 μl of Tris-EDTA (10 mM Tris–HCl pH 8 and 1 mM EDTA) and the concentration determined in a Nanodrop ND-1000 Spectrophotometer (Labtech International, UK).

Table 1 Primers used in this study

Primer name	Primer sequence (5'→3')
MIHA-VAP-1f	TGGGCTGATAAATGCACTTA
MIHA-VAP-1r	GTGTCCAATGTCCAATACCT
MIHA-FAR-1f	GGCTAGGGTTAATAAGATTTG
MIHA-FAR-1r	CCTTCTGGTTTCAACAAGCT
MHI-VAP-1f	TTATGGAGAGATTTCTATGC
MHI-VAP-1r	GTGTCCAATGTCCAATACCT
MHI-FAR-1f	GATTTGGTCCGCCTGAGGTT
MHI-FAR-1r	CGGTAATCTTGGGGAAGTTG
β-actinf	GATGGCTACAGCTGCTTCGT
β-actinr	GGACAGTGTTGGCGTAAAGG
M13f	CGCCAGGGTTTTCCCAGTCACGAC
M13r	TCACACAGGAAACAGCTATGAC

Amplification of *vap-1* and *far-1* genes

PCR amplifications were performed in a mixture containing 25 ng of *M. hispanica*, *M. arenaria, M. incognita,* or *M. javanica* DNA as template and five units of Taq DNA polymerase (Promega, UK), in 1x Go Taq Reaction Buffer, 1.5 mM $MgCl_2$, 10 mM dNTP's, and 10 μM of each primer, *vap-1* primers were MIHA-VAP-1f/MIHA-VAP-1r and MIHA-FAR-1f/MIHA-FAR-1r for *far-1* (Table 1). Amplifications were carried out using the following conditions: 3 min at 95ºC, 40 cycles at 95ºC for 30 s, 40ºC for 30 s and 72 °C for 2 min and a final extension at 72 °C for 5 min. The PCR reaction was analysed on a 1.0 % agarose gel in 1x TAE buffer stained with GreenSafe (NZYTech, Portugal). The amplified products were purified with the QIAquick Gel Extraction Kit (QIAGEN, UK) and sequenced by

standard procedures at Eurofins MWG Operon (Ebersberg, Germany). The sequences designated as *Ma-vap-1*, *Mhi-vap-1*, *Mi-vap-1*, *Mj-vap-1*, *Ma-far-1*, *Mhi- far-1*, *Mi-far-1* and *Mj-far-1* were deposited in GenBank as KF030969, KF030970, KF030971, KF030972, KF030973, KF030974, KF030975 and KF030976, respectively. *Meloidogyne hispanica* specific primers (MHI-VAP-1f/MHI-VAP-1r and MHI-FAR-1f/MHI-FAR-1r) were designed from conserved sequences between this species and *M. incognita*, located in the 3'UTR region, as described before (Table 1).

RNA extraction and developmental expression analysis

Total RNA was extracted from *M. hispanica* eggs, J2 and adult females. The specimens were placed in liquid nitrogen and homogenized, separately, using the sample preparation system MP Fast Prep-24, speed at 4.0 m/s (MP Biomedicals, California, USA). Afterwards, the RNA was isolated using the RNeasy Mini Kit including RNase-Free DNase Set (QIAGEN, UK). The concentration and purity of the RNA was determined in a Nanodrop ND-1000 Spectrophotometer. Total RNA (180 ng) of each developmental stage was reverse transcribed into cDNA using the SuperScript II Reverse Transcriptase (Invitrogen, UK), according to the manufacturer's instructions, and the concentration determined. After the reverse transcriptase reaction, a standard PCR was performed with the *M. hispanica vap-1* and *far-1* specific primers (Table 1). Actin genes were amplified from each sample as positive control (Table 1). The PCR mixture, containing 25 ng of synthesized cDNA template and five units of Taq DNA polymerase (Promega, UK), in 1x Go Taq Reaction Buffer, 1.5 mM $MgCl_2$, 10 mM dNTP's and 10 μM of each primer, was first heated for 3 min at 95ºC, and then submitted to 39 temperature cycles (95 °C for 30 s, 40 °C for 30 s, and 72 °C for 1.5 min) with a final extension at 72 °C for 5 min.

Genomic clone

Amplified *M. hispanica* cDNA fragments were purified, as described above, cloned into pGEM-T Easy vector (Promega, UK), and transformed into *Escherichia coli* DH5-alfa by electroporation, in a Micro-Pulser (Bio-Rad, Hercules, California, US). One positive clone of each *Mhi-vap-1* and *Mhi-far-1*genes was selected and amplified with the primers M13f/M13r (Table 1).

mRNA in situ hybridization

For in situ hybridization, the DNA fragment used as probe was amplified from the cloned cDNA of *M. hispanica* J2 with the designed specific primers MHI-VAP-1f/MHI-VAP-1r and MHI-FAR-1f/MHI-FAR-1r. 10 ng of each purified PCR product (QIAquick PCR purification Kit, QIAGEN, UK) was the template in an asymmetric PCR to synthesize digoxigenin (DIG)-labelled sense and antisense single-stranded cDNA probes with PCR DIG Probe Synthesis kit (Roche Applied Science, Indianapolis, USA) (Lee et al. 2006). In situ hybridization was performed with *M. hispanica* J2 as described by De Boer et al. (1998).

Phylogenetic analysis

The *vap-1* and *far-1* sequences of *M. hapla* were obtained from *M. hapla* genome website (http://www.pngg.org/cbnp/index.php?option=com_wrapper&Itemid=45) and *far-1* sequence of *G. pallida* was obtained from GenBank nucleotide database. The *vap-1* and *far-1* sequences of the *M. arenaria*, *M. hispanica*, *M. incognita* and *M. javanica* isolates were aligned with the ones from *M. hapla* and *G. pallida* sequences, and truncated to obtain a common start and end point. The alignment allowed the identification of the additional coding sequence of each sequence and the removal of introns. The amino acid sequences were deduced, from the 206 bp and 396 bp DNA sequences for the partial *vap-1* and *far-1* sequences, respectively. Protein phylogenetic trees were constructed using the Neighbor-Joining (Saitou and Nei 1987) and Maximum-Likelihood (Jones et al. 1992) algorithms, and topology of the trees were generated from evolutionary distances computed using the Poisson correction method (Zuckerkandl and Pauling 1965), included in MEGA5 (Tamura et al. 2011). The topology of the trees generated was evaluated by performing bootstrap analysis (Felsenstein 1985) of 500 resamplings of the data set. All positions with less than 75 % site coverage were eliminated. The protein alignment was used to determine the nucleotide position in the DNA sequences alignment, that was further used to perform DNA sequences phylogenetic analyses as described above, but the evolutionary distances were computed using the Jukes–Cantor correction method included in MEGA5 (Jukes and Cantor 1969; Tamura et al. 2011).

Results

Bioinformatics analyses and amplification of *vap-1* and *far-1* genes

The protein homology search of VAP-1 (accession Nº. ABO38109) and FAR-1 (accession Nº. CAA70477.2) in the databank, using tblastn, revealed 76 % (Minc17158) and 53 % (Minc08986) protein identity, respectively to *M. incognita* and 85 % (MhA1_Contig2874) and 88 % (MhA1_Contig113) to *M. hapla*. Based in analysis of EST from *M. incognita* and *M. hapla*, a pair of primers was designed for each region which permitted the successful amplification of a fragment of approximately 280 bp for *vap-1* and 700 bp for *far-1*, from all *Meloidogyne* species used in this study (data is only shown for *M. hispanica* J2, Fig. 1).

Transcription analysis of *Mhi-vap-1* and *Mhi-far-1* genes

Reverse transcription polymerase chain reaction was used to evaluate the expression of the genes *Mhi-vap-1* and *Mhi-far-1* in three *M. hispanica* developmental stages (eggs, J2 and females), and specific bands of approximately 200 bp and 400 bp were, respectively amplified in these developmental stages (Fig. 2). The cDNA fragments were amplified in all of the nematode samples. However, the expression of *Mhi-vap-1* was higher in J2 when compared with those obtained in eggs and females. For *Mhi-far-1*, the expression was equally higher in all the developmental stages (Fig. 2).

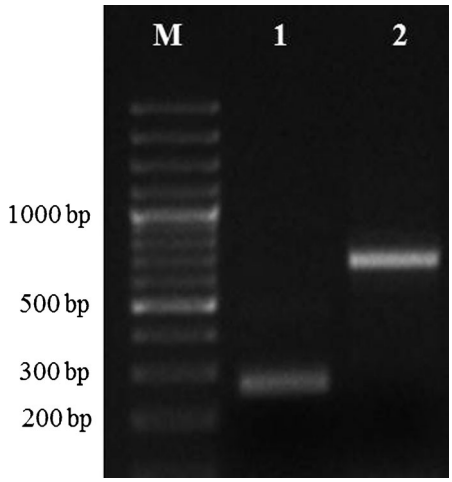

Fig. 1 DNA amplification products of *Meloidogyne hispanica* second-stage juveniles using MIHA-VAP-1f/MHIHA-VAP-1r (1) and MIHA-FAR-1f/MIHA-FAR-1r (2) primers. Lane M, DNA marker (GeneRuler 1 kb Plus DNA ladder, Fermentas)

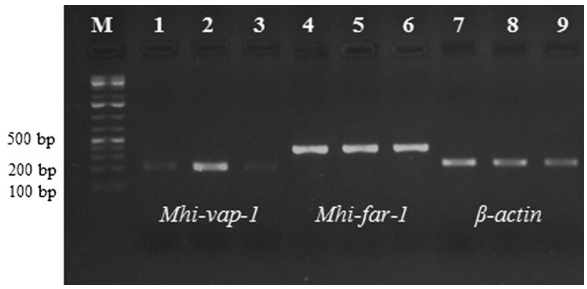

Fig. 2 Expression of the *Mhi-vap-1* and *Mhi-far-1* genes detected by reverse transcriptase mediated PCR amplification of cDNAs from *Meloidogyne hispanica* eggs, second-stage juveniles (J2) and females. Lanes 1, 4 and 7 cDNA templates from eggs; lanes 2, 5 and 8 J2 cDNA; lanes 3, 6 and 9 females cDNA; lane M, DNA marker (GeneRuler 1 kb DNA ladder, Fermentas). As a positive control, cDNA templates were amplified with the primers of *β*-actin gene

In order to localize the expression of the genes *Mhi-vap-1* and *Mhi-far-1* in nematodes, in situ hybridization experiments were performed. Sense and antisense cDNA probes were used against *M. hispanica* J2. The genes specific antisense cDNA probe specifically hybridized with mRNA accumulated within the subventral oesophageal gland cells of J2 (Fig. 3b and d). No hybridization signal was detected in the nematode when using control sense probes (Fig. 3a and c).

Phylogenetic analyses

Using the primers designed in this work (MIHA-VAP-1f/r and MIHA- FAR-1f/r), *vap-1* and *far-1* were identified and successfully amplified in *M. hispanica* and in three additional *Meloidogyne* species. The sequences obtained were used for further phylogenetic analyses of the predicted amino acid and partial gene sequences (Figs. 5 and 6). Analyzing the multiple alignments obtained to VAP-1 and FAR-1, the MHI-VAP-1 sequence differed by two amino acid positions from *M. incognita* and 11 from *M. hapla* while MHI-FAR-1 differed from *M. hapla* six positions (Fig. 4). *Globodera pallida*, GP-FAR-1, showed 43 amino acid differences in alignment with the other *Meloidogyne* species (Fig. 4b). The MHI-VAP-1 amino acid sequences displayed sequence identities ranging from 77.2 (*M. hapla*) to 100 % (*M. arenaria* and *M. javanica*) when compared with the other species and the MHI-FAR-1 from 63.9

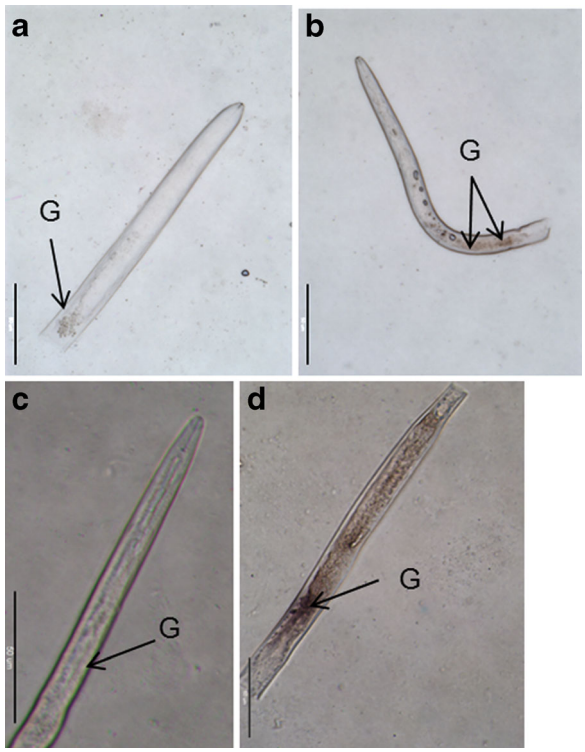

Fig. 3 *Meloidogyne hispanica* second-stage juveniles (J2) sections hybridized with digoxigenin-labeled sense and antisense cDNA probes derived from the *Mhi-vap-1* (**a** and **b**) and *Mhi- far-1* (**c** and **d**) genes. **a** and **c** - Alkaline phosphatase staining is absent in J2 that have been incubated with the sense probe. **b** and **d** - Alkaline phosphatase staining shows specific binding of the antisense probe to the cytoplasm of the subventral oesophageal gland cells (G). Scale bars=50 μm

(*G. pallida*) to 100 % (*M. arenaria*, *M. incognita* and *M. javanica*) (Tables 2 and 3). The phylogenetic analyses revealed that VAP-1 shared high protein homology and phylogenetic relations with *M. hispanica*, *M. arenaria*, and *M. javanica* (Fig. 5a), whereas FAR-1 was closely related to the four RKN species, *M. hispanica, M. arenaria*, *M. incognita* and *M. javanica* (Fig. 5b). In both proteins, *M. hapla* was the most divergent RKN species (Fig. 5).

The topology of the VAP-1 tree was identical to the one obtained from DNA sequences (Figs. 5a and 6a). The topology of the FAR-1 protein and DNA trees exhibited some differences, specifically on the cluster formed by *M. hispanica, M. arenaria*, *M. incognita* and *M. javanica* (Figs. 5b and 6b). These results are congruent with the differences observed between the identity values determined for the protein and DNA alignments (Tables 3 and 5). *Mhi-vap-1* sequence exhibited sequence similarity values ranging from 83.7 % (*M. hapla*) to 100 % (*M. arenaria* and *M. javanica*) (Table 4) and formed a closed cluster with *M. arenaria* and *M. javanica* (Fig. 6a). *Mhi-far-1* displayed sequence similarity values of 55.5 % towards *G. pallida* and from 87.7 to 100 % towards *Meloidogyne* spp. (Table 5) and formed a well-supported clade with *M. incognita* with 100 % bootstrap (Fig. 6b). *Meloidogyne hapla* was the most divergent *Meloidogyne* spp. (Fig. 6a and b).

In fact, the protein alignment identity values decreased slightly when compared to the values obtained from DNA sequences alignment (Tables 3 and 5), most probably related with the presence of synonymous mutation, thus explaining the difference of the phylogenetic results.

Discussion

The effector genes *vap-1* and *far-1* (encoding proteins VAP-1 and FAR-1, respectively) have been identified for the first time in *M. hispanica* and the partial sequences of these genes were successfully amplified. The phylogenetic analyses, conducted on VAP-1 and FAR-1 and on partial genes sequences, determined that *M. hispanica* was most closely related with *M. arenaria*, *M. incognita* and *M. javanica*, and *M. hapla* was the most divergent of the *Meloidogyne* species. The presence of synonymous mutation was also observed indicating a conservation of the protein sequences within the *Meloidogyne* species, most probably due to its functional specificity.

Two types of VAPs have been identified in nematodes: a short single domain type, of approximately 220 amino acids, and a longer double domain type of approximately 425 amino acids (Bin et al. 1999; Hawdon et al. 1999; Gao et al. 2001). *Meloidogyne hispanica* MHI-VAP-1 is representative of the single domain venom allergen-like protein, the most common type found in nematodes (Gao et al. 2001).

Multiple sequence alignment showed that MHA-VAP-1 contains the most variant amino acids residues compared with *M. hispanica*, *M. arenaria* and *M. javanica*. MHI-VAP-1 partial predicted amino acid sequence has 100 % homology with MA-VAP-1 and MJ-VAP-1, indicating a strong conservation of these proteins.

Previous phylogenetic studies demonstrated that FAR homologues from the animal parasitic nematodes *Onchocerca*, *Brugia*, *Wuchereria*, *Loa*,

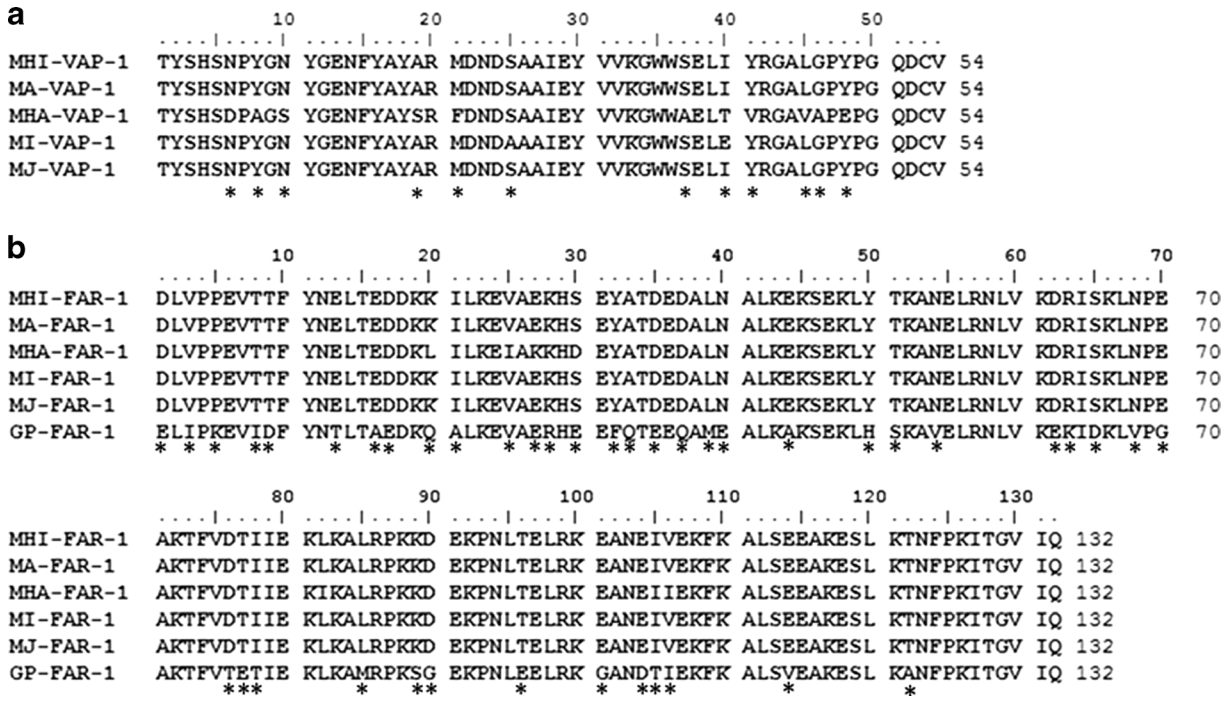

Fig. 4 Multiple sequence alignment of *Meloidogyne hispanica* venom allergen-like protein, MHI-VAP-1 (KF030969) (**a**), and fatty acid and retinol binding protein, MHI-FAR-1 (KF030974) (**b**), amino acid sequences with homologues from other phytoparasitic nematodes. MA-VAP-1 (KF030971) and MA-FAR-1 (KF030973) from *M. arenaria*; MHA-VAP-1 (MhA1_contig2874) and MHA-FAR-1 (Mh10g200708_contig113) from *M. hapla;* MI-VAP-1 (KF030970) and MI-FAR-1 (KF030975) from *M. incognita*; MJ-VAP-1 (KF030972) and MJ-FAR-1(KF030976) from *M. javanica* and GP-FAR-1 (CAA70477.2) from *Globodera pallida.* Differences between amino acids are indicated by asterisks

Acanthocheilonema, Ascaris suum, Toxocara canis, and *Litomosoides*, and the plant-parasitic nematodes *G. pallida, G. rostochiensis, M. javanica, M. arenaria, H. schachtii, P. vulnus* and *R. similis*, and the free-living nematode *Caenorhabditis elegans* were closely related and they all share conserved amino acid sequences in its primary and secondary structures (Prior et al. 2001; Garofalo et al. 2002; Iberkleid et al. 2013). Mj-FAR-1 grouped closely with FARs belonging to other parasitic nematodes (animal, sedentary, and migratory plant-parasitic nematodes). However, FAR proteins clearly distinguished and grouped different nematode species according to their trophic group and nematode parasitism strategy. The highest predicted amino acid identity of Mj-FAR-1 was found amongst *M. incognita, M. arenaria* and *M. hapla* and the lowest observed between *M. javanica* and *M. chitwoodi* (Iberkleid et al. 2013). Although, there is a strong conservation for this group of proteins, *M. hapla* in this study showed to be the most divergent when compared with *M. hispanica*, which might account for a particular mode of parasitism adaptation and/or reproduction. *Meloidogyne hapla* reproduce by facultative meiotic or mitotic parthenogenesis while *M. hispanica*, *M. arenaria*, *M. incognita* and *M. javanica* reproduce by obligatory mitotic parthenogenesis (Chitwoodi and Perry 2009).

The high degree of conservation in the lipid-binding characteristics of FAR proteins and their presence at the host parasite interface, across multiple families of para-

Table 2 Pairwise sequence identities among *Meloidogyne hispanica* (MHI-VAP-1, KF030969), *M. arenaria* (MA-VAP-1, KF030971), *M. hapla* (MHA-VAP-1, MhA1_contig2874), *M. incognita* (MI-VAP-1, KF030970) and *M. javanica* (MJ-VAP-1, KF030972) sequences of VAP-1 protein using the Poisson correction method included in MEGA5

	MHI-VAP-1	MI-VAP-1	MA-VAP-1	MJ-VAP-1
MI-VAP-1	96.2			
MA-VAP-1	100	96.2		
MJ-VAP-1	100	96.2	100	
MHA-VAP-1	77.2	74.9	77.2	77.2

Table 3 Pairwise sequence identities among *Meloidogyne hispanica* (MHI-FAR-1, KF030974), *M. arenaria* (MA-FAR-1, KF030973), *M. hapla* (MHA-FAR-1, Mh10g200708_contig113), *M. incognita* (MI-FAR-1, KF030975), *M. javanica* (MJ-FAR-1, KF030976) and *Globodera pallida* (GP-FAR-1, CAA70477.2) sequences of FAR-1 protein using the Poisson correction method included in MEGA5

	MHI-FAR-1	MI-FAR-1	MA-FAR-1	MJ-FAR-1	MHA-FAR-1
MI-FAR-1	100				
MA-FAR-1	100	100			
MJ-FAR-1	100	100	100		
MHA-FAR-1	95.3	95.3	95.3	95.3	
GP-FAR-1	63.9	63.9	63.9	63.9	61.7

sitic nematodes, support the hypothesis that this nematode restricted family of proteins play a crucial role in the life cycle, and in the parasitism of their host (Bath et al. 2009). Only six differences were observed in the predicted amino acid sequences between MHA-FAR-1 and MHI-FAR-1, MA-FAR-1, MI-FAR-1 and MJ-FAR-1.

The cDNA transcription analysis demonstrated that *Mhi-vap-1* and *Mhi-far-1* genes were transcribed in eggs, J2 and females. Moreover, a high expression of MHI-VAP-1 was evidenced in *M. hispanica* J2 and a very low level of expression was detected in eggs. In contrast, almost equal expression of MHI-FAR-1 was detected in eggs, J2 and females, which suggest a potential role during the different developmental stages.

The localization of the *Mhi-vap-1* and *Mhi-far-1* transcripts in J2, by in situ hybridization, revealed that there is a specific binding of the antisense probe to the subventral oesophageal glands. These results suggest that, in *M. hispanica* VAP-1 and FAR-1 might be natural components of the nematode secretions which are released through the stylet by the J2.

Remarkable similarities of VAP proteins to allergens from hymenopteran insect venoms were found and the in situ hybridization showed that homologues of this gene are present in the gland cells of *H. glycines* and *Ditylenchus destructor* (Gao et al. 2001; Peng et al. 2013). Furthermore, the venom allergen AG5-like protein and the *Mi-vap-2* gene were expressed exclusively in the oesophageal glands of pre-parasitic and parasitic J2 of *M. incognita* (Ding et al. 2000; Wang et al. 2007) while the *Mhi-vap-1* was detected in oesophageal glands of pre-parasitic J2 being weakly transcribed in eggs and females. Animal parasitic allergen genes have been associated with the induction of a host immune response (Chen et al. 2010) and the effector Gr-VAP-1, localized in the subventral oesophageal glands of *G. rostochiensis* pre-parasitic J2, has also been implicated in resistance (Lozano-Torres et al. 2012).

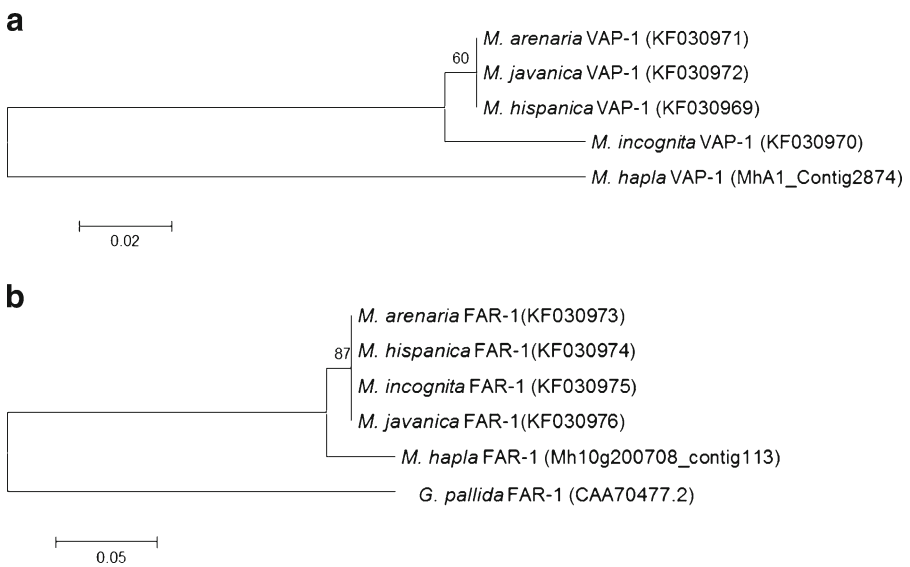

Fig. 5 Phylogenetic trees constructed on the basis of the predicted venom allergen like protein (VAP-1) sequences from *Meloidogyne hispanica*, *M. arenaria*, *M. hapla, M. incognita* and *M. javanica* (**a**) and fatty acid and retinol binding protein (FAR-1) sequences from *M. hispanica*, *M. arenaria*, *M. hapla, M. incognita, M. javanica* and *Globodera pallida* (**b**). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches

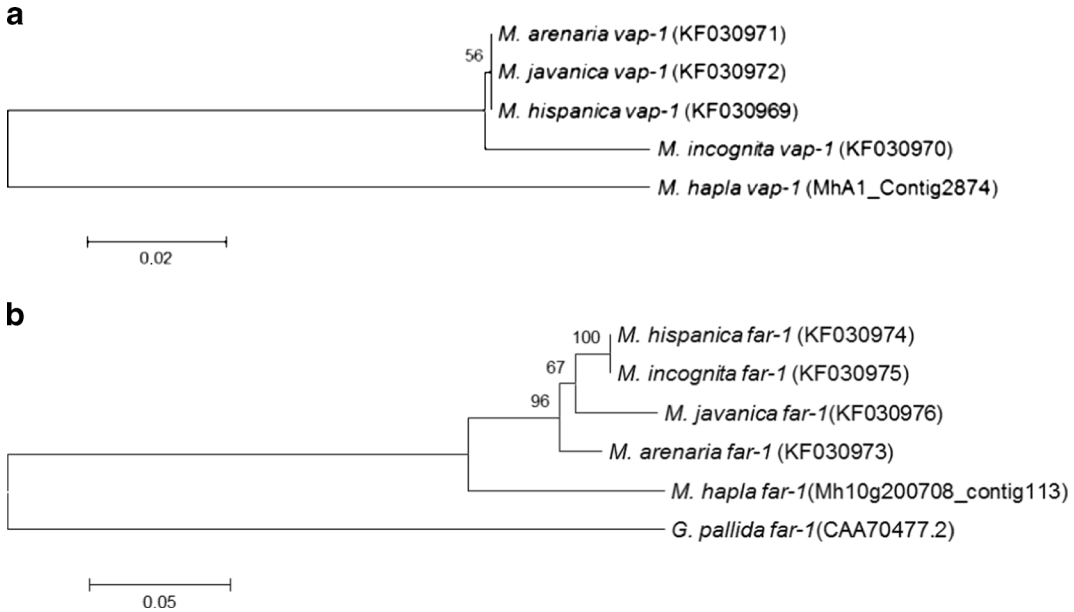

Fig. 6 Phylogenetic analysis of the venom allergen like protein (*vap-1*; A) and fatty acid and retinol binding protein (*far-1*; B) gene sequences. *Meloidogyne hapla* and *Globodera pallida* were included for comparison. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches

Using RT-PCR, this study shows that equal expression of MHI-FAR-1 was detected in eggs, J2 and females however, quantitative real time PCR indicates that for *M. javanica* the lowest level of expression for *mj-far-1* transcripts was detected within eggs and the highest in J2 within the first hours after root infection, which suggest a potential role of MJ-FAR during the different parasitic stages (Iberkleid et al. 2013). Using in situ hybridization, this work localized the *Mhi-far-1* in the subventral oesophageal glands of J2 and the same localization, using different approaches, was identified for a protein similar to the Gp-FAR in the oesophageal glands of *M. incognita*, another FAR protein of the plant parasitic nematode *D. africanus* was also identified in the oesophageal glands (Bellafiore et al. 2008; Haegeman et al. 2009).

However, in contrast with this work, *G. pallida* and *M. javanica* FAR-1 proteins have been shown to be highly expressed in the migratory and parasitic J2 with transcription in the nematode surface and also within the adult female body, using immunolocalization studies with the same antiserum (Prior et al. 2001; Iberkleid et al. 2013). Although *M. hispanica far-1* formed a well supported clade (100 %) with *M. incognita* it showed only 67 % bootstrap with *M. javanica* which could

Table 4 Pairwise sequence similarities between *Meloidogyne hispanica* (*Mhi-vap-1*, KF030969), *M. arenaria* (*Ma-vap-1*, KF030971), *M. hapla* (*Mha-vap-1*, MhA1_contig2874), *M. incognita* (*Mi-vap-1*, KF030970) and *M. javanica* (*Mj-vap-1*, KF030972) sequences of *vap-1* gene using MEGA5*

	Mhi-vap-1	*Mi-vap-1*	*Ma-vap-1*	*Mj-vap-1*
Mi-vap-1	97.5			
Ma-vap-1	100	97.5		
Mj-vap-1	100	97.5	100	
Mha-vap-1	83.7	81.4	83.7	83.7

* Analyses were conducted using the Maximum Likelihood model

Table 5 Pairwise sequence similarities between *Meloidogyne hispanica* (*Mhi-far-1*, KF030974), *M. arenaria* (*Ma-far-1*, KF030973), *M. hapla* (*Mha-far-1*, Mh10g200708_contig113) *M. incognita* (*Mi-far-1*, KF030975), *M. javanica* (*Mj-far-1*, KF030976) and *Globodera pallida* (*Gp-far-1*, CAA70477.2) sequences of *far-1* gene using MEGA5*

	Mhi-far-1	*Mi-far-1*	*Mj-far-1*	*Ma-far-1*	*Mha-far-1*
Mi-far-1	100				
Mj-far-1	95.8	95.8			
Ma-far-1	96.9	96.9	94.8		
Mha-far-1	87.7	87.7	87.1	88.3	
Gp-far-1	55.5	55.5	53.6	55.9	53.6

* Analyses were conducted using the Maximum Likelihood model

account for a particular mode of parasitism adaptation of this gene in *M. hispanica* and *M. incognita*.

We also showed in this paper that the localization of *Mhi-far-1* is similar to *M. incognita* FAR protein but differs in *M. hispanica* in comparison with *M. javanica* (Bellafiore et al. 2008; Iberkleid et al. 2013).

The FAR-1 protein binds to linolenic and linoleic acid, which are precursors of plant defence compounds in the jasmonic acid signaling pathway (Prior et al. 2001; Curtis 2007). It was recently detected in the *M. javanica* cuticle surface and along the adjacent host root tissues and a continuous secretion of this protein into the intercellular space between the nematode body and the host cells was proposed (Iberkleid et al. 2013). The authors also reported that tomato plants over expressing of *Mj-far-1* are highly susceptible to nematode infection indicating that the FAR protein might be involved in the manipulation of host lipid-based defences playing an important role in the parasitism of RKN (Iberkleid et al. 2013).

The localization of the expression of the genes *vap-1* and *far-1* in the subventral oesophageal glands suggest a potential parasitic function for these genes in *M. hispanica*. Further work is being conducted in order to assess whether the silencing of these genes affects *M. hispanica* behavior and whether they are essential for successful infection.

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