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Plant parasitic nematode proteins and the host–parasite interaction

Rosane H. C. Curtis

Abstract

This review focuses on the proteins and secretions of sedentary plant parasitic nematodes potentially important for plant–nematode interactions. These nematodes are well equipped for parasitism of plants. Having acquired the ability to manipulate fundamental aspects of plant biology, they are able to hijack host-cell development to make their feeding site. They feed exclusively from feeding sites as they complete their life cycle, satisfying their nutritional demands for development and reproduction. Biochemical and genomic approaches have been used successfully to identify a number of nematode parasitism genes. So far, 65 204 expressed sequence tags (ESTs) have been generated for six *Meloidogyne* species and sequencing projects, currently in progress, will underpin genomic comparisons of *Meloidogyne* spp. with sequences of other pathogens and generate genechip microarrays to undertake profiling studies of up- and down-regulated genes during the infection process. RNA interference provides a molecular genetic tool to study gene function in parasitism. These methods should provide new data to help our understanding of how parasitic nematodes infect their hosts, leading to the identification of novel pathogenicity genes.

Keywords: Globodera *sp.;* Meloidogyne *sp.; nematode secretions; monoclonal antibodies; nematode genes*

BACKGROUND

Currently, plant parasitic nematodes are a major limitation on crop yield and quality causing estimated losses of $70 billion per annum [1]. The sedentary plant parasitic root-knot nematodes (*Meloidogyne* spp.) and cyst nematodes (*Globodera* spp. and *Heterodera* spp.) are amongst the world's most damaging agricultural pests attacking a wide range of crops. Due to their economic importance and their intimate host–parasite relationship, this review focuses on these nematodes. Current approaches to combat agricultural losses are the use of nematicides, cultural techniques and resistant varieties that may be used in an integrated manner. Nematicides include some of the most hazardous compounds used in agriculture and alternative control is required urgently, because of health and environmental concerns over their use. The complex nematode life cycle consists of eggs and a distinct free living pre-parasitic stage in the soil and parasitic stages inside the root tissue. Once hatched from eggs,

the second-stage juvenile (J2) of cyst and root-knot nematodes does not feed in soil and thus must have a behavioural strategy that makes efficient use of its lipid reserves to find a host plant; if these reserves are depleted by more than 65% of the original level the juvenile is unable to invade plants and establish a feeding site [2]. Therefore, to establish a successful parasitic relationship with plants, nematodes rely on behavioural strategies based on their well developed nervous system, including specific sense cells and also on special structures (such as the stylet and large pharyngeal glands) for efficient root-cell penetration and modification, and food withdrawal and digestion.

Biology of cyst and root-knot nematodes

The J2s of cyst forming nematodes are attracted to, and penetrate, plant roots to migrate intracellularly towards the vascular cylinder, where they establish an intimate nutritional relationship with their host through the development of syncytial feeding sites.

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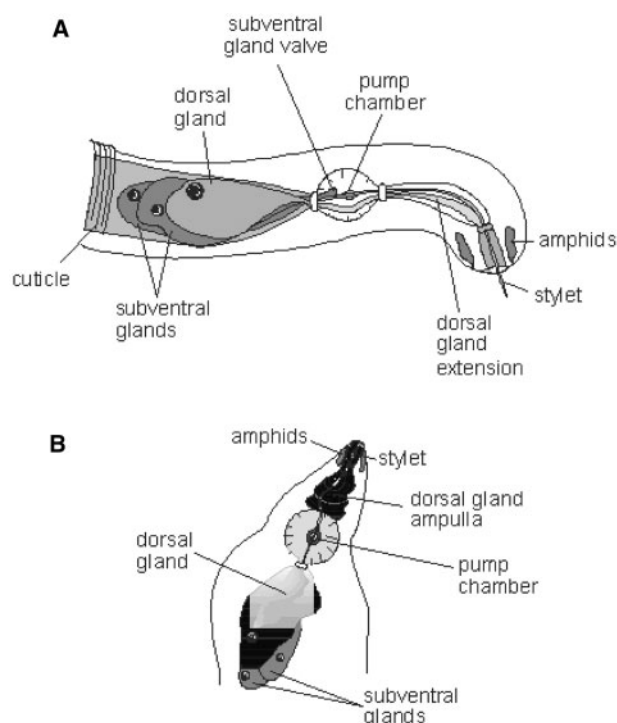
Figure I: Diagram of the anterior part of the body of a JR (**A**) and an adult female (**B**) of a typical sedentary plant parasitic nematode.

During nematode development, the syncytium continuously increases in volume by incorporation of neighbouring cells through cell wall breakdown. The syncytium becomes a large multinucleated hypertrophied cell generated by the fusion of as many as 200 neighbouring protoplasts after partial cell wall dissolution [3]. The juveniles undergo three additional moults before reaching the adult stage. When feeding commences, the juvenile body grows and becomes saccate and immobile (Figure 1A and B). The vermiform males regain their mobility and leave the root to migrate in the soil, where they are attracted to the females by a pheromone and fertilization occurs. The fertilized females produce eggs, most of which remain inside their bodies. They become a protective cyst, when they die and under favourable conditions the J2 will hatch and migrate towards a new host root [3].

In contrast, J2 of root-knot nematodes after penetrating the root epidermis migrate intercellularly between cortical cells until they find a suitable root cell to form their feeding site. Root cells around the nematode's head are stimulated to go through repeated rounds of mitosis uncoupled from cytokinesis, leading to multinucleated giant cells [4]. The juveniles moult three times and although males

can be formed, reproduction of most root-knot nematodes is by parthenogenesis. Eggs are deposited outside the female body and stay in a protective proteinaceous matrix secreted by the female until they are ready to hatch as J2 and then continue the nematode's life-cycle which takes approximately 25–30 days from eggs to adults.

Syncytium and giant cell maintenance require repeated stimulation from the nematode and cyst, and root-knot nematodes depend entirely on functional feeding cells to complete their life cycles.

Nematode invasion of roots and their migration to their feeding sites results in changed root architecture and significant reductions in nutrient and water uptake and consequent crop yields. The host–parasite relationship is governed by a complex network of interactions and in susceptible interactions there is a subtle interplay between parasite survival strategies and host defense mechanisms. Understanding the complexity of the molecular signal exchange and response during infection of plants is important to identify vulnerable points in the life cycle of the parasite, which can be used to target disruption of nematode–host-recognition, nematode migration and feeding inside root tissue. This information will be useful in defining those processes that are essential for pathogenesis by the nematode.

Nematode signals potentially important for the plant–nematode relationship

Feeding cell formation is presumably initiated in response to signal molecules released by the parasitic J2, but the nature of the primary stimulus is unknown, as is the host target for the presumed nematode ligand(s), which must be transduced to elicit the feeding site. The most widely held hypothesis is that the necessary metabolic re-programming of root cells is triggered by specific nematode secretions, which presumably interact with membrane or cytoplasmic receptors in the plant to switch on cascades of gene expression that alter cell development [5–7].

As nematodes set up their feeding site they alter plant gene expression, leading to the re-differentiation of the parasitized cell [8]. Secretions of plant parasitic nematodes, which are released into the plant tissue, may play a role in pathogenesis. They probably have a role in: (i) penetration and migration of second stage juveniles (J2) in the plant tissue, (ii) modification and maintenance of plant cells as feeding sites,

(iii) digestion of host cell contents to facilitate nutrient acquisition by the nematodes (iv) suppression of host responses [9–11]. Secretions from the nematode pharyngeal glands, surface cuticle and the amphids, the main nematode chemosensory organs, are the primary signals at the host interface and they are considered to contain nematode pathogenicity factors [11].

The complexity and secretory function of the three pharyngeal glands, one dorsal and two subventral glands are reported in ultrastructural studies [12]. Secretions from the dorsal gland are transported through a cytoplasmic extension and are released in the pharynx close to the base of the stylet, whereas the two subventral glands empty their granules behind the pump chamber (Figure 1A and B). Significant changes in morphology of the pharyngeal glands accounts for a role for the pharyngeal secretions in the initiation and main-tenance of the feeding cells [13, 14]. Triggers for feeding site induction are believed to originate mainly in the dorsal glands and there is some evidence for the role of the subventral glands in the early events of parasitism, i.e. plant invasion and migration of J2 through plant tissues [15, 16].

The two bilaterally symmetrical amphids in the nematode head are the main nematode chemosen-sory organs involved in host-recognition processes. Host signals play an important role in the life cycle of sedentary plant-parasitic nematodes and this is frequently synchronized with that of their plant hosts to optimize the chances of successful invasion [5, 8]. Nematodes rely on chemoreception to find a host in the soil and when a root is encountered, its surface is explored for a suitable penetration site. At this stage, plant chemicals in the rhizosphere originating from root diffusates or sites of previous penetration, can influence nematode behaviour. Chemoreception might also be involved in helping nematodes locate suitable root cells to form their feeding sites [8, 9].

Several other roles have been considered for the secretions that are found in the amphids of plant-parasitic nematodes. These include capture and transport of chemotactic stimuli to the sensilla membranes [17], protection of the sensory dendrites at the base of the pore [18] and osmoregulation [19]. Secretions from the nematode amphids have also been implicated in forming the nematode feeding plug, that functions to seal the orifice of insertion of the nematode stylet into the root cell [9]. The amphids might also be involved in other aspects of the host–parasite interaction, since a putative avirulence gene from the root-knot nematode encodes a secreted amphidial protein [20].

The nematode cuticle surface is a dynamic structure and plays important physiological roles in: locomotion, the onset of moulting, nutrition, resistance to dehydration and physical protection [21]. The external cuticular layer of nematodes is the epicuticle, covered in many species by a surface coat (SC). The SC is composed mainly of proteins, carbohydrates and lipids [22]. One of the most interesting features of the nematode SC is its dynamic nature; there is a continuous turn-over of the surface associated antigens, that involves shedding and replacement of the surface antigens [23]. In plant-parasitic nematodes this was demonstrated for the pre-parasitic juveniles of *Meloidogyne* spp., the hypodermis is the suggested site of production of surface antigens and the turnover rate of secreted proteins is approximate 1.5 h [24–29].

The SC of the J2 of plant-parasitic nematodes is considered to be involved in interactions with micro-organisms in the soil and rhizosphere, as well as with the host plant. Phytohormones such as auxin and cytokinin as well as other molecules present in root diffusates, have been shown to trigger a rapid alteration of the surface cuticle of sedentary plant-parasitic nematodes [30, 31]. Therefore, phytohormones act as signalling molecules that prepare *Meloidogyne* sp. for root invasion by inducing changes in the nematode cuticle and nematode behaviour that are probably essential for host infection [30, 31]. This significant increase in the lipophilicity of the nematode surface cuticle might play an important role in altering the permeability barrier of the nematode cuticle and therefore control the uptake of water, ions and lipids in the nematode, which are important for cell signalling [32].

The nematode surface has also been implicated in disguising the invading nematode from the host and in protecting the nematode from plant defense responses [27].

Biochemical approaches for the identification and characterization of nematode secretions involved in the plant–nematode interactions

Identification of nematode secretions is essential to understand the processes involved in nematode invasion of roots and establishment of feeding cells in their host.

Plant nematodes are small (0.5 mm long by 20 μm wide) obligate parasites, they are difficult to produce in large numbers and the parasitic stages live inside plant roots making the direct biochemical analysis of their secretions an extremely difficult task. Nevertheless, a number of approaches have been used successfully to identify and partially characterize nematode secretions. Nematodes were incubated with serotonin agonists such as 5-methoxy-N, N-dimethyltryptamine (DMT) to increase pharyngeal pumping which artificially induces the nematodes to secrete. *Globodera rostochiensis* secretions produced in this way were concentrated by ultrafiltration and at least 10 proteins were detected by SDS-PAGE ranging from 15 kDa to 70 kDa and the presence of proteases and superoxide dismutase (SOD) were demonstrated in activity gels. The SOD may have a role in protecting the nematode from the plant resistant response, neutralizing oxygen free radicals produced in the oxidative burst associated with infection [26, 27]. A small peptide (3 kDa) which was isolated from secretions of *G. rostochiensis* has the ability to co-stimulate the proliferation of tobacco leaf protoplasts in the presence of synthetic auxin and cytokinin [33].

Biochemical methods for identification of protein–protein interactions, such as immunoprecipitation and purification through chromatographic columns are generally time consuming, however, the rapid advances in protein analytical technologies, makes mass spectrometry-based interactive proteomics a method of choice for analyzing functional protein complexes. A combination of 2D-gel electrophoresis with micro-sequencing has led to the identification of two endoglucanases and a novel protein in the secretions of the cyst nematode *Heterodera schachtii* and a calreticulin and a 14–3–3 protein in the secretions of *Meloidogyne incognita* [34, 35]. The last two proteins have multiple functions including regulation of signaling and metabolic pathways and the control of the cell cycle [36].

The use of monoclonal antibody (Mab) technology led to the identification of a number of secretory proteins produced in the pharyngeal glands, amphids and cuticle. This technology was very useful and enabled the localization of the secretions in the nematode body of different nematode developmental stages and *in planta* [5, 11, 37].

However, only a few nematode genes encoding secretions were identified by screening nematode cDNA expression libraries with antibodies [27, 38–42]. An annexin gene (*Gp-nex-1*) and putative collagen gene (gp-*col*-8) were isolated from a *G. pallida* expression library by screening with a polyclonal and a Mab, respectively, both antibodies reacted with antigens present in the amphids of *Globodera* spp. [39–41]. Annexin is a calcium-dependent phospholipid binding protein and it was localized in secretions from the nematode amphids. The gene gp-nex-1 has a potential phosphorylation site that could be phosphorylated by protein kinase C and this might play an important physiological role in this secreted protein. Monoclonal antibodies directed against amphidial secretions were shown to interfere with nematode invasion of plants and therefore secretions from the amphids might be involved in host–recognition processes [43]. The gene *gp-col-8* was very similar to cuticular collagen genes and the putative collagen protein was present in the amphids of J2 and in the hypodermis of adult females. Also, this collagen protein was immunolocalized in the cuticle of the first stage juvenile from within the egg and of the parasitic J2 inside the root tissue. Therefore, it seems to play a role in the construction of the cuticle of only two moulting developmental stages [44].

Polyclonal antibodies raised against nematode secreted/excreted products were also used to probe nematode expression libraries and a peroxiredoxin (rGrTpX), a retinol and fatty acid-binding protein (Gp-FAR–1) and two novel genes (*gp-sec-2* and *gp-sec-3*) were isolated and localized on the surface cuticle of *Globodera* spp. The nematode peroxiredoxin protein was shown to catalyse the breakdown of hydrogen peroxide *in vitro*, whilst the Gp-FAR–1 was shown to bind to linolenic and linoleic acid which are precursors of plant defense compounds and jasmonic acid signalling. Thus, these proteins might be involved in protecting the nematode from plant defense responses [27, 45].

The Mabs reactive to nematode secretions were used to affinity purify the proteins for amino-acid sequencing [46, 47]. The first nematode pharyngeal secretion to be identified in this way was the enzyme B-1,4-endonuclease. The corresponding genes were then cloned from cDNA libraries and represented the first cellulase genes cloned from an animal [15].

The Mabs reactive to amphidial and cuticular secretions were used to purify nematode antigen for amino-acid sequence analysis, preliminary

results show strong homology of the peptide sequence with a metallopeptidase (R. Curtis, unpublished data), *in situ* hybridization of DNA probes to the nematode gene encoding this enzyme shows expression in the nematode amphidial glands [47].

Nematode secretions immunolocalized *in planta*

Nematode secretions have been demonstrated *in planta* but not inside the nematode feeding sites. Nematode cellulases and pectinases, which are secreted from the pharyngeal glands were immuno-localized *in planta* along the juvenile's migratory path through the root cortex [16, 48]. The role of these enzymes in plant nematode pathogenesis probably involves cell wall loosening to facilitate migration of J2 through the root tissue.

Secretions from the surface cuticle of *Meloidogyne* spp. were immunolocalized in the migratory J2 and sedentary stages *in planta* using a Mab raised to excreted/secreted products obtained by incubation of live second stage juveniles of *Heterodera avenae* with 5-methoxy dimethyltryptamine for 16 h at room temperature [49, 6]. The secretion vesicles were exuded as globules of different sizes over the whole body of these nematodes (Figure 2A–C). Figure 3A and B shows that they were secreted abundantly into the apoplast and were in close contact with the root cells forming the feeding site which were surrounded by these secretions [49]. Preliminary results show that the amino-acid sequence of the purified secretion has homology with metalloenzymes (R. Curtis, unpublished data). This abundant cuticular secretion envelops the adult females and the giant cells and it might have a role in protecting the nematodes from harmful root compounds by masking the cuticle from recognition [49]. A calreticulin secreted from sub-ventral glands of root-knot nematodes has also been immunolocalized *in planta* throughout parasitism [37]. These are calcium binding proteins which regulate cell calcium homeostasis and act mainly as chaperone proteins, in protein export from the nucleus and in cell adhesion [50]. Several roles have been allocated to calreticulins secreted in the host by animal parasites including regulation of host defense responses to parasitism [37].

The calreticulin and metalloproteinase are likely to be involved in the molecular interaction between nematodes and plants.
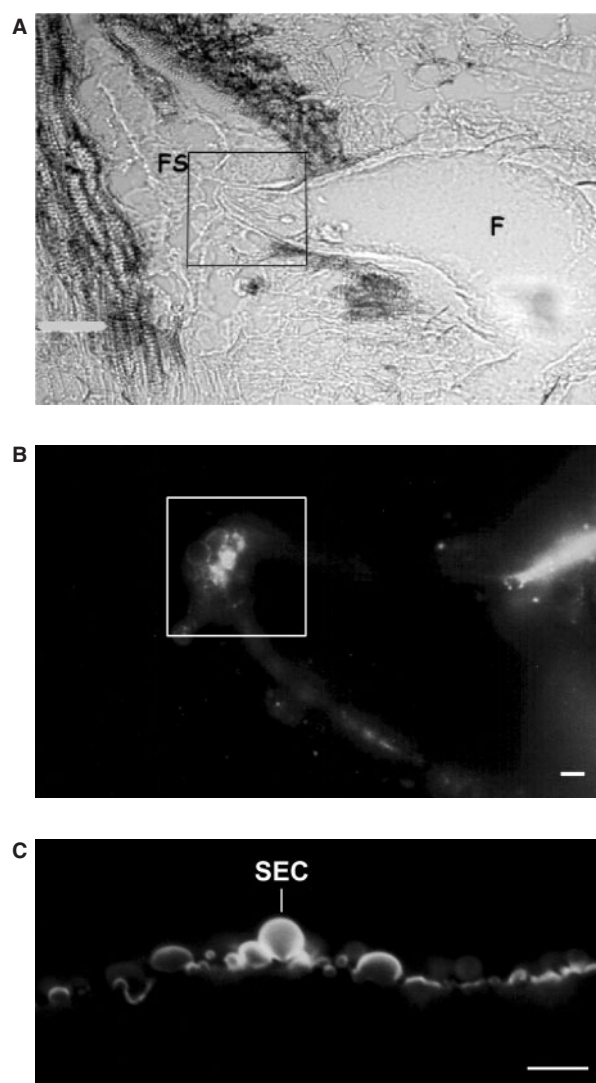

Figure 2: Immunolocalization *in planta* of cuticular secretions of *M. incognita*. (**A**) Bright field photograph of a cryostat section of 3 week old infected root of an aubergine plant showing the adult female and its feeding site, (**B**) immunofluorescence of the previous section showing reactivity of a monoclonal antibody to secretions abundantly produced by the adult female inside the root tissue. The secretions are in close contact with the root tissue and accumulate between the nematode head and the nematode feeding site, (**C**) higher magnification of the female cuticle showing details of the vesicles exuded *in planta*. F, adult female; FS, feeding site; SEC, secretions. Scale bar = 10 μm.

Molecular approaches for identification of nematode parasitism genes

Expressed sequence tag (EST) sequencing projects have been initiated (http://www.nematode.net/ NemaGene/) as a cost-effective method for gene discovery. This strategy has been very successful and
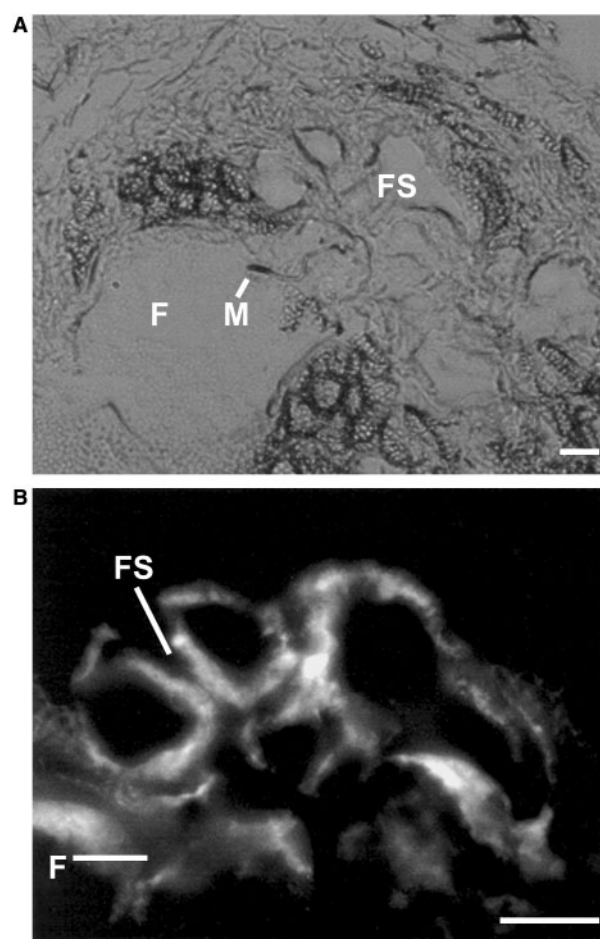
Figure 3: Nematode secretions surround the root cells forming the nematode feeding site. (**A**) Bright field photograph of a cryostat section of 3-week-old infected root of an aubergine plant showing the adult female and its feeding site, (**B**) immunofluorescence of the previous section and reactivity of monoclonal antibody to the nematode head and the outside of the root cells forming the feeding site. F, adult female; M, metacorpus and pump chamber; FS, feeding site. Scale bar = 10 μm.

approximately 82 330 ESTs have been generated for sedentary plant parasitic nematodes, several interesting genes have already been cloned, such as reviewed elsewhere [51, 52]. However, since these cDNA libraries were produced with pre-parasitic J2, which are easier to obtain in greater numbers than the post invasion stages of the nematode, the ESTs sequences characterized so far are biased towards genes expressed during penetration and migration of the nematode in the plant roots [52].

RNA fingerprinting has been used to analyse differential gene expression between pre-parasitic and parasitic stages of *M. incognita* and *G. rostochiensis*

and several pharyngeal gland-specific clones were identified with homology with Ran-binding proteins of unknown function [53, 54]. The same approach was used to identify a new class of ubiquitin extension proteins secreted by the dorsal pharyngeal glands of cyst nematodes [55]. The protein expansin, previously identified only in plants was identified in *G. rostochiensis* using complementary DNA-AFLP (amplified fragment-length polymorphism) and demonstrated to be expressed in the nematode subventral glands [56, 57]. Nematode expansins could be used to loosen cell walls during nematode invasion of the host plant and together with cell wall degrading enzymes may assist the rapid penetration of nematodes into the root tissue.

A number of approaches have been used to isolate parasitism genes specifically expressed in the nematode pharyngeal glands. Differential screening of gene expression using cDNAs from excised nematode pharyngeal glands and the nematode tail region yielded a full-length clone with homology to a bacterial chorismate mutase [58]. This enzyme initiates the conversion of chorismate, the end product of the shikimate pathway, to the aromatic amino acids, phenylalanine and tyrosine. Secretion of nematode chorismate mutase into the cytosol could potentially alter the spectrum of chorismate-dependent compounds, which among other functions, are involved in cell wall formation, hormone biosynthesis and synthesis of defense compounds in plants. Another secreted protein identified with differential hybridization is a pectate lyase, which may soften root tissue during nematode migration [48].

No novel gland specific genes were isolated with the analysis of the nematode transcriptome using cDNA libraries produced from material obtained from direct micro-aspiration of pharyngeal glands [59, 60]. However, by combining EST analysis of gland specific libraries with high-throughput *in situ* hybridization of the genes encoding secretory proteins, 53 genes encoding putative secreted proteins were identified, of which 41 were new gland-expressed candidate genes and 38 were novel sequences [61, 62].

Although, 85% of the *M. incognita* genes identified so far have homology with *C. elegans*, it is unlikely that genes with specific roles in parasitism are represented [51]. Nematode genes involved in vital physiological functions can also be used as targets to disrupt the nematode life-cycle and in this case

C. elegans will be valuable as a model for functional analysis of such genes.

Currently genome sequencing projects for *M. hapla* and for *M. incognita* are underway in the USA and in France, respectively. These data will allow genomic comparisons with sequences of other pathogens and contribute to our understanding of how plant parasitic nematodes cause disease, leading to the identification of parasitism genes.

Functional analysis of nematodes genes

Many candidate parasitism genes in plant nematodes are novel genes with no homologues in the databases and many of them are not present in *C. elegans*, which makes it very difficult to transform the sequence data obtained into useful biological information.

RNA interference (RNAi) has been widely used to characterize gene function in *C. elegans*. This technique is based on the ability of double stranded RNA (dsRNA) to direct sequence specific degradation of homologous RNA molecules [63]. Cyst and root-knot nematodes are biotrophs and do not feed or ingest liquid until their feeding sites are established inside the root tissue. A protocol for RNAi has been devised for cyst and root-knot nematodes and the delivery of dsRNA to the nematodes is achieved by incubating the nematodes with compounds which induce pharyngeal pumping such as octopamine [64–66]. For instance, knockout of cellulases reduced the ability of *G. rostochiensis* J2 to invade roots and a secreted amphid protein was shown to be essential for host location [67]. Transgenic soybeans transformed with an RNAi expression vector containing a cDNA clone of the major sperm protein gene from *H. glycines*, significantly reduced the reproductive potential of this nematode with 68% reduction in eggs g(-1) root tissue [68]. RNAi is a powerful technique for investigating the function of nematode genes and it will be very useful for functional analysis of novel genes for which no homologues exist. Such method will help identify potential nematode targets to use in novel approaches to control plant parasitic nematodes.

SUMMARY

Significant progress has been made to identify proteins involved in the plant–nematode interactions. The possible role of some of the secreted pharyngeal proteins identified is to assist nematode invasion and migration in the root tissue. Some genes encoding for cuticular secretions might be involved in protection of nematodes from host-defense responses. However, many plant nematode genes have no known homology with other genes and their role is unknown. Functional analysis of these genes using RNAi will help to elucidate the function of such genes only if there is a recognizable phenotype. A large number of plant nematode genes will be identified in the sequencing projects currently in progress. This sequence data will enable comparative genomic analysis of free living and parasitic nematodes, leading to the identification of novel parasitism genes and a better understanding of how nematodes cause disease in plants.

Key Points

- Plant nematodes use a combination of expansin and cell degrading enzymes to assist their migration in the root tissue.
- Secretions from the surface cuticle may be protecting the nematode from host-defense responses.
- Many plant nematode genes lack homology to known sequences and RNAi experiments might help investigate the function of these novel genes.
- Interfering with nematode chemoreception may reduce nematode invasion of plants.

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