

Getting to the roots of parasitism by nematodes

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Most phytoparasitic nematodes infect plant roots and some species have evolved sophisticated interactive relationships with host cells to sustain a sedentary parasitic habit. The recent isolation of multiple 'parasitism genes' expressed specifically within the esophageal gland cells of sedentary phytonematodes suggests that an arsenal of different secreted parasitism proteins from the nematode might have direct effects on recipient host cells. These include cell wall modifications and potential interactions with signal transduction receptors in the extracellular space, as well as direct introduction of proteins into host cells that might influence cellular metabolism, the cell cycle, selective protein degradation, a localized defense response and regulatory activity within the host cell nucleus.

Similar to animal parasites, plant-parasitic nematodes (phytonematodes) have evolved a range of parasitic habits, with the majority of the species being soil-dwelling and parasites of plant roots [1,2]. The common adaptation for all nematode parasites of plants is a stylet (Figure 1), which is a hollow, protrusible spear in the oral cavity used to pierce plant cell walls to inject secretions and withdraw host cell nutrients [3,4]. The single dorsal and two subventral esophageal gland cells of Tylenchid and Aphelenchid phytonematodes have evolved into enlarged secretory cells [4] that produce secretions released from the stylet and these play a central role in plant parasitism (Figure 1). In species of root-knot (*Meloidogyne* sp.) and cyst nematodes (*Heterodera* and *Globodera* spp.), the subventral gland cells are active early during the parasitic process, whereas the dorsal gland enlarges and becomes more active as parasitism progresses [3]. Infective, second-stage juveniles (J2) of these species induce elaborate modifications in selected root cells to form enlarged, multinucleate feeding cells (Figure 2) that serve as the sole food source for growth and reproduction of the subsequent sedentary parasitic stages [1,2,5]. These feeding cells have strikingly similar phenotypes to host cell modifications induced by some animal-parasitic nematodes, most notably changes in muscle cells to host the intracellular parasite *Trichinella spiralis* and changes in intestinal epithelia of the crypts of *Trichuris* spp. [6,7]. Likewise, adaptations of the esophagus (stichocytes) for

secretion by these animal-parasitic nematodes might have mechanistic roles in the parasitic process [6].

All host plant cell responses occur around the nematode 'head', suggesting that specific nematode molecules at this interface mediate the parasitic process. Although secretions from the nematode esophageal gland cells injected through the stylet are the principal molecules involved in parasitism, secretions from the chemosensory amphids and localized changes in surface molecules might also play a role in the parasitic process [8–10]. Genes that promote parasitism of a host have been collectively termed 'parasitism genes', and these encode secretions from a nematode that play a direct role in parasitism [3]. The protein products of these genes are considered members of the parasitome [11], a subset of the nematode secretome [12].

Approaches to parasitism gene analysis

Although a few genetic loci that condition parasitic ability have been identified in plant nematodes, the identification of nematode parasitism genes by forward genetics has proven difficult [3]. To date, the successes in identifying nematode parasitism genes have resulted from efforts focused on isolation of nematode factors (secretions) that promote responses in host cells and, more recently, on the isolation of expressed genes from parasitic nematodes [3,6,11,13,14].

Isolation of parasitism proteins

Peptide sequence from an antigen that was affinity purified with an esophageal-gland-specific monoclonal antibody (MGR 48) was used to identify the first parasitism gene from a phytonematode [15], encoding a β -1,4-endoglucanase (cellulase). Subsequent studies demonstrated the expression of cyst nematode endoglucanase genes and their products specifically within the subventral gland cells, cellulolytic activity of the enzymes, and secretion of cellulases from the stylet *in planta* [16,17] during migration of infective J2 through host plant roots (Figure 3). These studies confirmed and refined earlier reports of secreted cell-wall-modifying enzymes from phytoparasitic nematodes [18]. A significant discovery with the nematode endoglucanases was their strong similarity to prokaryote (glycosyl hydrolase family 5) endoglucanases, with little similarity to endoglucanases from eukaryotes and no similarity to any gene in the nematode *Caenorhabditis elegans* [15]. Transmission

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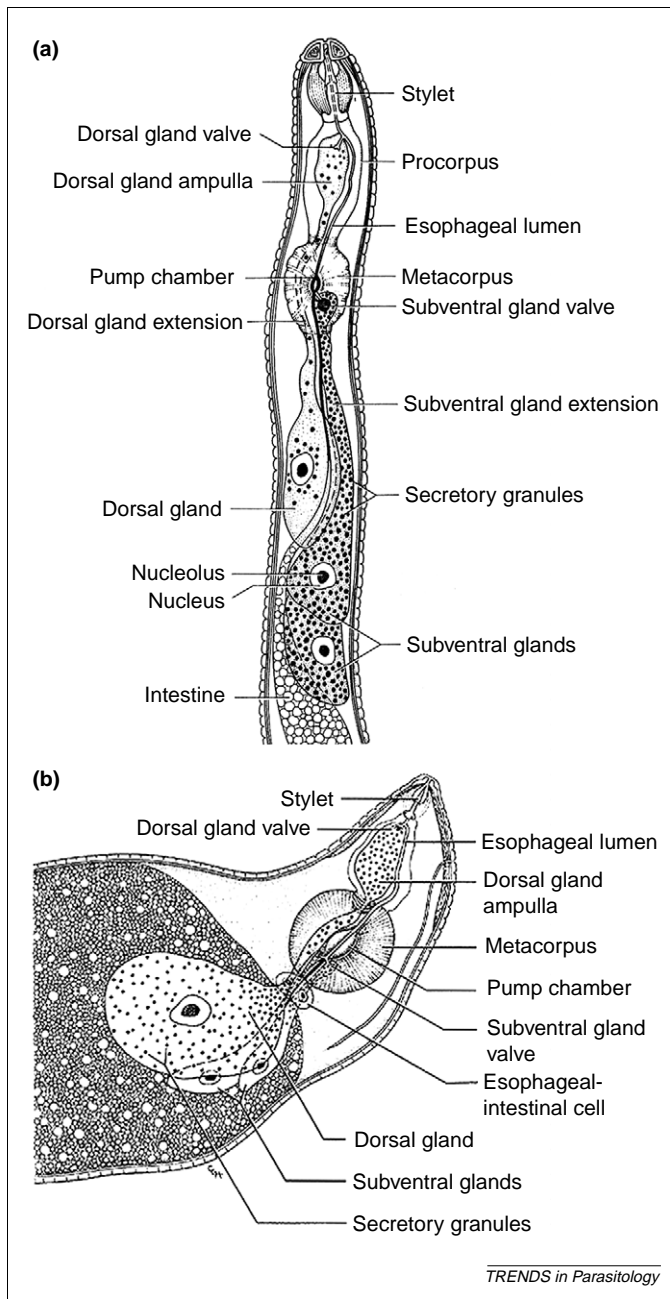


Figure 1. Illustration of the single dorsal and two subventral esophageal gland cells that produce secretions that are introduced into host tissues through the stylet (feeding spear) of plant-parasitic nematodes. (a) A pre-infective second-stage juvenile with numerous secretions packaged within granules in the subventral gland cells. Reproduced, with permission, from Ref. [4]. (b) A late parasitic stage female with enlarged dorsal gland cell packed with secretory granules and reduced subventral gland cells. Reproduced, with permission, from Ref. [64].

electron microscope studies have confirmed the absence of microbial symbionts within the nematode esophageal gland cells [19,20], and genomic studies of the cyst nematode endoglucanase genes verified that the cloned genes were of nematode origin [21]. This was the first report of endogenous cellulases in animals and provided the first evidence of potential horizontal gene transfer from a prokaryote to a nematode [15]. Similarly, a variant of nucleoside diphosphate kinase secreted by *T. spiralis* that was implicated in parasitism had only been previously identified in prokaryotes [22]. The similarity of phytoparasitic nematode genes encoding endoglucanases

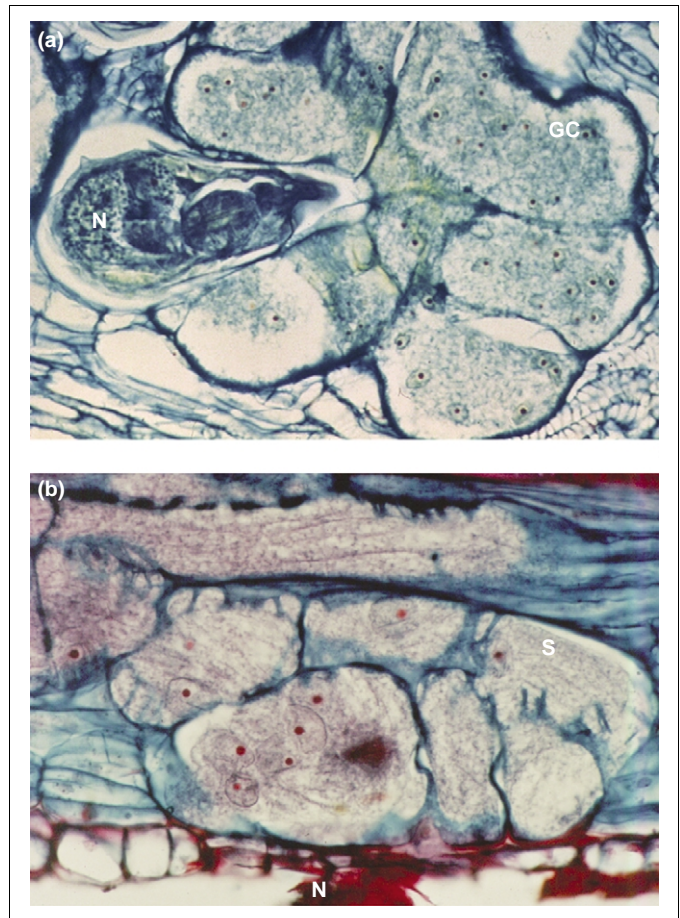


Figure 2. Cross-sections of feeding cells induced in plant roots by sedentary endoparasitic nematodes. (a) Multinucleate giant cells (GC) induced by the root-knot nematode *Meloidogyne incognita*, derived from karyokinesis uncoupled from cytokinesis of plant cells adjacent to the nematode (N) head. Reproduced, with permission, from Ref. [11]. (b) A multinucleate syncytium (S) induced adjacent to the head of the cyst nematode (N) *Heterodera glycines*, formed by dissolution of cell walls to incorporate neighboring plant cells into the feeding site (photo courtesy of Burton Y. Endo).

and other cell-wall-modifying enzymes to corresponding genes in bacteria has supported the hypothesis that this group of parasitism genes might have arisen by gene transfer from prokaryotes to ancestors of contemporary phytoparasitic nematodes [3,6,15].

Differential gene expression

Profiles used to identify genes differentially expressed in several nematode life stages and different nematode tissues led to the discovery of two other gland-expressed parasitism genes that had their highest similarity to prokaryote genes [23,24]. Global analyses of gene expression using expressed sequence tags (ESTs) of hatched J2 of root-knot and cyst nematodes combined with cluster analyses have also been used to identify and group nematode genes based upon predicted function, including potential parasitism gene candidates [25–28]. A dynamic profile of gene expression during parasitism by comparing ESTs throughout the life cycle of the soybean cyst nematode *Heterodera glycines* is now being generated and submitted to the public database (T.J. Baum and J.P. McCarter, unpublished); this is similar to the parasitic-cycle EST set available for the filarial parasitic nematode *Brugia malayi* [29].

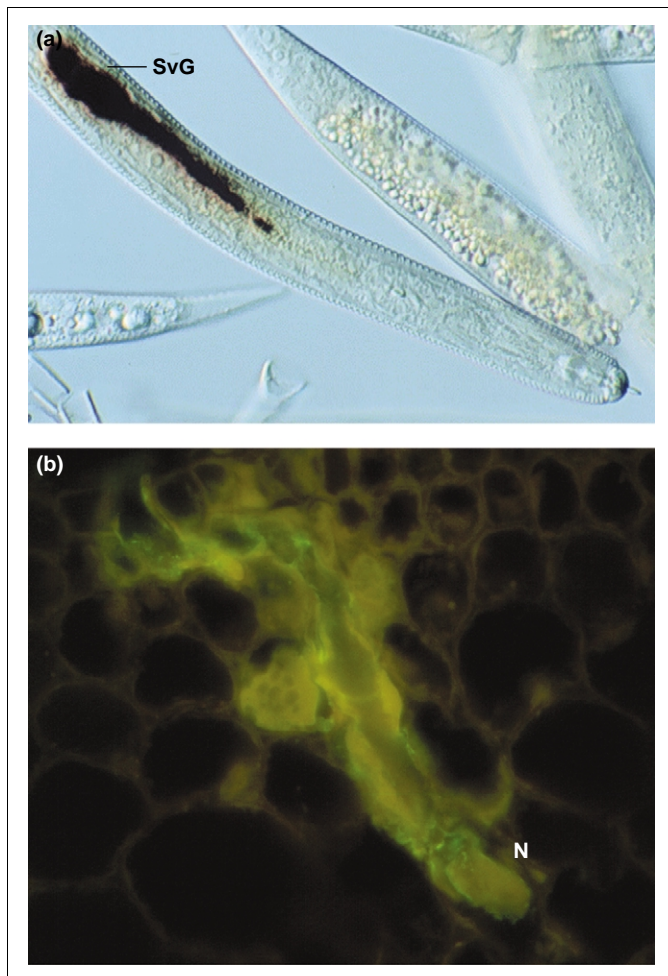


Figure 3. Expression of β -1,4-endoglucanases (cellulases) secreted from cyst nematodes (N). (a) mRNA *in situ* hybridization of a HG-eng-1 probe to transcripts expressed specifically within the two subventral esophageal gland cells of an infective juvenile of *Heterodera glycines*. Reproduced, with permission, from Ref. [33]. (b) Immunolocalization (green fluorescence) of HG-ENG-2 secreted by an infective *H. glycines* juvenile (N) during intracellular migration within a plant root. Reproduced, with permission, from Ref. [16].

A targeted approach to identify expressed parasitism gene candidates from cyst nematodes focused upon regulated activity in the esophageal gland secretory cells [30]. In the potato cyst nematode *Globodera rostochiensis*, the subventral gland cells of J2 inside egg shells produce secretory granules when soaked in water, whereas similar activity in the dorsal gland of J2 is only initiated when eggs are soaked in potato (host plant) root diffusate [31]. A cDNA-amplified fragment length polymorphism (AFLP) analysis of these stages was successful in isolating multiple genes expressed specifically within the esophageal gland cells of the nematode [30]. Identification of a predicted secretion signal peptide on the N-terminus of products of several of these gland-expressed genes identified them as potential parasitism factors [30].

cDNA libraries from nematode secretory cells

The direct approach of micro-aspirating the contents of esophageal gland cells of parasitic phytonematode stages to generate cDNA libraries of gland-cell-expressed genes has recently provided an array of parasitism gene candidates that profile the entire nematode parasitic cycle

[32–36]. Initial attempts to select for candidates from gland-cell cDNA libraries using a secretion signal peptide selection system [32], or microarrays [33], or suppression subtractive hybridization [34] yielded several parasitism gene candidates. Extensive EST analyses of gland-cell libraries from *H. glycines* and *Meloidogyne incognita* were generated using SMART™ cDNA technology (Clontech Laboratories, Palo Alto, CA). These were combined with secretion signal peptide prediction and mRNA *in situ* localization to yield >50 unique cDNA clones in *H. glycines* [35] and at least 37 different clones in *M. incognita* [36] that were identified as encoding products potentially secreted from the esophageal gland cells into host tissues during parasitism. The presence of homologs of most of the previously identified nematode parasitism genes (above) among the *H. glycines* and *M. incognita* gland-cell ESTs validated the new methods employed [35,36]. Interestingly, over 70% of the parasitism genes identified in the *H. glycines* and *M. incognita* gland cell EST analyses had no significant similarity to any reported genes, and few new parasitism gene candidates with database homologs had similarity to prokaryotic genes beyond the described cell-wall-modifying enzymes [35,36]. In addition, strikingly few similarities existed between the parasitism gene candidates of *H. glycines* and *M. incognita*, suggesting that the molecular tools used by cyst and root-knot nematodes to parasitize plants might differ considerably. Very few of the putative parasitism genes identified to date had homologs in *C. elegans*, reinforcing the hypothesis that some genes present in parasitic nematodes that are relatively divergent or absent from the *C. elegans* genome could be considered as potential adaptations for parasitism [3,37].

Also of note is the absence of a consensus trans-spliced leader (SL1, SL2) sequence [38] from most of the parasitism gene transcripts isolated from plant nematodes, suggesting potential novel origins of parasitism genes and the limited utility of using spliced leader-based primers to obtain many parasitism genes. In total, the number and variety of putative functions of the phytoparasitic nematode parasitism gene candidates identified to date paint a more-complex picture of the potential mechanisms of parasitism directed by nematodes than previously envisioned [3].

Gene function

Identifying the functions of different nematode parasitome members remains an essential but daunting task. Experimental limitations imposed by obligate parasites, including the use of mutants for forward genetics and the few technologies available for genetic manipulation of parasitic nematodes, are slowly being circumvented. The potential to clone and express nematode parasitism genes and/or their regulatory elements within host cells and tissues to assess effects on host gene expression and phenotype [39] provides a measure of gene function in the absence of concomitant foreign signals. This could be accomplished in plant and animal hosts, including *C. elegans* [40], but the utility of *C. elegans* for this purpose might be limited by genes that have evolved or have been acquired to promote parasitism directly [3,37].

The potential to knockout a target parasitism gene or its product [3] provides a mechanism to assess host changes in the absence of one signal, providing a more-comprehensive functional analysis. Target gene knockout by RNA interference (RNAi) using introduced complementary double-stranded RNA (dsRNA), as developed [41] and utilized extensively in *C. elegans*, represents a powerful functional approach for nematode parasitism genes. Recent success in stimulating parasitic nematodes to take up dsRNA *in vitro* from surrounding 'soaking' medium has provided target gene RNAi in parasitic nematodes [42–44], including residual RNAi effects in later parasitic stages. The potential to direct the expression of dsRNA within host cells and tissues for ingestion, and RNAi of target nematode genes, raises the possibility for *in vivo* functional analyses of nematode parasitism genes, with the exciting dual potential of nematode management applications.

Establishment and maintenance of parasitism

The physical and, to a much lesser extent, biochemical mechanisms involved in parasitism by nematodes have been described and summarized [1,2]. The response of host cells and tissues to direct signals from the nematode versus the indirect response to host signals stimulated during the interaction is difficult to dissect. Inducible host signals probably include the potential role of local phytohormone accumulation in feeding site formation by plant-parasitic nematodes [45] and the inflammatory response during innate immune activity in response to some animal-parasitic nematodes [6]. The function(s), for example, of homologs of genes encoding secreted venom allergen-like (VAP) proteins from *Ancylostoma* [46] that have been identified in plant-parasitic nematodes [47,48] are unknown and might promote both direct and indirect host responses during parasitism. However, the emerging isolation of parasitism genes from nematodes offers an unparalleled opportunity to identify and dissect the fundamental mechanisms of host infection by nematodes (Figure 4), and these effects must be considered within the dynamic complex of signals and responses between the host and parasite.

Penetration and migration

Endoparasitic nematode species must penetrate host tissues directly, using mechanical and/or biochemical methods. Secreted proteases from parasitic nematodes appear to aid in the penetration and migration through animal tissues [6]. For plant-parasitic nematodes, a cell wall composed primarily of cellulose poses a formidable barrier to penetration. Thrusts of the nematode stylet combined with esophageal gland secretions mediate penetration and migration through plant tissues [1,2]. Plant-parasitic nematodes possess an arsenal of hydrolytic enzymes for digesting cell wall polymers. Genes encoding secreted cell-wall-modifying enzymes have been localized to nematode esophageal gland cells including enzymes that degrade the pectic polysaccharides (pectate lyases and polygalacturonases) comprising the middle lamella between plant cells [49,50] and enzymes that degrade the cellulose (endoglucanases) and hemicellulose (xylnase) structural components of the cell wall [3,6,11].

Interestingly, the cell-wall-modifying enzymes appear to be active only in the subventral gland cells and, in the case of the cyst nematode endoglucanases, they are only active during nematode migration within roots [3,6,16,17], whereas plant endoglucanases upregulated in feeding sites probably modify the walls comprising these specialized cells [17]. The recent discovery (L. Qin, PhD Thesis, Wageningen University, 2001) that a gene encoding a secreted homolog of plant expansins [51] is expressed in the subventral glands of the potato cyst nematode is novel, and the observed plant cell wall expansion activity of the encoded protein (L. Qin, pers. commun.) suggests a unique mechanism for nematodes to 'loosen' cell walls to accommodate intracellular migration of infective juveniles through plant tissues.

Feeding cell formation

Nematodes orchestrate elaborate root cell modifications leading to the development of specialized feeding cells [1,2,5,6]. Upon hatching, pre-parasitic J2 have an arsenal of parasitism proteins already packaged in secretory granules within their subventral gland cells [4]. Although many subventral gland secretory proteins consist of cell-wall-degrading enzymes used by J2 to facilitate the invasion of roots [6], other parasitism proteins synthesized in the subventral gland cells [23,24,35,36,47,48] might have other roles in the parasitic processes – for example, the induction of feeding cells. The growth and predominant activity of the dorsal gland cell during and after feeding cell formation suggests both a role in feeding site induction and regulation of feeding cells [4]. On the basis of the predicted similarities of some plant nematode parasitism genes with genes of known function, we can begin to speculate on an early mechanistic model of feeding cell formation by sedentary endoparasitic nematodes (Figure 4).

Peptide signaling

One picture that is emerging is the potential role of secreted nematode peptide signaling molecules in feeding cell formation. Phytohormones are well known to regulate many processes in the growth and development of plant cells, and have been implicated as having roles (direct or indirect) in the parasitic interaction between nematodes and plants [45]. By contrast, small peptides represent a relatively newly recognized group of signal molecules in plants [52]. Plant peptide signal molecules regulate a variety of physiological processes, and a recent protein domain search has identified the predicted product of the parasitism gene encoding *H. glycines* SYV46 [32] to be similar to the CLAVATA3/ESR-related (CLE) class of plant signal peptides [53]. Plant CLAVATA3 exists in several isoforms, and if bound to extracellular CLAVATA1, which is a receptor-like kinase, CLAVATA3 promotes differentiation of stem cells in shoot meristems [54]. The potential for nematodes to have evolved a mechanism to mimic this type of peptide signaling and thereby regulate feeding cell differentiation in plants is extraordinary, yet is consistent with the hypothesis of de-differentiation of plant cells to become nematode feeding sites [1,2,5]. Similarly, a parasitism gene candidate (clone 16D10) in *M. incognita* [36] encodes a 13 amino acid secretory peptide that might also

homologs among the parasitism genes isolated from cyst nematodes [35,57] suggests that this multifunctional enzyme is a key player in modulating host cell phenotype by parasitic nematodes.

Cell-cycle augmentation

Nematode parasites of animals and plants can influence the cell cycle in host tissues, either by apparent arrest or re-entry into alternate phases of the cell cycle [5,6]. Genes have been identified that encode a family of Ran-binding protein in the microtubule-organizing center (RanBPMs) [58] predicted to be secreted from the potato cyst nematode *G. rostochiensis*, and roles in modulating the cell cycle of feeding sites have been considered (L. Qin, PhD Thesis, Wageningen University, 2001). Secreted RanBPMs might interact with heterologous associated proteins in plant cells to increase stabilization of the microtubule network involved in spindle fiber formation and hamper the transition from interphase to mitosis, resulting in the apparent shunting of the M-phase observed in nematode-induced syncytia (L. Qin, pers. commun.). The recent identification of genes similar to RanBPM within the gland-cell libraries of *H. glycines* (B. Gao and R.S. Hussey, unpublished) supports the *G. rostochiensis* results. Confirmation of gene expression in the gland cells and secretion of the gene product *in planta* would further suggest the potential of secreted RanBPMs as a mechanism for parasitic nematodes to mitigate the host cell cycle directly.

Nuclear localization

Nuclear localization signals (NLS) were predicted in *H. glycines* and *M. incognita* parasitism proteins [35,36] using PSORT II [59], presenting a tempting hypothesis that these proteins might be secreted from the nematode and have direct regulatory effects within the nucleus of the recipient plant cell. The detection of antigens from *T. spiralis* in the nucleus of host animal muscle cells [6] presents a parallel to this potential phenomenon in phytonematodes. To test potential nuclear localization, *H. glycines* parasitism genes with NLS motifs have been transiently expressed as fusion proteins of β -glucuronidase and green fluorescent protein in onion epidermis cells [60]. Localization of three *H. glycines* proteins in the plant nucleus has been observed to date (A. Elling and T.J. Baum, unpublished), supporting the hypothesis that secreted NLS-containing nematode parasitism proteins might have activity directly within the nucleus of host cells.

Protein degradation

Selective protein degradation by the ubiquitin-proteasome pathway plays a pivotal position in cell signaling and cellular regulation [61], and the potential secretion of putative members [S-phase kinase-associated protein 1 (Skp-1), RING-H2 and ubiquitin [35]] of this complex into host cells by nematodes might represent a mechanism of cellular regulation and mitigation of host defense to promote parasitism by nematodes. The Skp-1–cullin–F-box (SCF)-type E3 complex modulates a variety of cellular processes including cell signaling and cell cycle by selective protein degradation and interaction with

phytohormone proteins [61]. The RING-H2 class contains monomeric E3 proteins that facilitate the transfer of ubiquitin to target proteins for subsequent degradation within the cell [61]. The potential for nematodes to target specific host cell proteins for degradation by ubiquitin secreted directly into feeding cells would be a truly unique mechanism evolved for parasitism. The *H. glycines* ubiquitins [35] have two novel features: they contain a signal peptide and a novel 19 amino acid extension peptide at the C-terminus. By contrast, plant ubiquitin extension proteins are not secretory proteins and their extension proteins are ribosomal proteins [62]. Because ubiquitin is an abundant protein in the plant cell, the secretion of ubiquitin *per se* by *H. glycines* might not affect the parasitized cell. An alternative role could be that the novel secretory ubiquitin extension proteins of *H. glycines* serve as a chaperone for the unique extension peptide that might function as a peptide signal within the host cell.

Feeding tubes

Secretions of cyst, root-knot and a few other sedentary endoparasitic nematodes produce a 'feeding tube' that appears to serve as a molecular sieve for host cell contents that enter the stylet orifice during ingestion [2,63]. The crystalline feeding tube of sedentary phytonematodes is a unique structure with no known counterpart in other parasitic interactions. A new feeding tube is produced through the stylet orifice directly into host cell cytoplasm at the end of each secretion cycle before the ingestion phase, and the cycles are repeated throughout the duration of parasitism. The timing of feeding tube production would allow secretion of relatively large molecules from the stylet orifice before its formation but could limit ingested molecules to $\approx < 40$ kDa after its formation [2]. Since the stylet does not pierce the plasmalemma of the feeding cell, the destination of different secreted molecules from the nematode might be directed to the extracellular space or directly into host cell cytoplasm through the opening in the plasmalemma at the stylet orifice (Figure 4). Localization studies will be fundamental to infer the functions of different parasitism gene products secreted by nematodes into host plants.

Conclusions

The multitude of alterations in gene expression [5] leading to the phenotypes induced in host cells for parasitism by plant nematodes was consistent with an initial hypothesis that a few signals from the nematode probably induce regulatory machinery in affected plant cells that subsequently mediated the phenotype of feeding cells [3]. However, if the large number of putative parasitism genes recently discovered in plant nematodes, especially those encoding esophageal gland secretions that may be introduced directly into host plant tissues, truly function to direct different processes required for parasitic success, the emerging model of regulation of parasitism by nematodes is much more complex than originally hypothesized. At first glance, this appears to be an inefficient way 'to do business' but, if genuine, represents a true marvel of co-evolution. Confirming the function of nematode

parasitism genes will be a significant, and exciting, challenge for future research.

The first isolations of nematode parasitism genes encoding plant-cell-wall-modifying enzymes and CM that were most similar to those of bacteria lead to initial speculations that many nematode parasitism genes might have been acquired by ancient horizontal gene transfer [3,6]. However, database searches with the relatively large volume of parasitism genes recently isolated from plant nematodes do not support a much greater potential for horizontal gene transfer from prokaryotes as a mechanism of parasitic adaptation; rather, an extraordinary form of molecular mimicry might have evolved in plant nematodes to modulate host cellular processes to the benefit of the nematode. On one side, inherent mechanisms of host defense might be specifically disrupted to allow gross modifications in feeding cell phenotype to occur unabated (except in cases where recognition of specific nematode avirulence products betrays the worm to plant-resistance genes). The compatible side of the interaction suggests a dynamic network of signals from the nematode to regulate specific cellular processes combined with the potential to tap the regulatory machinery of the plant to transduce secondary responses that are required for parasitic success. These models are based upon hypotheses derived by database similarities of nematode parasitism genes, but their true activity must be investigated through functional analyses. What of the majority of nematode parasitism genes isolated that have no known homolog among other organisms or even among themselves (i.e. root-knot and cyst nematodes share relatively few common parasitism gene sequences)? Do these novel genes represent classes of genes that are unique and essential to parasites and are they therefore potential 'roots' of parasitism?

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