

# Secrets in secretions: genes that control nematode parasitism of plants

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The most evolutionary advanced adaptations for plant parasitism by nematodes are the products of parasitism genes expressed in their esophageal gland cells and secreted through their stylet into host tissue to control the complex process of parasitism. Molecular analyses of nematode parasitism genes are revealing the complexity of the tools that enable the nematode to attack plants, and the results paint a more elaborate picture of host cellular events under specific control by the parasite than previously hypothesized. Interestingly, the majority of the parasitism genes discovered encodes proteins unique to plant-parasitic nematodes. Identifying the nematode parasitome, i.e., the complete profile of parasitism gene products secreted through the nematode stylet during the parasitic cycle, is the key to understanding the molecular basis of nematode parasitism of plants. Such knowledge will identify vulnerable points in the parasitic process that can be interfered with to achieve nematode control to limit nematode-induced yield losses in crops.

**Key words:** esophageal gland cells, nematode parasitism, parasitome, parasitism genes, secretion.

**Secredos em secreções: genes tque controlam o parasitismo de nematóides de plantas:** As mais avançadas adaptações evolucionárias para o parasitismo de plantas por nematóides são os produtos dos genes de parasitismo expressos nas células da sua glândula esofágial e secretados através do estilete no tecido do hospedeiro, com a finalidade de controlar o complexo processo de parasitismo. Análises moleculares de genes de parasitismo de nematóides têm revelado a complexidade das ferramentas que os permitem atacar as plantas e também obter uma visão mais elaborada do que antes se tinha por hipótese, dos eventos celulares do hospedeiro sob controle específico do parasita. Interessantemente, a maioria dos genes de parasitismo de nematóides descobertos, codificam proteínas específicas para nematóides parasitas de plantas. Identificar o parasitoma do nematóide, isto é, o perfil completo dos produtos dos genes de parasitismo secretados através do estilete durante o ciclo parasítico, é a chave para a compreensão da base molecular do parasitismo de nematóides de plantas. Este conhecimento permitirá identificar os pontos vulneráveis no processo parasítico que podem sofrer interferência, a fim de se controlar o nematóide e limitar as perdas de produção que eles causam em plantas cultivadas.

**Palavras-chave:** células da glândula esofogial, genes de parasitismo, parasitoma, parasitismo de nematóides, secreção.

## INTRODUCTION

Plant-parasitic nematodes have evolved diverse parasitic relationships with their host plants to obtain nutrients that are necessary to support their development and reproduction. These biotrophic parasites, depending on species, feed from the cytoplasm of unmodified living plant cells or have adapted to modify root cells into

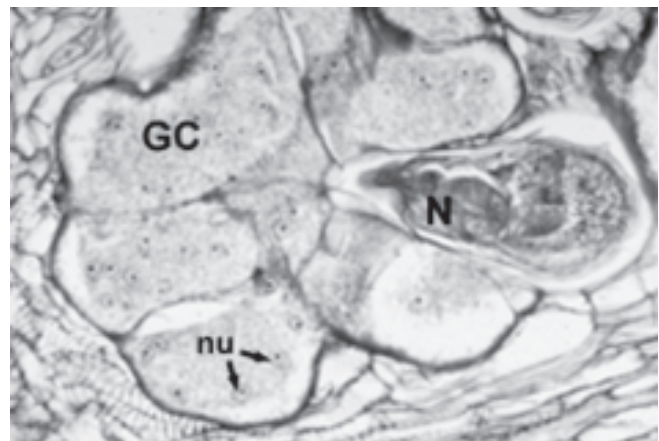
elaborate discrete feeding cells (Hussey and Grundler, 1998). All plant-parasitic nematodes have evolved a hollow, protrusible feeding spear, called a stylet, to penetrate the wall of a plant cell, inject gland secretions into the cell, and withdraw nutrients from the cytoplasm. Migratory feeding nematodes remove cytoplasm from the host cell, frequently causing cell death, and then move to

another cell to repeat the feeding process. Evolutionarily more advanced nematode species become sedentary and feed from a single cell or a group of cells for prolonged periods of time. For this sustained feeding, the sedentary parasites dramatically modify root cells of susceptible hosts into elaborate feeding cells, including modulating complex changes in plant cell gene expression, physiology, morphology, and function (Bird, 1996; Gheysen and Fenoll, 2002). The drastic phenotypic changes of root cells during feeding cell formation are the result of nematode-mediated changes, directly or indirectly, in the developmental program of the parasitized cells (Williams and Hussey, 1996). An understanding of the molecular signaling events in this process will not only provide fundamental knowledge of nematode parasitism and regulation of plant gene expression, but it will also suggest vulnerable points in the parasitic process that can be interfered with to achieve nematode control to limit nematode-induced yield losses in crops.

The evolutionary adaptations of nematodes for plant parasitism led to the development of the protrusible stylet as well as marked morphological and physiological modifications of the esophagus (Bird, 1971; Maggenti, 1987; Hussey, 1989). Secretory gland cells in the nematode esophagus are the principal sources of secretions involved in plant parasitism, and these gland cells enlarged considerably as nematodes evolved from microbial-feeding nematodes to become parasites of higher plants. Likewise the function of the secretions produced by the esophageal gland cells also evolved to enable nematodes to feed on plant cells and modify them into complex feeding cells (Hussey, 1989; Davis *et al.*, 2000). Recent discoveries also suggest that some genes encoding esophageal gland secretions of plant-parasitic nematodes may have been acquired via horizontal gene transfer from prokaryotic microbes (Smant *et al.*, 1998; Davis *et al.*, 2000).

This treatise focuses primarily on discoveries made in identifying parasitism genes in cyst and root-knot nematodes because these nematodes induce the most dramatic and evolutionarily advanced changes observed in host cell phenotype (Hussey and Grundler, 1998). Cyst and root-knot nematodes have evolved to alter gene expression in specific root cells to modify them into very specialized and metabolically active feeding cells, called syncytia or giant-cells, respectively. Cell fusion following cell wall degradation gives rise to the syncytia whereas

abnormal cell growth following repeated mitosis uncoupled from cytokinesis produces the giant-cells. These large multinucleate feeding cells possess thickened walls remodeled to form elaborate ingrowths and a dense granular cytoplasm with increased subcellular organelles and small vacuoles (figure 1). A number of genes with known or putative functions have been found to be up-regulated or silenced in these feeding cells, suggesting that root-knot and cyst nematodes induce transcriptional changes in the parasitized cells (Bird, 1996; Gheysen and Fenoll, 2002).

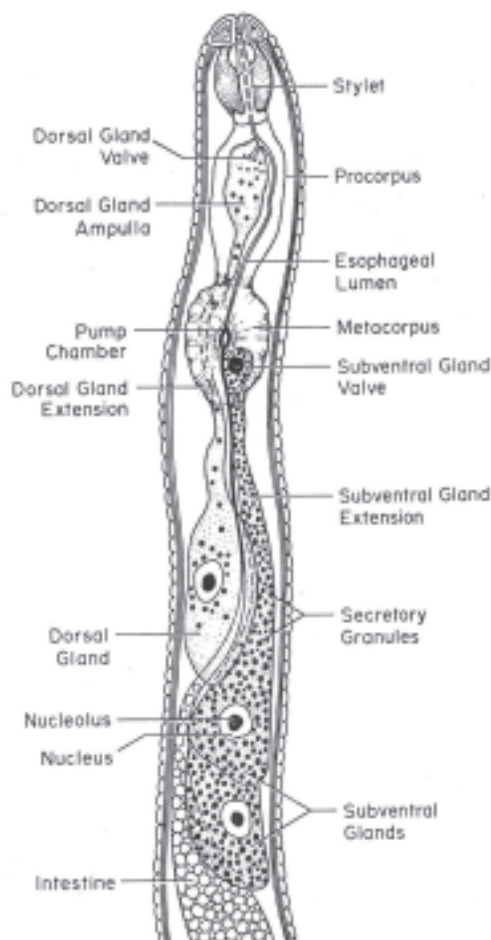


**Figure 1.** Multinucleate (nu) feeding cells (giant-cells, GC) induced in tobacco roots by the root-knot nematode (N).

### Esophageal gland cells

The largest group of plant-parasitic nematodes, the Tylenchids, are well adapted for plant parasitism. In addition to the stylet, tylenchid nematodes have a well-developed esophagus designed for feeding on plants (Hussey, 1989) (figure 2). The esophagus has a muscular metacorpus with a triradiate pump chamber and three large transcriptionally active secretory gland cells, one dorsal and two subventral (Endo, 1984; Hussey and Mims, 1990). Each gland is a single large specialized secretory cell that contains a large lobed nucleus with a prominent nucleolus, abundant Golgi bodies, rough endoplasmic reticulum, secretory granules, and other organelles typical of secretory cells (Burgess and Kelly, 1987). A cytoplasmic extension of each gland cell extends forward in the esophagus and terminates in a storage ampulla that is connected to the esophageal lumen by an elaborate valve (Anderson and Byers, 1975; Endo, 1984; Endo and Wergin, 1988; Hussey and Mims, 1990). The valve of the dorsal gland cell is located near the base of the stylet while the valves of subventral gland cells release secretions into the lumen of

the esophagus immediately posterior to the metacorpal pump chamber. Secretory proteins are synthesized in the nuclear region of the gland cell and stored in spherical Golgi-derived membrane-bounded granules that are transported along microtubules in the cytoplasmic extension to the ampulla. The association of neural processes and neurosecretory cells with the gland cytoplasmic extension and ampulla indicates that the regulated secretion of glandular proteins is controlled by the nervous system. During secretion, the gland cell is triggered to rapidly release the secretory proteins stored in the granules by exocytosis into the membranous end-sac of the valve where the proteins pass through a duct to enter the lumen of the esophagus to be injected through the stylet into host tissue. Secretory granules vary in size, morphology, and content among nematode species and between the dorsal and subventral gland cells within a specific life stage.



**Figure 2.** The anterior end of a second-stage juvenile of a plant-parasitic nematode (from Hussey, 1989).

Ultrastructural and morphological changes in esophageal gland cells are correlated with the developmental phases in the life cycle of sedentary endoparasitic nematodes. The subventral gland cells are the most active glands in infective second-stage juveniles, but following the onset of parasitism (penetration into host plant tissues and establishment of feeding sites), the dorsal gland cell is stimulated to increase synthesis of secretory proteins to become the predominate gland in the parasitic stages (figure 3; Bird, 1983; Hussey and Mims, 1990). These changes in the esophageal gland cells during the parasitic cycle indicate various roles for the gland secretory proteins during different stages of parasitism.

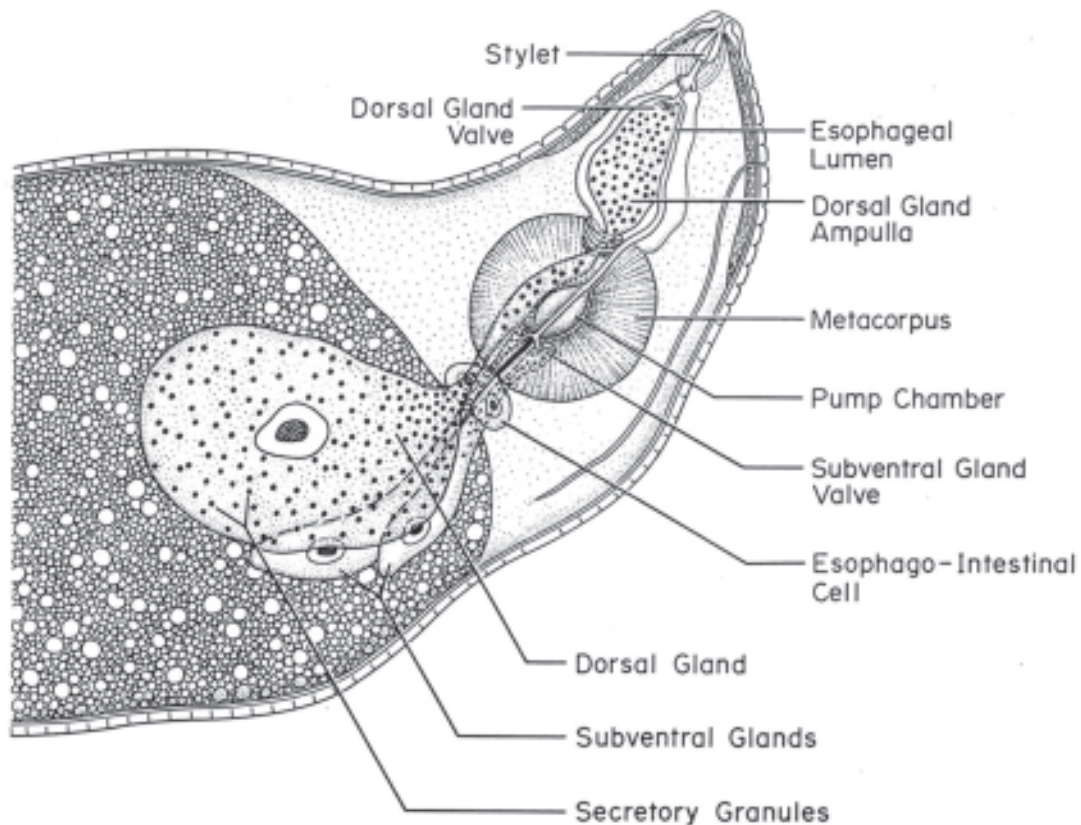
### Esophageal gland cell secretions

The most evolutionary advanced adaptations for plant parasitism by nematodes are the products of parasitism genes expressed in their esophageal gland cells and secreted through their stylet into host tissue (Davis et al., 2000). These stylet secretions have a direct role in infection and parasitism of plants, and developmental changes in the secreted proteins occur during the parasitic cycle (Hussey, 1989; Davis et al., 2000). Herein, the secreted products of the parasitism genes expressed in the nematode's esophageal gland cells are considered collectively as the "parasitome", a subset of the secretome (secreted proteins) of a parasite that mediates parasitism (based upon the nomenclature in Greenbaum et al., 2001). These stylet secretions may function in nematode penetration and migration through root tissue, modification and maintenance of root cells as feeding cells, formation of feeding tubes, and/or digestion of host cell cytoplasm to facilitate nutrient acquisition by the nematode (Hussey, 1989). The secretions from sedentary endoparasites are particularly intriguing because of the complex changes in phenotype, function, and gene expression that they modulate in the parasitized plant cells. During parasitism of a plant cell, the nematode's stylet penetrates the cell wall but does not pierce the plasma membrane, which becomes invaginated around the stylet tip to provide an opening exclusively at the stylet orifice (figure 4; Rebois, 1980). Esophageal gland cell secretions injected through the stylet by sedentary parasites transform root cells in susceptible plants into metabolically active feeding cells. These gland secretions modify, directly or indirectly, gene

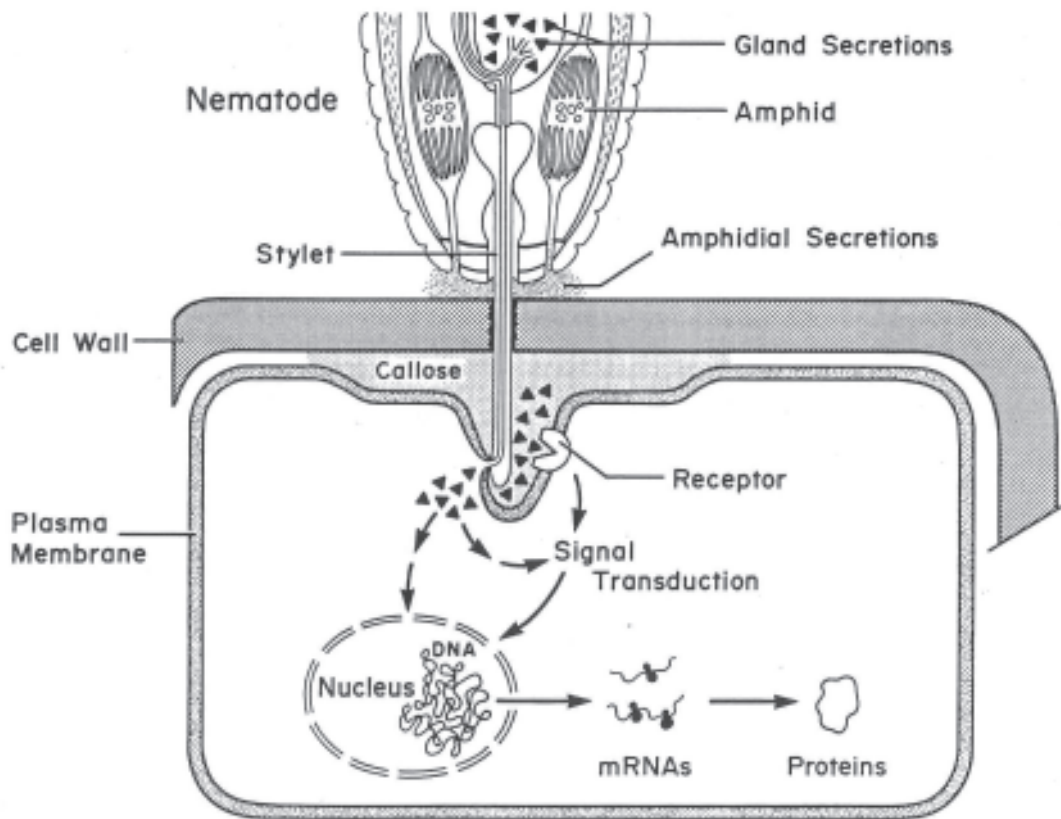
expression to induce profound morphological, physiological, and molecular changes in the recipient cells to enable them to function as a continuous source of nutrients for the nematode parasitic stages. Removal of the nematode at any point during the parasitic interaction results in degeneration of the feeding cells, suggesting the need for a constant and specific stimulus from the nematode to maintain the modifications in the parasitized cell. Although the mechanism(s) by which these nematodes alter plant gene expression is unknown, studies suggest that factor(s) in the nematode stylet secretions induce novel gene regulatory cascades causing the parasitized root cells to differentiate into the unique feeding cells (Hussey *et al.*, 1994; Gheysen and Fenoll, 2002). The gland secretions may be deposited outside the plasma membrane or injected directly into the cytoplasm of the recipient cell through the stylet orifice (figure 4). In either case, specific molecules in the secretions could bind to plant cell receptors to elicit a signal transduction cascade to modulate gene expression in the cell. Alternatively, the secretions could enter the nucleus to directly modify gene expression in the recipient plant cell.

Critical unresolved questions in the study of nematode esophageal gland cell secretions are the nature, number and functions of different members of the parasitome and their temporal expression during the parasitic cycle. The core of a secretory granule is typically a large volume of highly concentrated protein, and the number of different secretory proteins in the matrix can vary with gland cell type (Burgess and Kelly, 1987). Indeed, specific compartmentalization of a secretory protein within the matrix of granules formed in the subventral gland cells of *Meloidogyne incognita* second-stage juveniles was determined by the distribution of immunogold-labeling over the granules (Hussey *et al.*, 1990).

Development of monoclonal antibodies that bind to secretory antigens within the esophageal gland cells has been critical in the study of secreted proteins from cyst and root-knot nematodes (Hussey and Grundler, 1998; Davis *et al.*, 2000). The monoclonal antibodies have been used to monitor the developmental expression of different esophageal antigens at various stages of nematode development (Atkinson and Harris, 1989; Davis *et al.*, 1994; Goverse *et al.*, 1994; Smant *et al.*, 1997).



**Figure 3.** The esophagus of an adult female root-knot nematode (from Hussey *et al.*, 1994).



**Figure 4.** Schematic model of interactions of a sedentary parasitic nematode with its feeding cell. The anterior end of the nematode is shown with its stylet inserted through the plant cell wall but not piercing the plasma membrane, which becomes invaginated around the stylet tip. Gland secretions ( $\blacktriangle$ ) originating from the esophageal gland cells of the nematode may be deposited outside the plasma membrane and interact with a membrane receptor or injected directly into the cytoplasm of the recipient cell through a perforation in the plasma membrane at the stylet orifice (from Williamson and Hussey, 1996).

During feeding, sedentary endoparasitic nematode species (*Globodera*, *Meloidogyne*, *Heterodera*, *Rotylenchulus* species) also inject dorsal gland secretions that form unique tube-like structures called feeding tubes within the cytoplasm of the feeding cell (Rebois, 1980; Endo, 1991; Hussey and Mims, 1991). Feeding tubes function in the selective and efficient removal of nutrients from the cytoplasm of the large modified cells by the feeding nematode. Microinjection studies with fluorescent probes of different molecular weights showed that the walls of feeding tubes serve as a molecular sieve during nutrient uptake by the parasite (Bockenhoff and Grundler, 1994).

#### Parasitism genes

Although it is currently not possible to predict the number of members of the parasitome of plant-parasitic nematodes, only a small fraction of the estimated 15,000–20,000 genes (based on the ~19,000 genes of *Caenorhabditis elegans*) of a plant-parasitic nematode

should be expected to encode proteins that have a direct role in parasitism. The first members of a parasitome to be cloned from plant-parasitic nematodes were  $\beta$ -1,4-endoglucanases (cellulases) developmentally expressed in the two subventral gland cells of *Heterodera glycines* and *Globodera rostochiensis* (Smant et al., 1998; Yan et al., 1998). Two cellulase cDNAs in each cyst nematode species (*Hg-eng-1* and *Gr-eng-1*) encode a predicted secretion signal peptide, cellulase catalytic domain, small peptide linker, and a cellulose binding domain (CBD). A smaller cellulase cDNA in *G. rostochiensis* (*Gr-eng-2*) lacks the CBD, and one (*Hg-eng-2*) from *H. glycines* is missing both the peptide linker and CBD. The presence of a CBD presumably enhances cellulase activity toward crystalline cellulose. mRNA in situ hybridization and immunolocalization with anti-ENG polyclonal sera confirmed that *eng-1* and *eng-2* were expressed exclusively within the subventral esophageal gland cells of both nematode species (de Boer et al., 1998; Smant et al., 1998).

Genomic clones of *eng-1* and *eng-2* have intron/exon organization and putative promoter elements that are typical of eukaryotic genes (Yan *et al.*, 1998). The combined evidence demonstrates that the  $\beta$ -1,4-endoglucanases are endogenous in cyst nematodes, the first report of cellulase genes found in any animal (Smant *et al.*, 1998). Interestingly, the strong similarity of the cyst nematode cellulases to those from bacteria and their weak similarity to other eukaryotic genes (including *C. elegans*), suggests that the cellulase genes may have been acquired from prokaryotic microbes via horizontal gene transfer to an ancestor of cyst nematodes (Keen and Roberts, 1998; Smant *et al.*, 1998; Yan *et al.*, 1998; Davis *et al.*, 2000). Cyst nematode intracellular migration in roots and feeding from parasitized roots cells requires degradation of plant cell walls and this is facilitated by in planta secretion of cellulase by these parasites (Wang *et al.*, 1999). Additional cellulase genes more recently have been isolated from *H. glycines* (Yan *et al.*, 2001; Gao *et al.*, 2002a,b) and other plant-parasitic nematodes including *Globodera tabacum* (Goellner *et al.*, 2000, 2001), *Heterodera schachtii* (de Meutter *et al.*, 2001), *M. incognita* (Rosso *et al.*, 1999), and *Pratylenchus penetrans* (Uehara *et al.*, 2001). The expression of cyst nematode cellulases has been detected in juveniles within eggs, in hatched juveniles, in parasitic second-stage juveniles, but only rarely within third-stage juvenile males or females, and not in any subsequent female cyst nematode life stage (Smant *et al.*, 1997; de Boer *et al.*, 1999; Gao *et al.*, 2002a; Goellner *et al.*, 2000). Interestingly, cellulase expression is reinitiated in late-stage cyst nematode males, suggesting a primary role of cellulases secreted from the stylet of cyst nematodes in penetration, migration within, and emergence from plant root tissue. Cellulases secreted from cyst nematodes have not been detected within feeding cells, and recent evidence suggests that the extensive cell wall dissolution observed within synectia is due, in part, to up-regulation of plant endoglucanase genes within the feeding cells (Goellner *et al.*, 2001).

Differential screening of gene expression has been the most widely used method to clone parasitism genes expressed within the esophageal gland cells of plant-parasitic nematodes. Esophageal gland regions from second-stage juveniles of *M. javanica* were excised and cDNA was prepared from this tissue by reverse transcriptase-polymerase chain reaction (RT-PCR) (Lambert *et al.*, 1999). The cDNA pool was differentially screened against cDNA from the nematode tail region to

isolate genes that are up-regulated or expressed specifically in the esophageal gland region. A full-length cDNA clone that had homology to a bacterial chorismate mutase was obtained with this screening strategy (Lambert *et al.*, 1999). Expression of *Mj-cm-1* is localized within the subventral esophageal gland cells of parasitic *M. javanica* by mRNA in situ hybridization and with antisera generated to the product of *Mj-cm-1*. Chorismate mutase initiates the conversion of chorismate, the end product of the shikimate pathway, to the aromatic amino acids, phenylalanine and tyrosine. The secretion of Mj-CM-1 into the cytosol of a plant cell 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. Alternatively, these compounds (tyrosine) could be used by the nematode in cuticle formation.

RNA fingerprinting has been used to analyze differential gene expression between pre-parasitic and parasitic stages of *M. incognita*. A cDNA encoding for a secretory cellulose-binding protein (*Mi-cbp-1*) was isolated using this method (Ding *et al.*, 1998). *Mi-cbp-1* is specifically expressed in the subventral gland cells of *M. incognita*, and in vitro analysis confirmed the secretion of Mi-CBP-1 through the nematode stylet. The N-terminal region of the predicted peptide has no similarity to known proteins, but the C-terminus has strong homology to a CBD. Although in planta secretion seems likely, the potential role of CBP in plant-nematode interactions is uncertain. A recombinant CBD derived from the bacterium *Clostridium cellulovorans* modulates the elongation of different plant cells in vitro (Shpigel *et al.*, 1998). This finding may suggest a possible role of Mi-CBP-1 in plant cell modifications associated with root-knot nematode parasitism. A cDNA (*Mi-msp-1*) encoding a putative secretory venom allergen AG5-like protein with strong similarity to *Ancylostoma*-secreted protein 2 (Hawdon *et al.*, 1999) was also obtained from *M. incognita* using the RNA fingerprinting protocol (Ding *et al.*, 2000). A cDNA-amplified fragment length polymorphism (AFLP) analysis of different life stages of *G. rostochiensis* yielded three putative parasitism genes that are expressed in the dorsal gland cell (Qin *et al.*, 2000). Two of the candidate parasitism genes identified share homology with Ran-binding proteins and are hypothesized to be involved in feeding cell induction (Qin *et al.*, 2002).

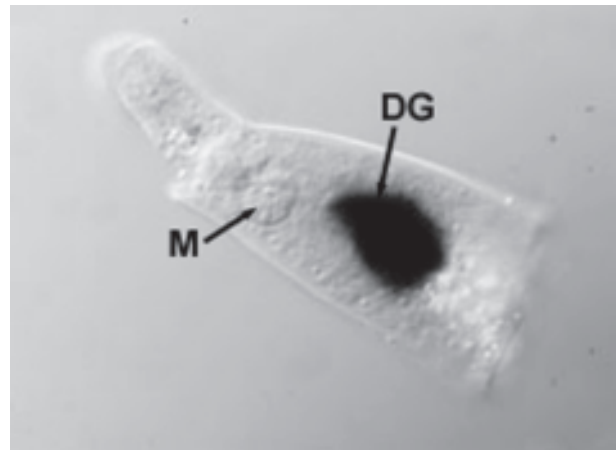
Whole nematode expressed sequence tag (EST) analysis also has been used to identify gland-expressed genes. However, this approach has limited potential because it predominately identifies only parasitism genes whose translation products are obviously related to parasitism, like cell-wall digesting enzymes (Popeijus et al., 2000a; Dautova et al., 2001). Analysis of ESTs from a pre-parasitic second-stage juvenile cDNA library of *G. rostochiensis* identified a full-length cDNA that encoded a predicted protein with a signal peptide at its amino terminus that had strong homology to Class III pectate lyases of bacteria and fungi (Popeijus et al., 2000b). A putative pectate lyase cDNA also has been cloned from *H. glycines* and *M. javanica* (de Boer et al., 2002a; Doyle and Lambert, 2002) as has been a polygalacturonase cDNA from *M. incognita* (Jaubert et al., 2002). Localization of transcripts of the pectate lyases to the subventral esophageal gland cells in nematodes indicates the potential for secretion of a pectate lyase from the nematode stylet during early stages of plant parasitism.

While ESTs generated from preparations of whole nematodes have resulted in the identification of a few obvious members of the parasitome, generating ESTs from specific nematode tissues (i.e., esophageal gland cells) that are likely to be involved in the host-nematode interaction should be the most fruitful strategy for cloning parasitism genes. Therefore, a cell-specific approach involving a direct analysis of gene expression in nematode esophageal gland cells should provide a comprehensive analysis of the nematode parasitome. The ability to generate cDNAs from individual cells by RT-PCR (Karrer et al., 1995) has been coupled with a microaspiration technique used to collect the cytoplasm of the esophageal gland cells (Shields et al., 1998; Gao et al., 2001a; Wang et al., 2001; Huang et al., 2002). mRNA isolated from transcriptionally-active gland cells of a range of parasitic stages has been pooled and used to generate esophageal gland cell-specific cDNA libraries that provided a comprehensive profile of nematode esophageal gland genes expressed in *M. incognita* and *H. glycines* during plant parasitism. A primary criterion used to identify members of the parasitome among the numerous gland cell-specific cDNA clones has been the presence of a putative secretion signal peptide on the encoded protein (von Heijne, 1986), suggesting that the nematode gene product could be actively secreted into plant tissues to influence the parasitic process. Screening a *H. glycines* gland cell cDNA library

using a signal peptide-selection vector expressed in yeast (Klein et al., 1996) yielded nine unique cDNA clones that encoded putative extracellular proteins, and four of these cDNAs hybridized in situ to transcripts within the dorsal gland cell of parasitic stages of *H. glycines* (Wang et al., 2001). Predicted proteins of two of the dorsal gland-specific clones had no similarity with known proteins in the databases. The other two predicted extracellular proteins had similarity with guanylyl cyclase from *H. glycines* (Yan and Davis, 2002) and ERp99 from *Mus musculus*. Microarray analysis of the same *H. glycines* gland cell library prior to signal peptide selection confirmed the developmental expression of these genes in nematodes and identified a few additional candidate parasitism genes (de Boer et al., 2002b). In another study, the generation of cDNA from the cytoplasm microaspirated from the esophageal gland cells using long-distance PCR (LD-PCR) was combined with suppression subtractive hybridization (SSH) using cDNAs generated from contents microaspirated from the intestinal region of *H. glycines* to remove “housekeeping” and structural genes and enrich for genes expressed preferentially in the esophageal gland cells (Gao et al., 2001a). SignalP analysis (Nielsen et al., 1997) indicated that the deduced proteins of ten cDNA clones were preceded by a signal peptide for secretion and PSORT II (Nakai and Horton, 1999) computer analysis predicted eight of these proteins as extracellular, one as nuclear, and one as plasmalemma-localized. In situ hybridization showed that four of the predicted extracellular clones were specifically expressed in the dorsal gland cell, one in the subventral gland cells, and three (proteinases) in the intestine in *H. glycines* (Gao et al., 2001a). One of the predicted extracellular proteins had strong similarity to salivary proline-rich glycoproteins from rat and human, whose functions remain unknown. The predicted extracellular protein of another dorsal gland cell-specific clone had no significant matches with any proteins in the databases. The predicted extracellular protein of the clone expressed in the subventral gland cells had strong similarity to Mi-MSP-1 from *M. incognita* (Ding et al., 2000) and secretory venom allergen AG5-like proteins, which are the major excretory/secretory proteins released by infective third-stage juveniles of the animal-parasitic nematode *Ancylostoma caninum* (Hawdon et al., 1999). Interestingly, another clone expressed in the subventral gland cells was predicted by PSORT II analysis to be nuclear localized, but since the predicted encoded

protein is preceded by a signal peptide for secretion, the product of this clone is a candidate for a *H. glycines* secretory protein that might enter the plant nucleus if secreted into a parasitized root cell. This possibility makes this clone a prime candidate for further functional analyses.

The signal peptide-selection (Wang *et al.*, 2001), microarray (de Boer *et al.*, 2002b), and SSH (Gao *et al.*, 2001a) analyses of gland-cell cDNA libraries provided a sampling of parasitism genes expressed within *H. glycines*, but the apparent complexity of the libraries suggested that a more comprehensive approach was necessary to obtain a complete profile of the nematode parasitome. The *H. glycines* gland cell library generated by LD-PCR (Gao *et al.*, 2001a) was macroarrayed on nylon membranes for indexing, and ESTs of 3,711 cDNA clones were analyzed (Gao *et al.*, 2002a). Deduced protein sequences of 261 unique cDNAs were preceded by a putative N-terminal signal peptide for secretion based on SignalP analysis (Nielsen *et al.*, 1997). The presence of the signal peptide identified these gland cell proteins as candidates for being secreted through the nematode's stylet and potentially having a biological function in *H. glycines* parasitism of soybean. High-throughput *in situ* hybridization analyses with these 261 clones was used to determine their developmental expression pattern in the parasitic cycle of *H. glycines*. *In situ* hybridization identified 56 unique clones that specifically hybridized with transcripts within the subventral (12 clones) or dorsal (44 clones, figure 5) esophageal gland cells of *H. glycines*. PSORT II (Nakai and Horton, 1999) predicted 15 proteins as nuclear localized and potential candidates for *H. glycines* secretory proteins that might target the plant nucleus if secreted into a parasitized soybean root cell. BLASTP analysis revealed 77% of the candidate parasitism genes to encode novel proteins specific to *H. glycines*. Some of the predicted proteins that had similarities with known proteins included a venom allergen like protein (Gao *et al.*, 2001b),  $\beta$ -1,4-endoglucanases (Gao *et al.*, 2002a,b), a pectate lyase, chorismate mutase, annexin, and a chitinase (Gao *et al.*, 2002c). Noteworthy, only 14% of the products of candidate parasitism genes were homologous with *C. elegans* proteins. As with previous gland cell library analyses, the developmental expression of the putative parasitism genes discovered differed among the *H. glycines* parasitic stages, suggesting that the regulation of some parasitism genes may require certain factors within soybean root tissue.



**Figure 5.** Hybridization of a digoxigenin-labeled antisense cDNA probe (dark staining) of a parasitism gene clone to transcripts expressed exclusively within the dorsal esophageal gland cell of a parasitic fourth-stage juvenile of *Heterodera glycines*. DG – dorsal gland cell, M – metacarpus.

In similar EST analyses of parasitism genes in root-knot nematodes, 37 unique clones from a gland-cell specific cDNA library were expressed within the subventral (13 clones) or dorsal (24 clones) esophageal gland cells of *M. incognita* (Huang *et al.*, 2002). In BLASTP analyses, 73% of the predicted proteins were novel proteins and those with similarities to known proteins included a pectate lyase, acid phosphatase, and hypothetical proteins from other organisms.

Molecular analysis of genes expressed in the esophageal gland cells is proving to be the most direct and efficient approach for identifying nematode parasitism genes. These direct molecular studies are providing for the first time new and surprising information on the complexity and dynamics of the parasitome of a multicellular parasite. Obtaining a comprehensive profile of the parasitome is critical for dissecting the molecular signaling events and regulatory mechanisms involved in nematode parasitism of crops by these economically important pathogens.

## CONCLUSIONS AND FUTURE RESEARCH

Significant progress is now being made in identifying parasitism genes expressed in nematode esophageal gland cells whose products are secreted into plant tissue to control the complex process of parasitism. These stylet secretions facilitate nematode migration in roots and mediate the modification of root cells into the elaborate feeding cells,



which are the sole source of nutrients for sedentary endoparasitic nematodes. Identifying the complete profile of parasitism genes expressed throughout the parasitic cycle of a nematode is the key to understanding the molecular basis of nematode parasitism of plants and defining what makes a nematode a plant parasite. The nematode parasitism genes being discovered are revealing the complexity of the tools a nematode possesses that enable it to attack plants and paints a more elaborate picture of host cellular events under specific control by the parasite than previously hypothesized. Interspecific and intraspecific comparison of the structure of parasitism genes encoding stylet secretions that induce feeding cell formation will also provide the knowledge that should lead to establishing a genetic basis for host range specificity among nematode species or races. Understanding this genetic variability will have an important positive effect on the development and deployment of sustainable nematode management strategies.

The role of the subventral gland cells in the infection process differs from that of the dorsal gland cell in plant-parasitic nematodes during the parasitic cycle. Parasitism genes expressed in the subventral gland cells primarily encode cell-wall digesting enzymes used by the nematode during migration in roots. However, this is apparently not the only role of secretions produced by the subventral gland cells as secretory proteins of unknown function and chorismate mutase are also synthesized in these gland cells. Nevertheless, the notion that the subventral gland cells primarily function during the early stages of the infection process is also supported by the morphological changes in these gland cells, which become smaller and contain fewer secretory granules in later nematode stages during the parasitic cycle (Hussey and Mims 1990).

Clearly one of the most surprising and interesting findings in the discovery of nematode parasitism genes is the large number of candidate parasitism genes that encode novel proteins. Remarkably, over 70% of the parasitism genes have no homology with functionally annotated genes in the databases. These pioneer parasitism genes seem to represent genes unique for nematode parasitism of plants, a hypothesis supported by the unique and complex interactions that sedentary endoparasites have with their host plants. These parasitism genes may have evolved from "basal" nematode genes while other parasitism genes, e.g. cell wall-degrading enzymes and chorismate mutase, may

have been acquired by horizontal gene transfer from prokaryotic microbes. Furthermore, the discovery that very few of the candidate parasitism genes have homologues in the bacterial-feeding *C. elegans* suggest that this model nematode has limited usefulness in studying nematode adaptations for plant parasitism.

Characterization of parasitism genes encoding stylet secretions is essential for understanding the molecular genetics of the nematode-host interactions. Beyond the subtle differences in molecular structure of parasitism gene products among nematode genotypes lies the larger question of how the products of the parasitism genes function and interact with host cell molecules to culminate in successful parasitism. The discovery of a comprehensive profile of a nematode parasitome represents a significant first step towards dissecting the molecular interactions of a nematode with its host. Several experimental approaches may be utilized in the functional analysis of nematode parasitism genes. Overexpression of parasitism genes in bacteria will simplify purification of secretory proteins for use in assays of biological activity, e.g. microinjection of parasitism gene product into root cells of susceptible and resistant hosts. Antibodies generated against overexpressed parasitism gene products can be used to immunolocalize nematode proteins secreted into root tissue during parasitism (Wang et al., 1999). Results of these immunocytochemical experiments on the spatial and temporal expression and localization of stylet secretions should provide evidence of their potential roles in pathogenesis. An alternative functional approach is to determine the effect of expression of cloned nematode parasitism genes on the phenotype of transformed plant tissues such as hairy roots (Mazarei et al., 1998). As individual nematode parasitism genes with observable effects on plant cell phenotype are identified, experiments to analyze the interacting effects of multiple parasitism genes on plant phenotype can be designed to understand how nematode parasitism genes work in concert. Several aspects of plant-parasitic nematode biology make classical genetic studies difficult with these organisms. For species like *M. incognita*, which reproduce by obligatory mitotic parthenogenesis, the opportunity to perform genetic crosses is not available. Therefore, proof-of-concept of gene function may be obtained by gene silencing studies determining the loss-of-function phenotype. The discovery that introduction of dsRNA can be used for RNA-mediated

interference (RNAi) of gene expression in *C. elegans* (Fire *et al.*, 1998) recently has been applied successfully to plant-parasitic nematodes (Urwin *et al.*, 2002). Specific nematode genes can be silenced by soaking pre-parasitic nematodes in dsRNA complementary to the coding region of the gene-of-interest. This RNAi method provides for the first time a molecular genetic tool that should allow us to study parasitism gene function in plant-parasitic nematodes.

Once parasitism genes essential for pathogenesis are characterized, this knowledge will allow several approaches to be implemented for developing transgenic resistant plants, including the expression of peptides (Reed *et al.*, 1997), plantibodies (Baum *et al.*, 1996; Rosso *et al.*, 1996; de Jaeger *et al.*, 2002), or dsRNA that specifically inhibit target nematode parasitism genes directly or their products (Davis *et al.*, 2000). The use of biotechnology to manage pests and pathogens in agriculture is coming to fruition, and the recent discoveries of nematode parasitism genes provide an unprecedented opportunity for limiting nematode damage to multiple crop plants.

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