MOLECULAR PLANT PATHOLOGY (2005) **6**(6), 577–588 DOI: 10.1111/J.1364-3703.2005.00306.X

Pathogen profile

Molecular aspects of cyst nematodes

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SUMMARY

Taxonomy: Superkingdom Eukaryota; kingdom Metazoa; phylum Nematoda; class Chromadorea; order Tylenchida; suborder Tylenchina; superfamily Tylenchoidea; family Heteroderidae; subfamily Heteroderinae; main genera *Heterodera* and *Globodera.*

Host range: Cyst nematodes comprise approximately 100 known species in six genera. They are pathogens of temperate, subtropical and tropical plant species and the host range of many species is narrow. The most economically important species are within the *Globodera* and *Heterodera* genera. *Globodera pallida* and *G. rostochiensis* are important pathogens of potato crops. There are many economic species in the *Heterodera* genus, including *Heterodera glycines* (soybean cyst nematode), *H. avenae* (cereal cyst nematode) and *H. schachtii* (sugar beet cyst nematode), the last of which attacks a range of Chenopodiaceae and Cruciferae, including *Arabidopsis thaliana*.

Disease symptoms: Field symptoms of severe cyst nematode infection are often stunting, wilting and chlorosis, but considerable yield loss can occur without obvious symptoms. The only unique indicator of cyst nematode infection is the presence of adult female nematodes attached to host roots after several weeks of parasitism.

Disease control: This is usually achieved by using integrated pest management involving cultural practices such as crop rotation, resistant cultivars if available and chemical control when economically justified.

INTRODUCTION

Cyst nematodes are obligate sedentary endoparasites of plants that form specialized and complex relationships with their hosts. Each developing female induces a syncytium in the plant root that involves a progressively increasing number of plant cells as she develops. Once established, the parasite loses locomotory ability and feeds from that one site. Adult males regain locomotion whereas the female remains sedentary and is developmentally committed to the feeding site for several weeks.

Cyst nematodes parasitize a range of temperate, tropical and subtropical plants and new species are still being identified from tropical and subtropical regions. The most economically important cyst nematode species are within the genera *Heterodera* and *Globodera*. Each species has a much narrower host range than typical root knot nematodes such as *Meloidogyne incognita*. The species that attack potato (*Globodera rostochiensis* and *G. pallida*), soybean (*Heterodera glycines*) and sugar beet (*H. schachtii*) are of particular economic importance. Further example species are pea, rice and carrot cyst nematodes. All are important to some growers of these crops. One widely distributed group is the cereal cyst nematode (*H. avenae*). This is a widespread pest of temperate cereal crops such as wheat, barley and oats that occurs in more than 50% of major European cereal-growing areas. Several sibling species of *H. avenae* parasitize cereals (Subbotin *et al*., 2003) and some populations of this complex are adapted to withstand dry conditions. They are severe endemic pathogens of wheat and barley in India plus cereals in large areas of southern Australia and elsewhere (Rivoal and Cook, 1993). India also suffers major crop losses of pulses and maize from infection with *H. cajani* and *H. zeae*, respectively (Evans and Rowe, 1998).

Yield loss due to nematode infection can be difficult to quantify as a lack of clear symptoms often allows the presence of nematodes to remain undetected by growers until the infestation is severe (Atkinson, 1996). However, it is estimated that in EU countries the combined annual yield loss to sugar beet cyst nematode is $95 million (Müller, 1999). Potato cyst nematodes occur in at least 64% of potato fields in England and Wales (Minnis *et al*., 2002) and are estimated to cause annual yield losses of approximately $80 million (Haydock and Evans, 1998). In 1998, the top ten soybean-producing countries, accounting for 97% of the world crop, suffered losses to soybean cyst nematode estimated at $1960 million (Wrather *et al*., 2001).

Control of cyst nematodes presents a particular problem as many or all of the eggs remain dormant within the protective cyst wall in soil for many years. Current control relies on a number of strategies, usually deployed in an integrated approach. Nematicides are widely used but some effective compounds have already been withdrawn

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due to their mammalian toxicity and environmental concerns. Of those chemicals remaining in use, the nematostat aldicarb (Temik) will be withdrawn from use by the EU in 2007 but others such as oxamyl (Vydate) and fosthiazate (Nemathorin) will remain available. Nematicide use imposes substantial costs and is often not economic for some crops such as soybean. It adds over 50% to the variable costs associated with potato production in the UK (Harkett, P., Unpublished data).

Natural resistance to cyst nematodes has been used in breeding programmes for a number of crop plants. Notable successes are the development of commercial soybean and potato cultivars resistant to *H. glycines* and *G. rostochiensis*, respectively (Starr *et al.*, 2002). Host resistance can be compromised, however, by virulence within nematode populations. Some sources of resistance, for instance the *Solanum vernei*-derived partial resistance to *G. pallida*, involve a complex trait inherited in a polygenic manner (Dale and Phillips, 1982). In other cases a single dominant resistance gene (R-gene) in the host plant interacts either directly or indirectly with a corresponding avirulence gene in the nematode. This highly specific 'gene-for-gene' interaction results in the initiation of a cascade of defence responses. Much research effort has been directed towards mapping resistance loci to linkage groups. This approach has led to the successful cloning of a number of resistance genes (Cai *et al.*, 1997; Ernst *et al.*, 2002; Paal *et al.*, 2004; Van der Vossen *et al.*, 2000) with the identification of others likely in the near future (Bakker *et al.*, 2004). In some cases the development of a commercial cultivar is rapid, as occurred for the potato cultivar Maris Piper that is resistant to UK populations of *G. rostochiensis*. Introgression into elite cultivars is a lengthy process for polygenic resistance such as that from *Solanum vernii* with efficacy against *G. pallida* (Dale and De Scurrah, 1998). There are also crops attacked by nematodes for which sources of natural resistance are unavailable. As more R-genes are cloned and transformation protocols improve for some crops, it has been suggested that there is the potential for such genes to be rapidly transferred to related elite cultivars or even to other plant species (Atkinson *et al.*, 2003; McDowell and Woffenden, 2003).

The relatively narrow host ranges of cyst nematodes ensure that crop rotation is an effective means of partial control but long rotations are required to decline populations of species such as *G. pallida* (Phillips and Trudgill, 1998). Other agronomic constraints are a lack of alternative crops or the needs of specialist growers to justify their investment in production of their chosen crop.

Life cycle (Figs 1 and 2)

Second-stage juveniles (J2) hatch in the soil from eggs contained within a protective cyst. For many cyst nematode species hatching

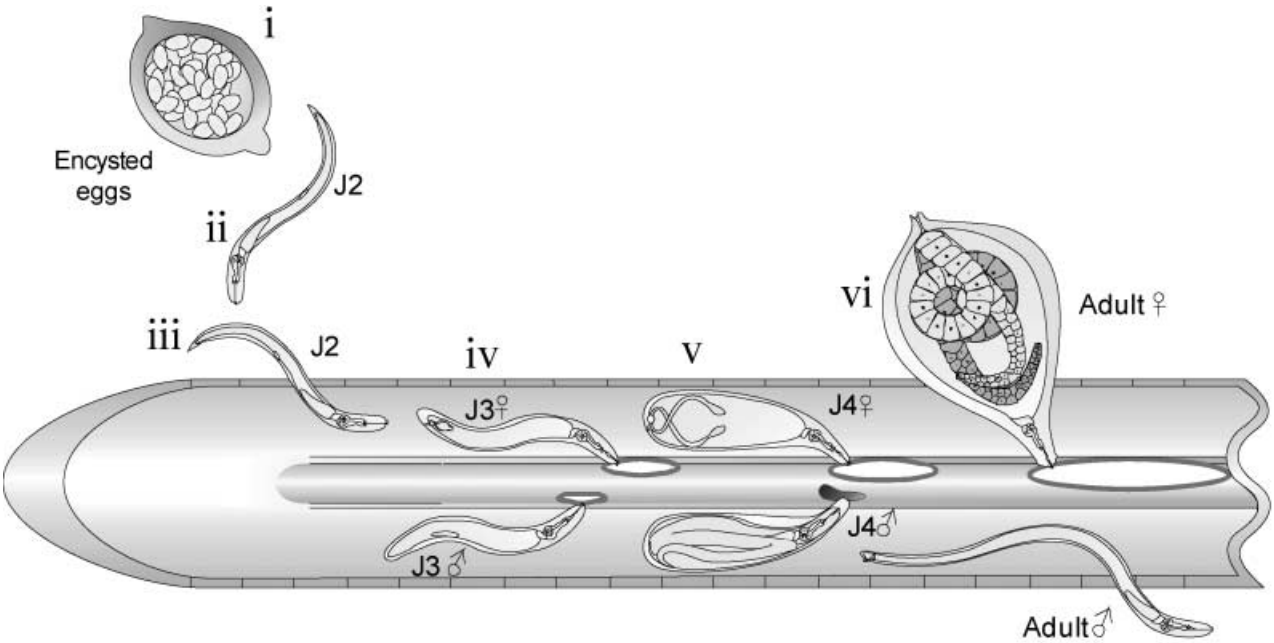

Fig. 1 Life cycle of an amphimictic cyst nematode, e.g. *H. glycines*. (i) Eggs may remain dormant in the soil protected within the tanned cyst for many years. (ii) Under favourable conditions the J2 hatches and migrates towards a host root. (iii) The J2 penetrates the root and migrates intracellularly through the cortex towards the vascular cylinder where it initiates formation of a feeding site. Sex is determined towards the end of the J2 stage. (iv & v) A multinucleate feeding site (syncytium) is established by cell wall dissolution. The female enlarges whilst the motile, vermiform adult male develops within the J4 cuticle. The male does not feed after the J3 stage and its syncytium begins to degrade. (vi) The male leaves the root and fertilizes the adult female, which grows to rupture the root surface. Eggs develop within the female body wall, which tans to form the cyst.

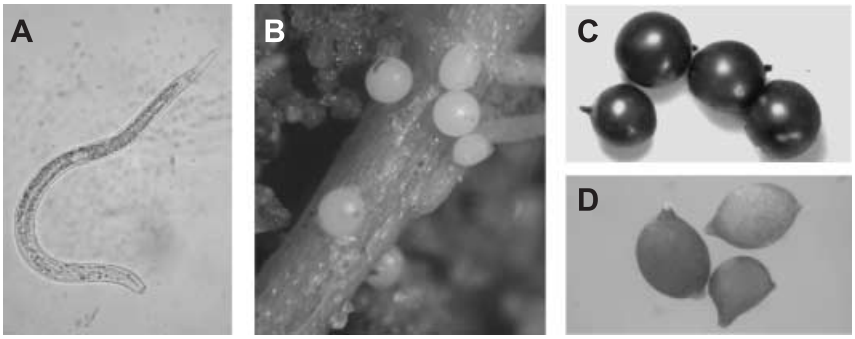

Fig. 2 Stages in the life cycle of a cyst nematode. (A) Hatched J2 of *G. pallida*. (B) Adult female *G. pallida* parasitizing a potato root. (C) Spherical cysts of *G. pallida*. (D) Lemon-shaped cysts of *H. glycines*.

occurs in response to root exudates from a suitable host plant, enabling the parasite to synchronize its life cycle with growth of the host. *G. rostochiensis* and *G. pallida* show a high dependence on host root diffusate for hatching to occur (Jones *et al*., 1998). By contrast, *H. schachtii* and *H. avenae* hatch in large numbers in the absence of any stimulus (Jones *et al*., 1998). A number of compounds that stimulate hatching have been purified from root extract or diffusate. Glycinoeclepin A was the first cyst nematode hatching factor to be characterized. Purified from kidney bean roots, this terpenoid compound induced hatching of *H. glycines* at very low concentrations (Masamune *et al*., 1982). Two other structurally related nortriterpenes with activity towards *H. glycines* (glycinoeclepins B and C) were subsequently purified (Fukuzawa *et al*., 1985). A hatching factor for *Globodera* sp. released from potato and tomato roots shows structural similarities to the glycinoeclepins and has been termed solanoeclepin A (Schenk *et al*., 1999). The hatched J2 is attracted towards a root, responding to gradients of a variety of stimuli. Some of the gradients that exist around roots, including those for CO_2, amino acids, pH and sugars, are likely to act as general, non-specific attractants for long-distance migration of nematodes in the soil (Perry, 1997). Specific semiochemicals that may be responsible for localized attraction of J2s to preferred sites of root invasion have yet to be defined, although a hydrophilic kairomone from white mustard that attracts *H. schachtii* has been partially purified (Rühm *et al*., 2003). The J2 penetrates the root epidermis using repeated thrusts of its protrusible, pointed stylet. Penetration occurs predominantly in the zone of elongation, just above the root tip, although other sites such as those where lateral roots emerge may also be selected (Wyss and Zunke, 1986). The J2 migrates intracellularly and crosses the cortical tissue towards the vascular cylinder. Cortical cell walls are disrupted by a combination of the mechanical action of highly coordinated stylet thrusts that produces a line of perforations (Wyss and Grundler, 1992) and the action of enzymes secreted by the nematode. The animal then forces its body through this line of weakness. Cell-wall-degrading enzymes such as β-1,4-endoglucanases (Gao *et al*., 2002; Goellner *et al*., 2000; Smant *et al*., 1998; Yan *et al*., 2001) and pectate lyase (De Boer *et al*., 2002; Gao *et al*., 2003; Popeijus *et al*., 2000b) produced in the subventral pharyngeal glands are secreted through the stylet and can be detected in the root (Wang *et al*., 1999).

After the invasion process is complete, a single cell is selected and penetrated carefully by the nematode stylet. Nematode secretions that are probably from the dorsal pharyngeal gland are secreted through the bore of the stylet into this initial feeding cell and induction of the syncytium is triggered. This involves a massive reprogramming of root cell development as neighbouring cells are incorporated into the syncytium through cell wall dissolution to form a large, multinucleate feeding structure (Fig. 3). The syncytium is highly metabolically active with dense granular cytoplasm and a proliferation of mitochondria, endoplasmic reticulum and free ribosomes. Endoreduplication of DNA (Niebel *et al*., 1996) leads to enlarged nuclei and nucleoli. The central vacuole fragments into numerous small vacuoles and cell walls adjacent to xylem vessels develop ingrowths enhancing the surface area through which solute uptake can occur (reviewed by Golinowski *et al*., 1997). The feeding cell acts as a nutrient sink, with solutes withdrawn by the nematode during its frequent feeding periods and replenished by the plant. A unique self-assembling structure called a feeding tube forms at the stylet tip each time it is re-inserted for a cycle of feeding. The blind-ended tube-like structure is thought to form from stylet secretions. It has an uneven electron-dense wall and a lumen with a direct connection to the stylet orifice (Endo, 1991; Sobczak *et al*., 1999). The feeding tube may provide a larger surface area to facilitate the transport of solutes from the syncytium to the stylet orifice. It also acts as a molecular sieve that in *H. schachtii* excludes dextrans of 40 kDa but not of 20 kDa (Böckenhoff and Grundler, 1994) and proteins of 23 kDa but not of 11 kDa (Urwin *et al*., 1998).

After the syncytium is induced, the juvenile cyst nematode undergoes three moults to reach the adult stage, with the J3 and J4 juvenile developmental stages each lasting 3–4 days at 25 °C (Von Mende *et al*., 1998). Cyst nematodes are sexually dimorphic. Males feed until the end of the J3 stage before a motile, vermiform adult male develops within the J4 cuticle. The adult male uses its stylet to emerge from the cuticle and to assist in its movement through plant cells during exit from the root. Cell-wall-degrading enzymes probably aid progression through the

root tissue (De Boer *et al.*, 1999). Females of some cyst nematode species are known to emit sex pheromones that attract the emerged males, but little is known about the chemical nature of these molecules. Vanillic acid was identified as an attractant for males of *H. glycines* (Jaffe *et al.*, 1989) and partial purification of components of the *H. schachtii* sex pheromone has been achieved (Aumann *et al.*, 1998; Jonz *et al.*, 2001). Development to an adult female is accompanied by enlargement and swelling to a saccate spherical (*Globodera* spp.) or lemon (*Heterodera* spp.) shape with at least the rear part of the female and her vulva rupturing from the root on to its surface prior to fertilization. Female size is correlated with the nutritional status of the plant and the extent of intraspecific competition between nematodes. Most cyst nematodes are amphimictic but a few such as *H. trifolii* (clover cyst nematode) and *H. oryzae* (rice cyst nematode) are parthenogenetic species (Evans, 1998). After fertilization the female cuticle is tanned by a polyphenol oxidase to form the tough, leather-like cyst. It is filled with eggs numbering up to 500 per female for *Globodera* sp. (Brodie *et al.*, 1993). Some species such as *H. glycines* also deposit a proportion of their eggs through the vulva into a gelatinous matrix. The rate of cyst nematode development is influenced by temperature with the life cycle, from egg to egg, being completed in around 30 days for most species. The life cycle of the corn cyst nematode *H. zeae* can be completed in 15–18 days at the high temperature of 33 °C required for optimum growth (Hutzell and Krusberg, 1990). By contrast, the life cycle of the potato cyst nematode may take up to 3 months (Turner and Evans, 1998).

Sex determination

The sexual fate of cyst nematodes first becomes apparent at the end of the parasitic J2 stage, shortly before the moult to J3. At this time the genital primordium begins to divide and elongate with a single, unbranched primordium destined to become the testis of the male and a branched primordium diagnostic of the developing ovaries of the female. However, the molecular mechanism of sex determination in amphimictic cyst nematode species remains unknown. Under favourable conditions that ensure a plentiful nutrient supply, the majority of juvenile nematodes develop to adult females. For *H. schachtii*, more than 90% of infecting juveniles develop as females under optimal conditions (Grundler *et al.*, 1991). When juveniles are exposed to adverse conditions such as a high level of infestation and associated intraspecific competition, the proportion of male nematodes in the adult population increases (Trudgill, 1967).

One suggestion is that sex is genetically determined so that under normal circumstances the ratio of adult males to females should be approximately 1 : 1. When nutrient supply is limiting, selective mortality of females with their higher food requirement (Müller *et al.*, 1981) leads to a greater proportion of males (Johnson and Viglierchio, 1969; Koliopanos and Triantaphyllou, 1972). Most studies support the concept of environmentally controlled, epigenetic sex determination in cyst nematodes. This could account for both the higher proportion of males in unfavourable conditions and the > 50% female population when there is adequate nutrition. In this model, the infective J2 has the potential to become either a male or a female. Nutritional cues received by the nematode during the first few days of feeding influence the developmental pathway that is followed. Sucrose concentrations were found to affect the sexual fate of *H. schachtii* growing on *Brassica rapa* roots (Grundler *et al.*, 1991). The high concentrations that favoured female development were accompanied by increased glutamine in the syncytium. Addition of glutamine to plants grown under otherwise unfavourable conditions also promoted female development. By contrast, methionine, phenylalanine, lysine and tryptophan all inhibited female development in otherwise favourable conditions (Betka *et al.*, 1991). Structural differences have been observed between the syncytia of male and female *H. schachtii*. Syncytia associated with male nematodes were less hypertrophied, with fewer, poorly developed wall ingrowths (Sobczak *et al.*, 1997). However, it is unclear if these differences are the cause or effect of nematode sexual identity. Further evidence for the role of nutritional stress in sex determination comes from studies of transgenic potato plants expressing a trypsin inhibitor. *G. pallida* parasitizing these plants had a population bias towards an excess of males, presumably due to inhibition of digestive serine proteinases (Atkinson, 1993). A similar reduction was observed in the number of female *H. schachtii* developing on Arabidopsis plants expressing cowpea trypsin inhibitor (Urwin *et al.*, 1998).

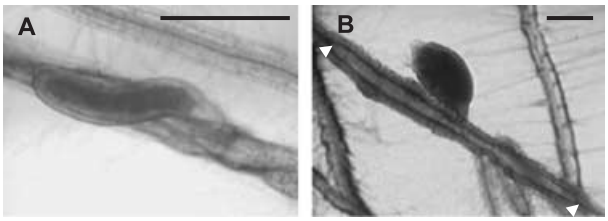

Fig. 3 *H. schachtii* parasitizing roots of *Arabidopsis thaliana*. (A) J3 stage nematode. (B) Female nematode—the extent of the syncytium is marked by arrowheads. Scale bar = 300 μm.

NEMATODE GENES INVOLVED IN PARASITISM

Penetration and migration

Study of the secretions produced by cyst nematodes helps define how nematodes interact with plant cells. The secretions of interest originate from three pharyngeal (oesophageal) glands, one located dorsally and two subventrally towards the posterior region of the

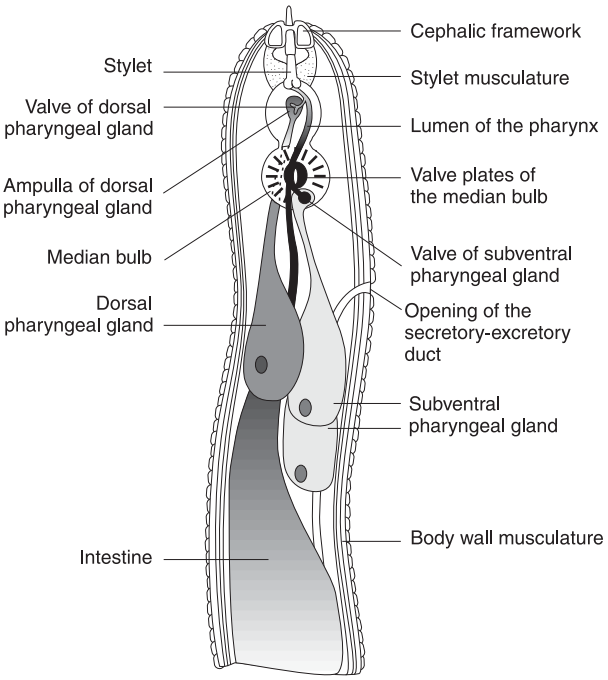

Fig. 4 Schematic representation of the anterior of a J2 cyst nematode showing the position of the pharyngeal glands.

pharynx (Fig. 4). Each gland is unicellular and consists of a lobe with the nucleus plus a cytoplasmic extension (Endo, 1984). The dorsal pharyngeal gland has a long cytoplasmic extension within the wall of much of the length of the pharynx. It provides secretory granules from the lobe to an ampulla close to the base of the stylet. The ampulla is refilled via forward movement of granules before the stylet is pushed into the syncytium and the ampulla empties. The two subventral glands empty just posterior to the chamber of the median bulb. Both subventral pharyngeal glands are highly active during invasion and migration of the J2 nematode but they decrease in size once the syncytium is initiated (Atkinson and Harris, 1989; Tytgat *et al.*, 2002). Correspondingly, genes encoding cell-wall-degrading enzymes are expressed in these two gland cells (Gao *et al.*, 2002; Popeijus *et al.*, 2000b; Smant *et al.*, 1998). A novel expansin-like protein capable of loosening plant cell walls is also secreted from these cells in the potato cyst nematode *G. rostochiensis* (Qin *et al.*, 2004). This protein may weaken non-covalent bonds and enhance the efficacy of hydrolytic enzymes. The dorsal pharyngeal gland cell increases in size after parasitism is established and remains active throughout the development of females. Release of secretions from this gland cell coincides with the onset of feeding (Wyss and Zunke, 1986). The secretions of the dorsal pharyngeal gland cell may be involved in both induction and maintenance of the syncytium (Hussey, 1989; Tytgat *et al.*, 2002).

Analysis of expression patterns of pharyngeal gland cell genes by *in situ* hybridization has helped to clarify the distinction in roles of the two types of gland cells suggested from morphological considerations and observation of feeding. Nevertheless, some of the proteins originating in the subventral pharyngeal glands have no clear role in the migration process. For instance, two putatively secreted venom allergen-like proteins are produced in the subventral pharyngeal glands of *H. glycines* (Gao *et al.*, 2001a). Although these proteins are presumed to play a role in the interaction of the nematode with its host plant, their biological function remains obscure. A nematode gene encoding a gland cell chorismate mutase (CM) was first described for the root-knot nematode *Meloidogyne javanica* (Lambert *et al.*, 1999). CM genes are similarly expressed in the subventral pharyngeal glands of both *H. glycines* (Bekal *et al.*, 2003) and *G. pallida* (Jones *et al.*, 2003) although a dorsal pharyngeal gland CM has also been reported for *H. glycines* (Gao *et al.*, 2003). This enzyme is usually found only in plants, bacteria and fungi and functions at a key branch point in the shikimate pathway, converting chorismate to prephenate. Chorismate is a precursor for a variety of plant compounds including aromatic amino acids, salicylic acid, indole-3-acetic acid (IAA) and a range of other secondary metabolites. The complexity of the biochemical pathways involving CM makes it difficult to define their biological function in cyst nematodes. Expression of the CM of a root-knot nematode *M. javanica* in soybean hairy roots suppresses both lateral root and vascular tissue formation. This phenotype could be rescued by the exogenous application of IAA (Doyle and Lambert, 2003). The nematode-introduced CM may deplete the pool of chorismate, suppressing levels of auxin and plant defence compounds (Bekal *et al.*, 2003; Doyle and Lambert, 2003). Auxin has been implicated in syncytial induction (Goverse *et al.*, 2000; Karczmarek *et al.*, 2004; Mazarei *et al.*, 2003; Wubben *et al.*, 2001) but a local increase in auxin is associated with induction of the feeding cell. Consequently, the function of the cyst nematode CM remains unclear. The CM genes form a polymorphic gene family in *H. glycines* and the polymorphisms of different inbred lines correlate with virulence on resistant soybean cultivars (Bekal *et al.*, 2003).

Syncytium induction and maintenance

Understanding of how cyst nematodes alter plant cell development is being enhanced by studies of gene expression in the dorsal pharyngeal gland. One powerful approach uses cDNA libraries constructed specifically from microaspirated gland cell contents of parasitic stages of *H. glycines* (Gao *et al.*, 2001b; Wang *et al.*, 2001). The technique of cDNA-amplified fragment length polymorphism (cDNA-AFLP) has also proved of value for isolating genes expressed in the dorsal pharyngeal gland of both *G. rostochiensis* (Qin *et al.*, 2000) and *H. schachtii* (Tytgat *et al.*, 2004). A profile of the 'parasitome' of *H. glycines* was obtained by combining expressed sequence tag (EST) analysis of a gland cell cDNA library with high-throughput *in situ* localization of

genes encoding secretory proteins (Gao *et al.*, 2003). Fifty-three genes encoding putative secreted proteins were identified, of which 41 were expressed specifically in the dorsal pharyngeal gland. Approximately 75% of these genes encode novel proteins with no known homologues. A subset of them may be uniquely required for parasitism by cyst nematodes or even specifically by *H. glycines*.

Function is not always evident even when putative homologues of the dorsal pharyngeal gland genes exist in other organisms. A novel type of secreted ubiquitin extension protein is expressed in the dorsal pharyngeal gland of both *H. glycines* (Gao *et al.*, 2003) and *H. schachtii* (Tytgat *et al.*, 2004). Ubiquitin extension proteins in which a ubiquitin monomer is fused to a C-terminal extension protein are common in eukaryotic cells. In those cases the extensions are ribosomal proteins, later cleaved from the ubiquitin which may act as a chaperone (Callis *et al.*, 1990). The C-terminal domains of the cyst nematode proteins are novel sequences of currently unknown function. A fusion of the *H. schachtii* protein with green fluorescent protein (GFP) was targeted to the nucleolus when expressed in tobacco Bright Yellow-2 (BY-2) cells (Tytgat *et al.*, 2004). The short C-terminal domain peptide may have a regulatory role in syncytial formation, with the ubiquitin domain acting as its chaperone. Many of the other secreted cyst nematode parasitism proteins also have nuclear localization signals suggesting that several function within the plant cell nucleus (Gao *et al.*, 2003).

Peptides have recently been recognized as important signalling molecules in plants, with roles in the defence response and control of cell division and expansion (Lindsey *et al.*, 2002). One such example is CLAVATA3 (CLV3), a 96-amino-acid secreted polypeptide involved in determination of cell fate at the shoot apical meristem of Arabidopsis. CLV3 is likely to be a ligand for the CLAVATA1 receptor kinase, interacting with its extracellular LRR domain (DeYoung and Clark, 2001). A motif-based database search identified homology between CLV3 and a polypeptide secreted from the dorsal pharyngeal gland of *H. glycines* (Olsen & Skriver, 2003). This is the only example of such a molecule from a non-plant species. Expression of the nematode gene (*Hg-SYV46*) in Arabidopsis under control of the CaMV35S promoter resulted in a phenotype similar to that reported for over-expression of CLV3 (Wang *et al.*, 2005). Similarly, *Hg-SYV46* expression was able to rescue a *clv3* mutant phenotype. Possibly, adaptive molecular mimicry has resulted in a nematode peptide that regulates differentiation of the syncytium. The *H. glycines* CLAVATA3 homologue may mimic or compete with the function of an endogenous plant peptide.

A number of related genes encoding small secreted peptides are expressed in the dorsal pharyngeal gland of *G. rostochiensis*. The peptides may have roles in cell cycle control during syncytium formation (van Bers, N., *et al.*, Abstract 150, ESN symposium 2004). A low-molecular-weight peptide secreted by *G. rostochiensis* does stimulate proliferation of tobacco protoplasts (Goverse *et al.*, 1999). A role in cell cycle control is also suggested for members of a *G. rostochiensis* multigene family with homology to RanBPM (Ran-binding protein in the microtubule organizing centre). RanBPM interacts with a number of other proteins to fulfil roles including cytoskeleton organization and signal transduction. The nematode proteins localize to the dorsal pharyngeal gland cell and once secreted into the plant may change the dynamic instability of microtubules in the developing syncytium (Qin *et al.*, 2002; Rehman, S., *et al.*, Abstract 148, ESN Symposium 2004). Other dorsal pharyngeal gland cell proteins for which homologues can be identified may modulate protein degradation and cell signalling in the syncytium (Gao *et al.*, 2003). Transgenic expression of these nematode genes in plants could help to elucidate their function.

GENOMIC STUDIES

Much wider genomic studies are underway for cyst nematode species than those aimed at identifying particular 'parasitism' genes. Generation of ESTs by single-pass sequencing of clones randomly selected from cDNA libraries is a rapid and cost-effective means of identifying genes. The availability of high-throughput approaches has led to a large increase in EST sequences for plant parasitic nematodes from only 22 ESTs in February 2000 (McCarter *et al.*, 2003) to more than 125 000 by March 2005 (dbEST release 11 March 2005). After pilot studies established the utility of the approach (Popeijus *et al.*, 2000a), large-scale EST collections are being made by the Genome Sequencing Center at Washington University using nematode material and cDNA libraries provided by members of the plant nematology research community. More than 37 000 cyst nematode EST sequences have now been generated (GenBank, March 2005) and can be searched at http://www.nematode.net (Wylie *et al.*, 2004). Over 24 000 of these sequences are from *H. glycines*, with smaller representation for *G. rostochiensis*, *G. pallida* and *H. schachtii*. Many ESTs are derived from preparasitic J2 nematodes owing to the technical difficulties associated with collecting sufficient material from parasitic stages. Consequently, genes involved in the penetration and migration stages of infection will be better represented than those expressed by established parasites. This situation is improving as stage-specific cDNA libraries have been sequenced for *H. glycines* and ESTs have recently been generated from a cDNA library of feeding females of *G. pallida*.

ESTs for each species are grouped into clusters based on sequence similarity and a consensus sequence is predicted. This increases the length of the derived transcript sequence as each cluster represents a putative gene. The cluster datasets together form 'partial genomes' that can be compared and subjected to data mining (Parkinson *et al.*, 2004). A recent analysis catalogued more than 7000 gene clusters for *H. glycines*, 2851 for *G. rostochiensis* and

977 for *G. pallida.* Assuming that most nematodes have about 20 000 protein coding genes, there are sequence tags for roughly one-third of the *H. glycines* gene complement, with less coverage for the other species (Parkinson *et al*., 2004).

About 30% of the putative *H. glycines* genes, 24% of *G. rostochiensis* and 43% of *G. pallida* have no significant sequence similarity to a gene outside that species. An even higher proportion of genes are unique to either the clade Tylenchina or the phylum Nematoda. Further analysis of the cyst nematode EST clusters will allow functional classification based on gene ontology assignments of conserved protein domains. Clusters can also be assigned to metabolic pathways using the KEGG database (Kanehisa *et al*., 2004). Many general pathways are likely to be conserved within nematodes but distinct routes of fatty acid biosynthesis have already been revealed (Parkinson *et al*., 2004).

Functional analysis of nematode genes

Nematode-specific or species-specific genes of unknown function with no putative homologues in current databases may provide insight into unique aspects of the plant–nematode interaction. They may also provide targets for the development of novel control strategies. A key challenge is the functional analysis of such genes. A major obstacle to their study is the obligate parasitic life cycle of cyst nematodes that extends for several weeks. By contrast, the free-living nematode *Caenorhabditis elegans* can be cultured on bacterial lawns with a life cycle of only 3 days. This ease of culture combined with stocks of mutant strains and techniques for transformation ensure that *C. elegans* is the model nematode species of choice (Bürglin *et al*., 1998). Targeted knockout of gene expression by RNA interference (RNAi) using double-stranded RNA molecules (dsRNA) corresponding to the gene of interest has proved to be a powerful approach for studying gene function in *C. elegans* (Fire *et al*., 1998). RNAi in *C. elegans* is facilitated by the ease of oral uptake of dsRNA, either from solution or after expression within its bacterial food source. Cyst nematodes do not ingest liquid or food until established within the host plant root. Delivery of the dsRNA to J2 cyst nematodes has been achieved by exposure to the neuroactive compound octopamine which induces pharyngeal pumping. As a consequence, their uptake of dsRNA leads to an RNAi effect (Urwin *et al*., 2002). Uptake of dsRNA by a cyst nematode causes a reduction in target gene transcript abundance and induces phenotypic effects for both *G. pallida* and *H. glycines.* Genes encoding cysteine proteinase, major sperm protein and a novel C-type lectin expressed in the hypodermis were initially targeted by dsRNA. Transcript abundance was reduced for all genes. Plant infection experiments showed that inhibition of cysteine proteinase gene expression did not affect infection, establishment or growth up to 14 days post infection (dpi). It did cause a 40% reduction in the number of nematodes on plants at 21 dpi plus a higher than expected number of males (Urwin *et al*., 2002). This is consistent with the importance of this class of proteinase as a digestive enzyme in adult females (Lilley *et al*., 1996). When the C-type lectin gene was targeted by dsRNA it resulted in 55% fewer *H. glycines* being recovered from plants at 14 dpi. An aminopeptidase gene of *H. glycines* has also been successfully targeted by RNAi using this method (Lilley *et al*., 2005). Current work in our laboratory suggests that RNAi effects can be achieved for a wide range of genes expressed in very different tissues of a cyst nematode (M. Bakhetia, personal communication). Modifications to the delivery of dsRNA have been reported during targeting of secreted proteins of *G. rostochiensis.* Knockout of β-1,4-endoglucanase reduced the ability of J2 nematodes to invade roots and a secreted amphid protein was shown to be essential for host location (Chen *et al*., 2005).

RNAi experiments are sufficiently sensitive to identify potential targets for development of transgenic resistance approaches. RNAi should also be a powerful tool for understanding the plant–nematode interaction and in particular investigating the novel nematode genes for which no homologue exists. The recently reported successful application of RNAi to root-knot nematodes extends the utility of this technique (Fanelli *et al*., 2005; Rosso *et al*., 2005). More accurate analysis of phenotypic effects than currently available may be required to enhance the value of the approach.

PLANT GENES INVOLVED IN PARASITISM

A susceptible plant–nematode interaction, leading to the successful establishment of a syncytium, involves concerted changes in plant gene expression. A variety of approaches have been used to identify plant genes with altered transcript abundance that are associated with syncytium development. Differential display (Hermsmeier *et al*., 1998, 2000), screening of subtractive cDNA libraries (Gurr *et al*., 1991) and promoter tagging by random insertion of a promoterless *gus* gene into the plant genome (Barthels *et al*., 1997; Puzio *et al*., 1998) have all led to the isolation of plant genes induced by cyst nematodes. Up-regulated genes have a range of proposed functions, including defence and stress responses, cell cycle control, cell wall modification, osmotic regulation, general metabolism and response to plant hormones. A smaller number of genes show reduced expression in response to nematode infection. There has been a recent comprehensive review of plant gene expression in nematode feeding sites (Gheysen and Fenoll, 2002) so only recent findings will be described here.

Microarray analysis of the nematode–plant interaction

Only a small number of the genes involved in initiation and maintenance of the syncytium are likely to have been characterized by previous approaches. A broader study of the transcriptional changes associated with both susceptible and non-host interactions will

reveal important details about how the syncytium is induced and maintained by the cyst nematode. Microarray technology allows such an approach to be realized. The transcript levels of thousands of genes can be monitored simultaneously and a comparative approach adopted. Both cDNA and oligonucleotide arrays have been used to study plant responses to pathogens (e.g. Dowd *et al.*, 2004; Schenke *et al.*, 2000; Whitham *et al.*, 2003). Differential gene expression during the early compatible interaction between soybean and *H. glycines* has been analysed using a cDNA microarray (Khan *et al.*, 2004). The 1300 sequences arrayed were selected from a subtracted soybean cDNA library enriched for genes up-regulated in an incompatible interaction. Many of the induced genes with an identified function were involved in stress/defence responses. An oligonucleotide array representing approximately one-third of the *Arabidopsis thaliana* genes was used to profile gene expression changes in roots during the early stages of cyst nematode infection (Puthoff *et al.*, 2003). At 3 dpi with *H. schachtii*, 82 genes were up-regulated and 46 down-regulated. Putative functions assigned to these genes by amino acid homology fell broadly into the categories of stress and defence responses, cell wall modification, nutrient transfer, general metabolism, signal transduction and phytohormone action. Only 12 genes showed differential expression following infection with the soybean cyst nematode *H. glycines* that invades, but cannot induce a functional syncytium in, Arabidopsis. None of the changes was unique to the incompatible interaction. The lack of a concerted induction of defence-related genes suggests that failure of *H. glycines* to establish is not due to an active defence response by the plant. This study provided the largest list to date of plant genes showing altered expression following cyst nematode infection (Puthoff *et al.*, 2003). Only a small proportion of nematode-infected tissue was present in the whole root samples used in that work. Some localized, non-abundant changes in expression may not have been detected with such samples. It is likely that with current improvements in technology many more genes will be identified. Affymetrix GeneChips representative of the complete Arabidopsis genome are now available (Redman *et al.*, 2004) and so have the potential to define patterns of expression for all genes expressed in roots. Secondly, samples taken from root sections limited to where syncytia occur provide a higher proportion of feeding cell biomass than in earlier work with whole roots and early infection time points. This approach has proved of value for studying gene expression in established syncytia of *H. schachtii*. It revealed changes in expression greater than two-fold for more than 1100 genes. About 450 genes showed increased expression and 700 a decrease in expression relative to uninfected root samples growing in identical conditions (Haeger, A., *et al.*, Abstract 140, ESN Symposium 2004). The ability to study localized changes in gene expression will be further enhanced by the use of laser capture microdissection (LCM) to isolate syncytial tissue specifically (Huang *et al.*, 2004).

The role of phytohormones in cyst nematode parasitism

Formation of nematode feeding cells may involve modulation of both auxin and ethylene response pathways. Auxin-insensitive mutants of tomato and Arabidopsis display resistance to *G. rostochiensis* and *H. schachtii*, respectively, suggesting a prominent role for auxin in the early stages of syncytium development (Goverse *et al.*, 2000). Ethylene-insensitive Arabidopsis mutants also support the development of fewer females whilst inhibition of ethylene signal transduction hinders successful parasitism (Wubben *et al.*, 2001). Conversely, mutant plants that over-produce ethylene are hypersusceptible to *H. schachtii* infection (Goverse *et al.*, 2000; Wubben *et al.*, 2001). Extensive syncytia form in such plants as a result of enhanced cell wall breakdown and larger female nematodes develop. Syncytium development may be accompanied by a nematode-induced, local increase in auxin concentration (Goverse *et al.*, 2000). This has been confirmed by GUS-reporter studies using auxin-responsive promoters (Mazarei *et al.*, 2003, 2004). Studies of the precise spatial expression of a synthetic auxin response element suggest auxin may play a role in the preconditioning of cells prior to their integration into the expanding syncytium (Karczmarek *et al.*, 2004). One of the genes encoding ACC synthase, a key enzyme in ethylene biosynthesis, is up-regulated by auxin. Raised auxin levels would therefore lead to a secondary increase in ethylene production. UDP-glucose-4-epimerase, a target of negative regulation by ethylene, is correspondingly down-regulated by *H. schachtii* infection (Wubben *et al.*, 2004).

There is no evidence that cyst nematodes secrete auxin into the initial feeding cell. They may introduce proteins that manipulate polar auxin transport both stimulating auxin influx to the developing syncytium (Mazarei *et al.*, 2003) and inhibiting its efflux (Goverse *et al.*, 2000). Alternatively, nematode proteins may alter directly the local auxin concentration by affecting conjugation, degradation or synthesis (Karczmarek *et al.*, 2004). A key part of the auxin response pathway involves the degradation of Aux/IAA transcriptional repressors by their interaction with an SCF-type (SKP1-CULLIN-Fbox) E3 ubiquitin ligase protein complex. Ubiquitin ligases tag proteins with polyubiquitin, marking them for degradation by the 26S proteasome. A RING-domain protein is associated with the SCF complex in Arabidopsis, facilitating transfer of ubiquitin to target proteins (Weijers and Jurgens, 2004). Genes encoding secreted SKP-1 (S-phase kinase-associated protein 1) and RING-H2-like proteins have been shown to be expressed in the dorsal pharyngeal gland cell of *H. glycines* (Gao *et al.*, 2003).

CONCLUSIONS

During the last decade there has been significant progress in the molecular characterization of the cyst nematode–plant

interaction. Technical advances have overcome many of the constraints associated with studying obligate parasites with small biomass. Genome-wide approaches such as high-throughput sequencing of large EST collections and the use of whole genome microarrays enable the study of many thousands of genes. Some of the newly discovered nematode genes, such as those encoding cell-wall-degrading enzymes, have an obvious role in the parasitic process. For many more the role is unclear, or the lack of any homology to known sequences prevents even speculation of their function. The challenge over the next few years will be the functional analysis of the large numbers of genes identified during sequencing projects. RNAi, combined with careful phenotypic analysis, could help elucidate the function of such genes. Transgenic expression of proteins putatively secreted into the syncytium may confirm an effect in the plant cell. *C. elegans* has been proposed as a model for functional analysis of parasitic nematode genes (Brooks and Isaac, 2002; Bürglin *et al.*, 1998). Although those genes with a specific role in parasitism are unlikely to be represented in *C. elegans*, many other cyst nematode genes have *C. elegans* homologues. Eighty-five per cent of *Meloidogyne incognita* gene clusters with database matches show homology to a *C. elegans* gene (McCarter *et al.*, 2003). Functional complementation of *C. elegans* mutants and analysis of cyst nematode gene promoters in transgenic *C. elegans* may provide useful information.

The identification of both plant and nematode genes involved in key stages of parasitism will aid the development of novel resistance strategies. It may be possible to deliver dsRNA molecules from the syncytium to the feeding nematode to disrupt the function of an essential nematode gene. Alternatively, dsRNA could be delivered from a nematode-inducible promoter to knock out expression of a plant gene required for syncytium formation. Some promoters with suitable characteristics for targeted expression of a nematode defence are available (Lilley *et al.*, 2004) and many more are likely to be identified by microarray studies.

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